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Current Research in Head and Neck Cancer

Molecular Pathways, Novel Therapeutic Targets, and Prognostic Factors

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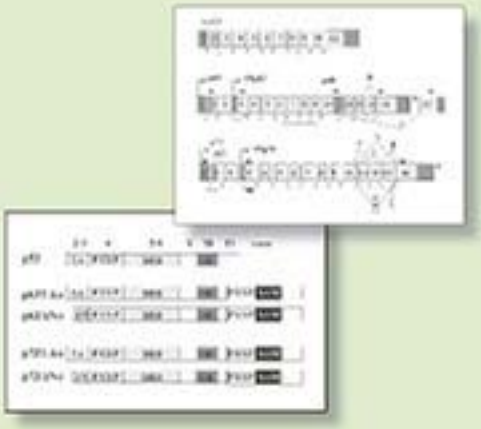
Henning Bier



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Current Research in Head and Neck Cancer

Molecular Pathways, Novel Therapeutic Targets, and Prognostic Factors

Volume Editor

Henning Bier *Düsseldorf*

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Contents

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Development of a Conditional Mouse Model for Head and Neck Squamous Cell Carcinoma

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Abstract

Last decade major advances have been made in the field of mouse model engineering. Newly developed conditional mouse models have overcome important drawbacks in conventional mouse models. Conditional mouse models are especially suited for the development of models of sporadic human carcinomas. These models can control gene (in)activation in a time and/or tissue-specific manner. Here, we review two important conditional mouse model systems, based on the Tet off/on and the Cre-Lox system. Furthermore possible applications of the Cre-Lox system in the development of a mouse model for HNSCC are being discussed. In the future, conditional mouse models for HNSCC can be used in the identification of new key genes in HNSCC tumorigenesis, and would furthermore serve as an indispensable tool for designing new treatment-modalities.

Despite advances in the management of head and neck squamous cell carcinoma (HNSCC), overall survival has hardly improved for the last 25 years. The development of a (mouse) model for HNSCC that closely mimics the human situation would be beneficial to detect new key genes in HNSCC tumorigenesis and serve as a tool for designing new treatment modalities.

Current model systems for head and neck cancer research mainly consist of in vitro cell cultures and in vivo models using (nude) mice, hamsters or rats.

Experiments with cultured cells are of course fast and cost effective in testing large amounts of different new drugs and combinations of drugs for cancer therapy and chemoprevention. However, most in vitro systems are devoid of tumor-host interactions, and their tumor cells have genetically adapted to the culture conditions. This makes extrapolation of in vitro results to the human situation very difficult.

So far, several animal models for HNSSC have been generated. The most commonly used model is the 7,12-dimethylbenz(a)anthracene-induced hamster buccal pouch model [1, 2]. This model consistently induces premalignant mucosal lesions and SCC after repeated application of the carcinogen for 14 weeks. Although this seems an attractive approach for carcinogenesis, these tumors do not genetically resemble human oral carcinomas. Unlike the human counterpart, 7,12-dimethylbenz(a)anthracene-induced tumors are relatively genetically stable and contain specific mutations of the Ha-*ras* gene [3].

Rats and mice develop oral SCC after treatment with the carcinogen 4-nitroquinoline-N-oxide. Generally, treatment protocols consist of exposure to 4-nitroquinoline-N-oxide via the drinking water for 2–6 months or painting on the tongue for 12–16 weeks [4, 5]. Unfortunately, the frequency and yield of tumor development is relatively low [6]. Also other carcinogens, like benzo[a]pyrene, N-nitroso-N-methylurea, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone or nitrosonornicotine, are used to induce tumors in the oral cavity of hamsters, rats and mice [7, 8]. However, p53 inactivation, an important hallmark in HNSCC development, is infrequent in all these chemically induced epithelial tumors [9]. Furthermore, the prolonged application of carcinogens makes these above-mentioned models laborious and time consuming.

Other in vivo models consist of (xeno)grafting of (human) cancer cells into immunologically compromised mice [10]. Recently, an orthotopic model has been developed which gave rise to invasion and metastasis [11]. However, models with immunocompromised mice have limitations, as these do not resemble multistep carcinogenesis and the influence of the immune system on tumorigenesis cannot be studied.

There is a need for an animal model of HNSCC that more closely resembles the disease as found in man from initiation and progression to metastasis. As there is an enormous genetic homology between man and mouse, it is likely that most genes that play a role in human cancer have their counterparts in mice. Therefore the transgenic mouse model could permit identification and characterization of mutations found during carcinogenesis and progression. In such a model, clonal expansion of premalignant cells can be assessed. It can also be used to assess the contribution of individual genes involved in tumorigenesis (genotype-phenotype relation). Finally this model system might permit to test novel chemopreventive and therapeutic substances closely related and specific to the genes affected. In this way it might be possible to interact at different time points during tumor progression to see which pathways are essential for tumor development and maintenance.

Nowadays, advances in mouse genetic engineering [12, 13] make it possible to fulfill much of the requirements stated above. Conditional mouse models

in general and their use in the development of a mouse model for HNSCC will be discussed below.

Conditional Mouse Models

In the last few decades, conventional transgenic mice (gene overexpression or knockouts) have played an invaluable role in the study of normal biological processes and disease. However, these conventional model systems have limitations. Firstly, in the 'all or nothing' conventional approach, the genetic modification is found in all cells of the mice. Frequently, a genetic alteration has a profound effect on normal development and can either lead to embryonic lethality or developmental adaptation. For that reason, the contribution of embryonic-lethal BRCA1 and BRCA2 in mammary tumorigenesis could not be studied in a classical mouse model [12, 14]. Secondly, because all cells of a specific tissue express the genetic lesion, tumor cells are surrounded by abnormal cells, which might influence tumor behavior. Thirdly, the contribution of some genetic alterations to a specific tumor type is biased by their strong predisposition to tumor formation in other tissues. Homozygous deletion of p53 almost inevitably leads to lymphoma and sarcoma, before onset of tumorigenesis in the desired tissue [12, 14]. To circumvent these problems, conditional transgenic technologies have been developed, which allows spatiotemporal control of gene deletion or (over)expression in mice. These systems can roughly be divided into two categories: (1) regulatable overexpression and (2) conditional genetic (in)activation. With these new conditional mouse tools, an increasing number of mouse models are being made, which cover important cancers in man, like lung, prostate, breast and pancreas carcinoma [14–17].

The Tetracycline Off/On System

Several in vivo systems exist, which can regulate transgene expression via transcriptional transactivation [reviewed in 13, 18]. We only discuss the best described and most frequently used: the tetracycline (Tet)-responsive system. It was originally developed by Gossen and Bujard [19; www.zmbh.uni-heidelberg.de/Bujard/Homepage.html], is nowadays commercially available [www.bdbiosciences.com/clontech/tet/index.shtml] and used worldwide in many laboratories. In the Tet system, the tetracycline repressor is modified in a transcriptional activator by fusing it with the activation domains of the herpes simplex virus VP16 protein. This fusion protein is called tetracycline transcriptional transactivator (tTA). The tTA specifically binds to a DNA binding site called TRE (tetracycline-responsive element), which consists of the tetracycline operator and a cytomegalovirus-derived minimal promoter. In the absence of tetracycline, tTA can bind to the TRE and activate transcription of a desired transgene.

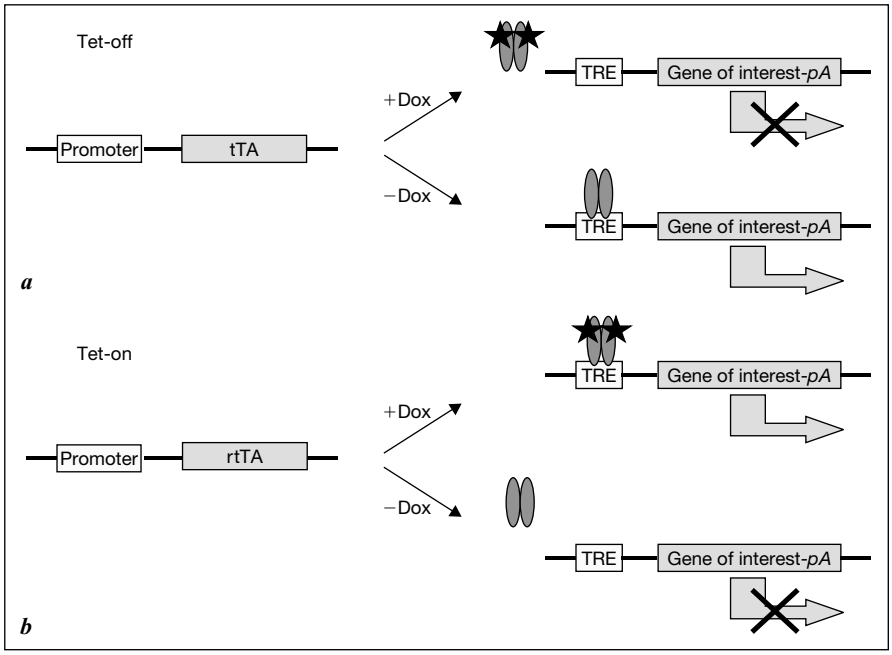

Fig. 1. Regulation of gene expression by the Tet system. ***a*** In the absence of the inducer doxycycline (Dox), tTA dimers bind to the TRE (containing 7 tandem-repeated tetracycline operator sequences fused with a minimal promoter), thereby driving expression of a transgene. In the presence of doxycycline, tTA dimers undergo conformational changes and cannot bind the TRE (Tet-off). ***b*** In the rtTA system, only in the presence of doxycycline, the rtTA dimers can bind to the TRE and activate transcription (Tet-on). *pA* = poly A signal.

Oppositely, addition of tetracycline inactivates tTA and causes transgenic downregulation (Tet-off; fig. 1a). A mutagenized version of tTA exists, 'reverse tTA' (rtTA), which only binds to the TRE in the presence of the ligand (Tet-on; fig. 1b). The Tet system is regarded as the method of choice for 'gain of function' studies of a specific transgene (oncogenic overexpression). Its big advantages are the reversibility of expression and a dose-response effect [19]. Combining this system with a tissue-specific promoter will allow regulatable transgene expression in a particular tissue or cell type.

Especially in mice, tTA and rtTA have a major difference in their kinetics of transgene induction [20]. Because of the low levels of doxycycline (Dox, a tetracycline analogue) required, induction of the rtTA system was rapidly achieved. In the tTA system, gene induction can take days, because of the long half-life of Dox in the animal. Furthermore, Dox has to be permanently

available for the mice as long as the transgene has to be shut down. However, rtTA retained some affinity for the TRE even in the absence of Dox, leading to leaky expression of the transgene, which obscured the role of rtTA in transgenic animals. Therefore, several altered versions of rtTA are developed with reduced basal activity and increased Dox sensitivity [21].

Over the years, both Tet systems have been used in the transgenic laboratory with variable results [13, 18, 19]. Especially, the reversal of oncogene expression after tumor formation has given spectacular information. In a majority of cases, tumors regressed after oncogene downregulation by the Tet system [22]. Suggesting that these oncogenes are not only required for tumor initiation but also tumor maintenance pinpoints them as valid therapeutic targets.

Site-Specific Recombination: The Cre-LoxP System

The site-specific recombination mechanism has opened a possibility for the development of mouse models of sporadic cancer. Best known is the Cre recombinase of the P_1 bacteriophage [23]. It catalyzes the recombination between two of its recognition sites, called LoxP (locus of crossover in P_1). The LoxP site is a 34-base-pair (bp) DNA sequence, containing two 13-bp inverted repeats flanking an 8-bp core sequence (fig. 2a). The 8-bp core sequence confers the directionality to the LoxP site. DNA segments flanked by 2 LoxP sites are the target of Cre. The relative orientation of the 2 LoxP sites determines the mode of recombination: inversion or excision of the intermediate DNA stretch.

The system is best clarified by two examples. (a) In the case of a tumor suppressor gene, LoxP sites are created around coding regions of a specific gene, resulting in a 'floxed' (flanked by LoxP sites) allele. LoxP sites are placed in such a way that they do not harm wild-type expression of the gene. After Cre expression the 'floxed' exons are excised, leading to an inactive tumor suppressor gene (fig. 2b). (b) In the case of an oncogene, transcription of the transgene is prevented by a 'floxed' stop signal. Only after Cre-mediated excision of this stop signal, transcription of the transgene comes about (fig. 2c).

One should bear in mind that, in contrast to the Tet system, the excision reaction of the Cre/LoxP system is irreversible. In that respect, it gives less plasticity than the Tet system. Another caveat is the reported Cre toxicity in vivo and in vitro [24–26]. Unexpected recombination events, like cell growth arrest and chromosomal aberrations, took place in some cases with Cre expression. In the mouse genome, pseudo LoxP sites have been identified, which could be sites of illegitimate Cre recombination [27].

Until now, there are only two reports dealing with Cre toxicity in vivo; their findings are probably correlated with very high Cre levels. Researchers have overcome this problem by using either controllable Cre or self-deleting Cre [24, 25, 28].

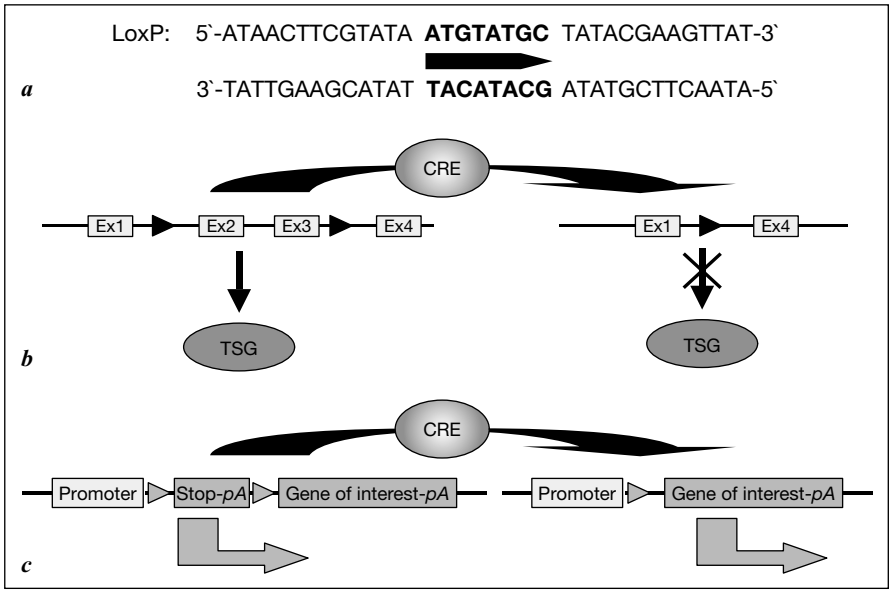

Fig. 2. Controlling gene expression by the Cre/LoxP system. ***a*** The LoxP site is a 34-bp consensus sequence, which consists of an 8-bp core flanked by two 13-bp palindromic sequences. The 8-bp core sequence determines the orientation of the LoxP site. ***b*** Example of a conditional allele; LoxP sites are created in the noncoding regions of a gene, on both sides of one or more exons (Ex), Cre expression will result in excision of the region between the 2 LoxP sites, leading to an inactive tumor suppressor gene (TSG). ***c*** Conditional transgene. Transcription of the transgene is prevented by a stop signal. This stop signal is usually an arbitrary gene with a polyA signal that causes termination of transcription. Cre expression elicits excision of the stop signal and subsequent transcription of the transgene.

Development of a Conditional Mouse Model for HNSCC

Genetic analyses uncovered many of the affected genes in HNSCC, like p53, $p16^{ink4a}$, E-cadherin, cyclin D_1 and c-*erbB2* [29]. Of these described genes, many conditional knockout/transgenic mice are already developed [12]. However, the establishment of tight tissue-specific control of Cre will greatly determine the success on a conditional mouse model for HNSCC. To date many Cre tools are described [12; www.mshri.on.ca/nagy/default.htm], which could help fulfilling this requirement: (1) Cre expression driven by tissue-specific promoters. Interesting candidates are the keratin 5 and 14 promoters (K5 and K14); both are directing expression to the basal cell layers of squamous epithelia [30]. However, expression of these promoters is not only restricted to the oral cavity,

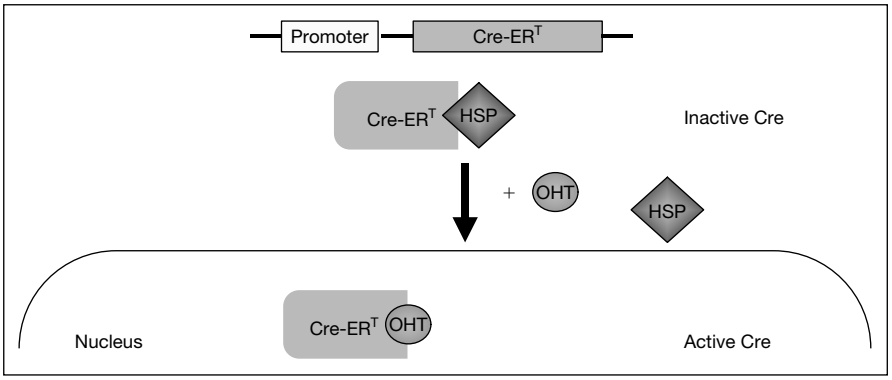

Fig. 3. Inducible Cre-LoxP system. The Cre recombinase is fused to a mutated ligand-binding domain of the ER. Expression of this fusion protein is driven by e.g. a ubiquitous or tissue-specific promoter. Without ligand binding, the inactive Cre-ER^T fusion protein is being sequestered by heat shock proteins (HSP) in the cytoplasm. Upon OHT treatment, OHT binds to the ligand-binding domain of Cre-ER^T. Subsequently, Cre translocates to the nucleus, where it exerts its recombinase activity.

e.g. K14-Cre mice are used to drive recombination in a conditional mouse model for breast cancer [13]. Using the K14-Cre mice, crossed with conditional p53 and nonconditional P16^{ink4a} mice in our laboratory resulted in the induction of cancers in the oral cavity as well as in the skin and breast. Opitz et al. [31] used the Epstein-Barr virus to drive transgenic expression of cyclin D_1 in stratified squamous epithelia. Combining these mice with p53-deficient mice resulted in oral-esophageal cancer. However, their model suffered from unwanted tumorigenesis of the lymphogenic compartment due to use of conventional p53 knockout mice. But then again, the ED-L2 promoter would be an excellent candidate to drive Cre in a conditional mouse model for HNSCC. (2) Somatic Cre delivery. Replication-deficient adeno- or lenti-Cre viruses are being employed to deliver Cre recombinase to tissues [15, 28]. Variation in infection grade per tissue is being noticed and adeno-Cre can have adverse effects on the immune response of the mouse. (3) Regulatable Cre. The archetype of this mode of regulation is called Cre-ER^T, a fusion protein between a mutated ligand-binding domain of the estrogen receptor (ER) and Cre recombinase. The activity of Cre-ER^T can be induced by 4-hydroxytamoxifen (OHT), but not by the natural ligand 17-estradiol (fig. 3). Mice have been made which exhibit expression of Cre-ER^T (RCM) in a wide variety of tissues [32]. With the use of these RCM mice, researches have spatial-temporal control on Cre activity in conditional mice. We have studied the efficiency of ligand-induced gene switching using the R26R reporter mice (fig. 4a), which permits

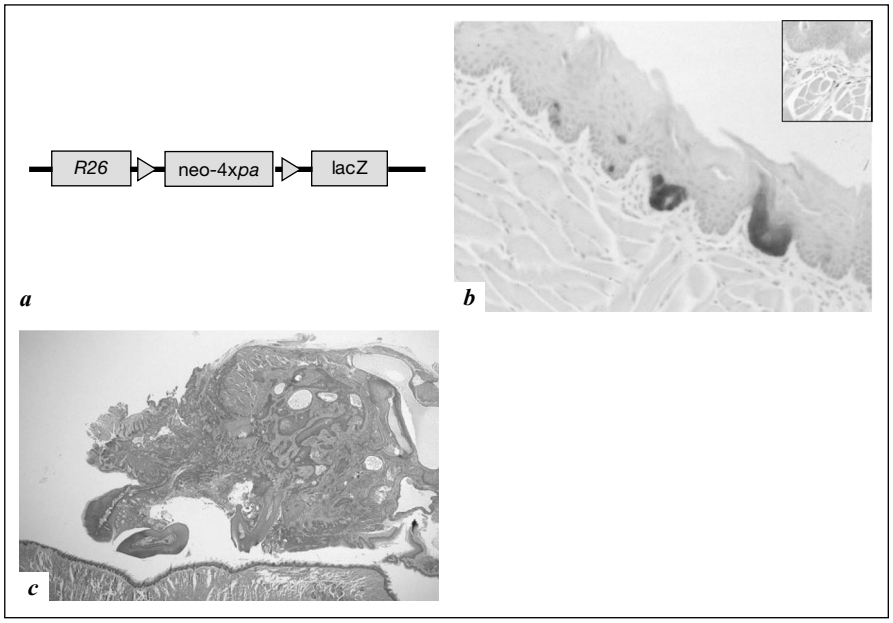

Fig. 4. Efficiency of ligand-induced gene switching in the oral cavity. ***a*** Schematic representation of the conditional reporter allele of R26R reporter mice that permits the visualization of Cre-mediated recombination via β-galoctosidase staining of tissue sections [33]. ***b*** OHT-inducible (RCM) mice were crossbred with R26R mice. After OHT application, specific gene switching could be monitored in the oral cavity. **Inset:** Also nonmucosal cells were switched after OHT treatment. ***c*** Induction of SCC in the oral cavity. RCM mice were crossbred with mice which harbor conditional p53 and nonconditional $p16^{ink4a}$ alleles. Painting of the tongues of these mice with OHT resulted in the formation of oral SCC.

the visualization of Cre-mediated recombination via β-galoctosidase staining of tissue sections [33]. After tamoxifen application, specific gene switching could be monitored in the oral cavity (fig. 4b). Subsequently, tamoxifen treatment of crosses with conditional p53 and nonconditional $p16^{ink4a}$ mice resulted in the induction of SCC in the oral cavity, although with a long latency and low incidence (fig. 4c).

However, Cre-ER^T showed residual recombinase activity in the absence of ligand, leading to germline deletions and making it impossible to use with certain conditional transgenic or knockout mice. Therefore, Cre-ER^{T2} has been developed which has no detectable background recombinase activity and is $\sim$10-fold more sensitive to OHT induction than Cre-ER^T [34]. Uniting Cre-ER^{T2} with a tissue-specific promoter like K14 or ED-L2 would give an even higher

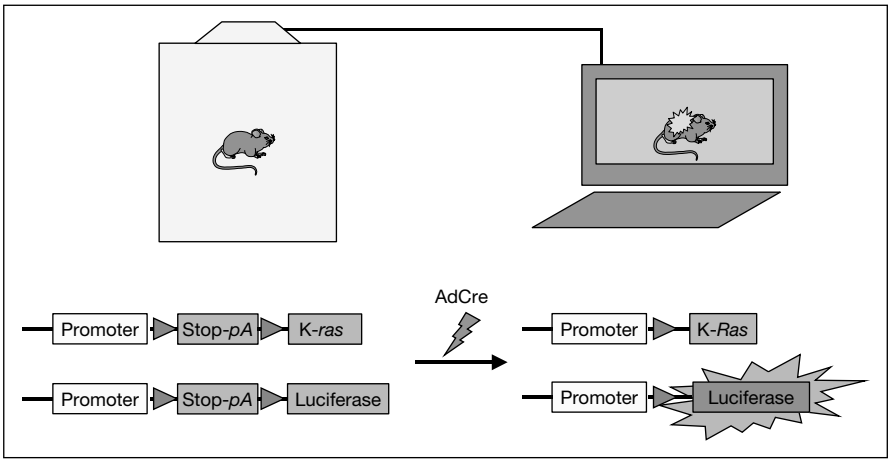

Fig. 5. In vivo imaging of tumor development. A luciferase reporter transgene permits the noninvasive visualization of spontaneous tumorigenesis in tumor-bearing mice. In these mice, Cre-mediated tumor formation is accompanied by activation of a luciferase transgene. Anesthetized mice are injected with luciferin, which serves as a substrate of luciferase. After a short incubation time, photon emission is measured with an ultrasensitive cooled CCD camera and transmitted to a personal computer with image analysis software. As an example, mice harboring conditional K-*ras* and luciferase (reporter) transgenes develop adenocarcinoma of the lungs after intrabronchial administration of adeno-Cre (AdCre). Development of lung tumors can be longitudinally monitored with this noninvasive approach [38].

degree of specificity to the spatial-temporal control in site-specific recombination in the oral cavity.

Concluding Remarks

Conditional mouse models have a great potential for the study of HNSSC. With the worldwide already existing mice with conditional tumor suppressor and oncogenes, efforts have to be concentrated on the tissue-specific expression of Cre. Development of double-layered systems could contribute to a tighter expression of Cre in the epithelium of the oral cavity. This newly developed (future) conditional mouse model for HNSCC can be used for testing new therapies. Furthermore, it will enable the study of tumor genetics using mouse expression arrays, mouse array CGH [35] and mouse spectral karyotyping (SKY) [36]. New imaging techniques using tumor-specific luciferase expression (fig. 5) [37, 38] will even broaden the impact of such a model.

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Hypofolatemia as a Risk Factor for Head and Neck Cancer

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Abstract

Head and neck cancer (HNSCC) includes squamous cell carcinomas of the oral cavity, pharynx and larynx. Approximately 38,500 cases of HNSCC are estimated to occur in the USA in 2004, with 11,000 deaths. HNSCCs represent about 3% of all malignant tumors in the USA. However, in other parts of the world, as India, Southeast Asia or Brazil, the disease is much more prevalent. The standard therapeutic approach, focused on surgery, irradiation and chemotherapy, alone or in combination, has been in part modified in the last 30 years, but the overall survival of HNSCC patients has not substantially improved. To characterize and thus identify high-risk mucosal areas and preclinical tumors, molecular abnormalities in head and neck carcinogenesis have been extensively studied. Metabolic aspects in head and neck carcinogenesis have been less extensively studied. Nevertheless, we know that metabolic alterations, often aspecific, are frequently associated with cancer. These may be secondary or may precede tumor development and favorite progression. In particular, based upon our results, a role for folate deficiency as a risk factor in head and neck carcinogenesis seems plausible. A chemoprevention protocol with folate is at present feasible and ethically correct and is already in progress at our institution. Homocysteine levels in cancer patients are probably largely affected by the HNSCC phenotype. An accumulation of homocysteine might reveal a genetic defect which is theoretically a target for pharmacological therapy, for example by antifolic drugs.

Head and neck cancer (HNSCC), as defined here, includes squamous cell carcinomas (SCCs) of the oral cavity, pharynx and larynx. Approximately 38,500 cases of HNSCC are estimated to occur in the USA in 2004, with 11,000 deaths [1]. HNSCCs represent about 3% of all malignant tumors in the

USA [1]. However, in other parts of the world, as India, Southeast Asia or Brazil, the disease is much more prevalent [2]. The standard therapeutic approach, focused on surgery, irradiation and chemotherapy, alone or in combination, has been in part modified in the last 30 years, but the overall survival of HNSCC patients has not substantially improved [1, 2]. Efforts toward early detection and prevention have not been entirely successful. For patients affected by early stage carcinomas, with a high disease-specific survival rate, second primary tumors represent the first cause of death [2–5]. On the other hand, patients with advanced head and neck cancer have a high risk of primary-treatment failure and death [2].

The best-established risk factors for HNSCC are behavioral ones, and a primary prevention can be easily obtained by suspension of wrong habits. Smoking and alcohol intake are the best-defined risk factors for HNSCC. A role has been proposed also for low vegetables intake [6], infections by Epstein-Barr virus (especially for the nasopharynx) and human papillomaviruses (for the oropharynx and other sites) [7, 8], betel quid chewing [9], marijuana smoking [10, 11], acid and biliary reflux [12]. A preventive surgery can be performed on early-diagnosed precancerous lesions of the larynx (leukoplakia, erythroplakia). But it is known that not all LSCC patients have a history of behavioral risk factors or clinically evident precancerous lesions. Molecular epidemiology should help us to recognize patients and/or areas of laryngeal mucosa with a high susceptibility to develop laryngeal cancer, and possibly to identify molecular targets for an effective secondary prevention (chemoprevention). To characterize and thus identify high-risk mucosal areas and preclinical tumors, molecular abnormalities in head and neck carcinogenesis have been extensively studied [13–15]. As a genetic predisposition to the development of HNSCC is highly probable, in the last few years a number of genetic polymorphisms have been evaluated in relation to the risk of developing cancers of the aerodigestive tract [16–18]. Metabolic aspects in head and neck carcinogenesis have been less extensively studied. Nevertheless we know that metabolic alterations, often aspecific, are frequently associated with cancer. These may be secondary or may precede tumor development and favorite progression.

In the present work, we focus our attention on compounds involved in the so-called methionine cycle, which leads to the production of S-adenosylmethionine, the body's universal methyl donor. Folate, a water-soluble B vitamin, which is plentiful in fresh vegetables and fruits, is present under a number of coenzymatic forms, whose main biochemical function in mammalian systems is to mediate the transfer of one-carbon units at different states of oxidation. It is fundamental in the synthesis of serine from glycine, in the synthesis of purine and pyrimidine bases and as methyl donor to create methylcobalamin, which is used for remethylation of homocysteine to methionine (fig. 1) [19]. Folate

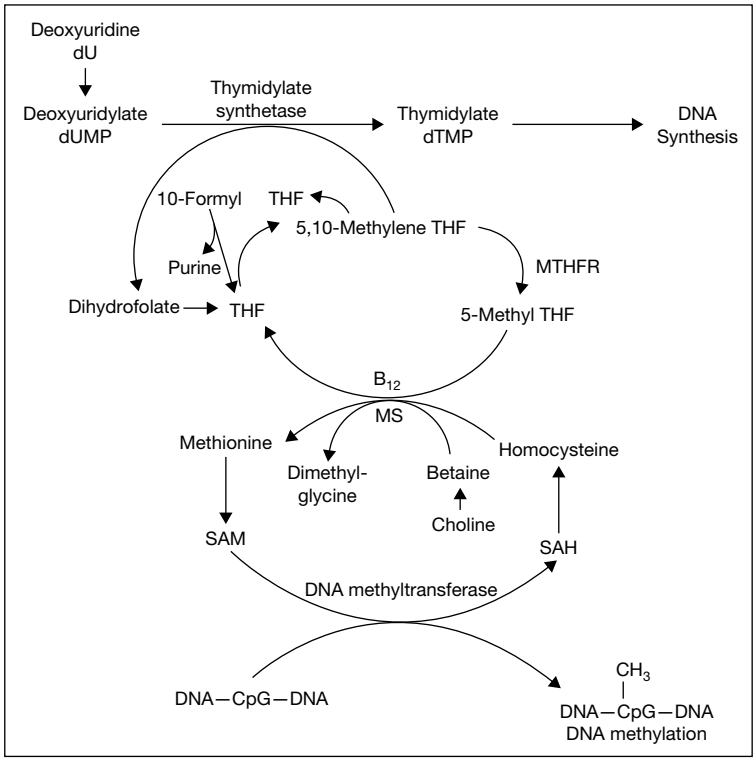

Fig. 1. Compounds involved in the methionine cycle. THF = Tetrahydrofolic acid; MTHFR = methylenetetrahydrofolate reductase; MS = methionine synthase; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; CpG = CpG islands, unmethylated regions with a higher GC content in eukaryotic DNA.

deficiency, almost always secondary to an insufficient dietary intake, has been reported to be the most common vitamin deficiency in the USA, affecting up to 10% of the general adult population [20]. Homocysteine, a sulfur-containing amino acid, is an intermediate metabolite of the methionine metabolism, located in an important metabolic crossroad (fig. 1), and its serum levels can be potentially affected by a large and nonspecific range of metabolic alterations [20]. Folate deficiency is often associated with an elevation of homocysteine, and folate supplementation reduces hyperhomocysteinemia [21]. Vitamin B_{12} (or cobalamin) is a coenzyme of methylmalonyl coenzyme A mutase and of methionine synthase (fig. 1) [19].

Alterations in the methionine cycle have been reported in several human malignancies. Maintenance of an adequate folate status from dietary sources and/or by synthetic folic acid supplementation has been associated with a

protective effect and reduced incidence of a variety of human cancers [22]. In a large prospective study, a high dietary folate intake was shown to protect, in particular heavy smokers, from lung SCC development [23]. Furthermore, folate and vitamin B_{12} supplementation has been described to induce the regression of bronchial squamous metaplasia [24, 25]. An inverse correlation between folate intake and pancreatic and breast cancer risk was also reported [26, 27]. A recent animal study evidenced a strong protective effect of folate in beagles treated with a gastric carcinogen [28]. A role for folate deficiency, first postulated in the 1960s [29], and for other alterations of methionine cycle metabolites (homocysteine, vitamin B_{12}) as a risk factor for cervical cancer has never been definitely demonstrated [30–33]. Colorectal carcinogenesis was extensively studied in relation to folate metabolism, and several epidemiological studies showed an increased risk associated with folate deficiency. Patients affected by colorectal cancer seem to have a lower folate dietary intake [34, 35], higher homocysteine and lower folate serum levels [36]. A role of folate deficiency in colorectal carcinogenesis is confirmed also by animal studies [37, 38]. Folate is at present considered one of the most promising chemopreventive agents for colorectal carcinogenesis [39]. Plasma homocysteine levels are increased also in patients with hematological tumors and in particular in children with acute lymphoblastic leukemia [40]. In ovarian carcinomas, the increase in homocysteine ascitic and cystic concentrations might derive from a biochemical defect of the methionine cycle in tumor cells [41], determined for example by an alteration in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene [42].

Several cellular effects have been described that may account for an involvement of such metabolites in carcinogenesis. Methionine cycle disruptions, by reducing intracellular S-adenosylmethionine, can alter cytosine methylation in DNA (fig. 1), leading to inappropriate activation of proto-oncogenes, repression of tumor suppressor genes and induction of malignant transformation. Alterations in DNA methylation and in particular a global hypomethylation and a regional hypermethylation, especially of promoters of tumor suppressor genes, have been described in human tumors [43]. In HNSCC, promoter hypermethylation of key genes in critical pathways, as *INK4A*, is common and has also recently been described [44]. Alternatively, abnormal DNA metabolism and a variety of cytogenetic lesions have been associated with folate deficiency in laboratory models as well as in human folate deficiency [45, 46]. Normal levels of the precursor nucleotides (dNTPs and NTPs) for DNA/RNA synthesis are directly dependent on intracellular folate availability [47], and dNTP pool imbalance seems of great relevance for the carcinogenic effects of folate deficiency [48]. A specific and important biochemical alteration, secondary to a defect in dTMP synthesis (fig. 1), is uracil misincorporation in DNA, which might be

sufficient to determine double-strand breaks, mutations and chromosomal aberrations [49, 50]. On the whole, dNTP pool imbalance reduces the efficiency of both DNA-synthesizing enzymes, with an increase in the background mutation rate, and of DNA-repairing enzymes, with an enhancement of mutagen-induced carcinogenesis [22, 51]. Convincing evidence from clinical studies indicates that moderate folate deficiency would not be independently mutagenic *in vivo* but probably interacts with other risk factors in promoting tumor progression. In fact, studies about lung and cervical carcinogenesis suggest that folate deficiency enhances an underlying predisposition due to environmental factors, as heavy cigarette smoking and human papillomavirus infection [52, 53]. On the other hand, hypofolatemia can interact also with genetic factors. Homozygous mutant individuals for a common MTHFR gene polymorphism (677 C → T; Ala → Val) have been reported to have a reduced risk of colorectal cancer [54, 55] and of adult acute lymphocytic leukemia [56]. The reason is probably that the normal more efficient enzyme reduces the quota of folate available for pathways involved in DNA synthesis and repair (fig. 1). Notably in subjects with low systemic folate status, the protective effect for colon carcinogenesis of the mutant TT genotype is lost anyway.

Therefore polymorphisms of MTHFR and probably of other enzymes involved in the methionine cycle may modify susceptibility to cancer. At present we are studying polymorphism 677 CT and other rare polymorphisms of MTHFR, and also the methionine synthase gene.

In a preliminary study, we have previously evaluated the concentrations of folate and homocysteine in the serum of patients with HNSCC, with statistically significant findings. In the present study, we evaluate folate, homocysteine and vitamin B_{12} serum levels in a larger series of HNSCC patients putting it in relation with the site, the local extension and the neck status. We also collected data about these methionine cycle metabolites in patients with laryngeal leukoplakia, a well-known preneoplastic lesion, in order to assess if such alterations are early or should be considered a late consequence of tumor progression.

Patients and Methods

Patients

144 consecutive untreated patients suffering from primary HNSCC (table 1) and 40 consecutive untreated patients with laryngeal leukoplakia were enrolled in our Department of Otolaryngology after obtaining their informed consent. Because most HNSCC patients (89.5%) and patients with leukoplakia (92.5%) were smokers, and cigarette smoking can determine alterations in homocysteine and folate status and could be a confounding factor [58], we compared cancer patients and patients with laryngeal leukoplakia, with 2 control

Table 1. Cancer patients: site and stage

	Early cancers ($T_{1–2}$, N_0)	Locally advanced ($T_{3–4}$, N_0)	Regionally advanced (every T, N_+)	Total
Oral	20	9	9	38
Laryngeal, glottic	46	5	1	52
Laryngeal, supraglottic	5	2	3	10
Laryngeal, transglottic	–	15	4	19
Pharyngolaryngeal	–	4	5	9
Oropharyngeal	2	3	4	9
Nasopharyngeal	1	2	4	7
Total	74	40	30	144

Table 2. Patients and controls

	HNSCC patients	Laryngeal leukoplakias	Nonsmoker controls	Smoker controls
Number	144	40	120	90
Age, years				
Mean	64	56	55	58
Range	37–93	39–77	35–70	30–78
Gender (F/M)	28/116	4/36	30/90	22/68
Smokers	129 (89.5)	37 (92.5)	–	90
10–20 cigarettes daily	38 (29.5)	8 (20)	–	25 (28)
More than 20 cigarettes daily	91 (60)	29 (72.5)	–	65 (72)
Smoking period <20 years	41 (32)	19 (47.5)	–	28 (31)
Smoking period >20 years	88 (57.5)	18 (45)	–	62 (69)
Drinkers	115 (80)	27 (67.5)	40 (33)	63 (70)
2 glasses (or less)/day	65 (45)	17 (42.5)	30 (25)	28 (31)
2–4 glasses/day	50 (35)	10 (25)	10 (8)	35 (39)

Figures in parentheses indicate percentages.

age- and sex-matched groups composed of 90 smokers and 120 nonsmokers (table 2). HNSCC patients were divided according to disease progression in early cancers (T_1–T_2N_0; 74 patients), locally advanced cancers (T_3–T_4N_0; n = 40) and regionally advanced cancers (N_+; n = 30; table 1). Control subjects were from the same geographical area as patients. Both control subjects and patients were enrolled after obtaining informed consent to the use of part of their blood samples for an experimental study. We excluded from our study subjects with an estimated habitual alcohol consumption higher than 35 g of alcohol, or higher

than 4 glasses of alcoholic beverages, per day, as it is well known and described that heavy drinking can alter folate absorption and metabolism, being at the same time a risk factor for HNSCC, and might thus have been a relevant confounding factor in our study. On the other hand, evidence exists that a low to moderate alcohol consumption does not determine any change in serum levels of folate and homocysteine [59]. No subject included in the study had received folate or vitamin B_{12} supplements in the last 6 months. In addition, both the patients and the controls had normal renal function. Characteristics of patients and controls are shown in table 2.

Methods

Homocysteine was measured by a fully automated AxSYM method (Abbott Diagnostics, Abbott Park, USA) according to the manufacturer's recommendations. The automatic method is based on the determination of S-adenosyl-*L*-homocysteine (SAH) obtained from the enzymatic conversion of homocysteine, previously reduced with dithiothreitol, to SAH by bovine SAH hydrolase. The Abbott AxSYM immunoassay is based on the fluorescence polarization immunoassay technology. After the addition of mouse monoclonal SAH antibody to the sample, S-adenosyl-*L*-cysteine fluorescein tracer, which competes with SAH for antibody-binding sites, is added. The homocysteine concentration is then quantified by the intensity of polarized fluorescent light. Serum vitamin B_{12} was measured by microparticle enzyme immunoassay and serum folate by ion capture assay on an AxSYM Analyzer (Abbott Diagnostics).

Statistical Analysis

The α level was fixed at 0.05. Statistical analysis was performed using STATA 6.0 by variance analysis and the Student-Newman-Keuls test.

Results

Detailed results are reported in table 3.

At variance analysis by F test for serum folate levels, differences between groups were shown to be highly probable ($F = 120$, $p < 0.001$). Comparing groups by the Student-Newman-Keuls test, statistically significant differences were found between cancer patients and smoker controls ($q = 19.048$; $p < 0.05$), between cancer patients and nonsmoker controls ($q = 23.644$; $p < 0.05$), between patients with laryngeal leukoplakia and smoker controls ($q = 11.59$; $p < 0.05$) and between patients with laryngeal leukoplakia and nonsmoker controls ($q = 14.052$; $p < 0.05$).

At variance analysis by F test for serum homocysteine, differences between groups were found to be highly probable ($F = 13.22$, $p < 0.001$). Comparing groups by the Student-Newman-Keuls test, statistically significant differences existed between cancer patients and smoker controls ($q = 6.464$; $p < 0.05$), between cancer patients and nonsmoker controls ($q = 7.681$;

Table 3. Serum folate and homocysteine levels in controls, cancer patients and patients with laryngeal leukoplakia

	Folate levels ng/ml	q score p value	Homocysteine levels, μmol/l	q score p value	Vitamin B_{12} levels, pg/ml	q score p value
Cancer patients	4.87 ± 2.26	q = 23.644	13.4 ± 10.2	q = 7.681	429 ± 281	–
Nonsmoker controls	9.7 ± 2.2	$p < 0.05$	8.7 ± 3.9	$p < 0.05$	480 ± 256	(no significance)
Cancer patients	4.87 ± 2.26	q = 19.048	13.4 ± 10.2	q = 6.464	429 ± 281	–
Smoker controls	9.1 ± 2.7	$p < 0.05$	9.1 ± 5	$p < 0.05$	472 ± 225	(no significance
Leukoplakia	5.46 ± 2.12	q = 14.052	8.45 ± 2.29	–	373 ± 152	q = 3.3
Nonsmoker controls	9.7 ± 2.2	$p < 0.05$	8.7 ± 3.9	(no significance)	480 ± 256	$p > 0.05$; (no significance)
Leukoplakia	5.46 ± 2.12	q = 11.59	8.45 ± 2.29	q = 0.691	373 ± 152	–
Smoker controls	9.1 ± 2.7	$p < 0.05$	9.1 ± 5	$p > 0.05$; (no significance)	472 ± 225	(no significance)
Cancer	4.87 ± 2.26	q = 0.691	13.4 ± 10.2	q = 5.594	429 ± 281	–
Leukoplakia	5.46 ± 2.12	$p > 0.05$; (no significance)	8.45 ± 2.29	$p < 0.05$	373 ± 152	(no significance)

$p < 0.05$) as well as between cancer patients and patients with laryngeal leukoplakia ($q = 5.594$; $p < 0.05$).

At variance analysis by F test for serum levels of vitamin B_{12}, differences between groups were not highly probable ($F = 2.4$; $p = 0.068$). Comparing groups by the Student-Newman-Keuls test for vitamin B_{12} serum levels, no statistically significant differences were found among the groups (table 3).

Serum levels of the three metabolites did not differ among patients with SCCs of the various sites in the head and neck either (table 1; data not shown).

Discussion

Lower serum folate and higher serum homocysteine levels were observed in HNSCC patients, compared to smoker and nonsmoker controls, thus confirming our previous results.

Patients with HNSCC of various sites in different stages have substantially the same folate levels, which are therefore not a marker of disease progression.

Folate serum levels are low both in patients with leukoplakia and in cancer patients, without statistically significant differences (table 3), and thus cannot be considered a diagnostic marker; nevertheless this suggests a role for low folate serum levels as a risk factor for head and neck multistep carcinogenesis, which is plausible considering also the function of folate in DNA synthesis and repair. As it was postulated for other malignancies, also in head and neck carcinogenesis hypofolatemia probably has not an independent role as initiating factor. Instead it presumably acts synergistically with other genetic and environmental factors, such as tobacco carcinogens (in fact almost all our patients are smokers anyway), making cells more susceptible to mutagens and increasing the rate of tumor progression (see Introduction).

Homocysteine serum levels are higher in cancer patients than in patients with leukoplakia, who do not significantly differ from controls. These data exclude a role for high homocysteine serum levels both as a risk marker, as they are not altered in the presence of preneoplastic lesions, and as a marker of neoplastic progression. Nevertheless the evaluation of serum homocysteine does not seem useful either for a diagnostic purpose because hyperhomocysteinemia is a quite frequent and nonspecific finding and because it would not be sensible in detecting HNSCC. In fact in HNSCC patients values of homocysteine in the serum are extremely heterogeneous as documented also by the high standard deviation (10.2 μmol/l) if compared with the other groups (table 3). It suggests that, in cancer patients, homocysteine levels do not depend exclusively on folate levels but are probably largely affected by the phenotype of head and neck carcinomas, which are a quite heterogeneous subset of tumors also from a molecular point of view. An accumulation of homocysteine might reveal a genetic defect of the methionine cycle in tumor cells as it has already been described in ovarian cancers [41, 42]. Such a metabolic defect theoretically offers a target for pharmacological therapy, for example by antifolic drugs, in a defined subset of HNSCC.

The hypothesis of an involvement of vitamin B_{12} in carcinogenesis has a scientific basis as described in the Introduction; nevertheless, in the present study focusing on HNSCC, differences in vitamin B_{12} serum levels lack significance.

Epidemiological data suggest an inverse association between the consumption of fruits and vegetables and the incidence of HNSCC [6]. The present data suggest that for HNSCC, as for other malignancies, the protective effect of dietary fruits and vegetables may be due, at least in part, to the presence of folic acid. Hypofolatemia might be a common risk factor for HNSCC and colon cancer, and this might at least partially explain the high incidence of colon second primary tumors in HNSCC patients [4].

A diet rich in folate may thus be a simple and low-cost preventive measure in the population. An increase in the recommended dietary allowances for folate

and for other micronutrients involved in DNA metabolism has been proposed [60]. In fact a folate intake in the recommended range is often insufficient to reach in the cell the optimal levels of the precursor nucleotides (dNTPs and NTPs) for DNA/RNA synthesis and repair [45, 47, 60].

The definition of a role for hypofolatemia as a risk factor for HNSCC opens intriguing perspectives for chemoprevention, defined [61] as an attempt to reverse, suppress or delay the progression towards invasive cancer, particularly relevant under a clinical viewpoint, also considering the peculiar behavior of such tumors. In fact second primary tumors, which develop at an annual rate of 3–7%, are the leading cause for cancer-related mortality in HNSCC patients [4, 5]. Furthermore precursor lesions (i.e. leukoplakia, erythroplakia), which can be directly identified by clinical examination and often precede the development of malignancy, supply a definite target for secondary prevention and an immediate experimental verification during clinical trials, as the response to treatment can be very simply assessed. Retinoids, even if with a noninnocuous toxicity profile, have been initially proposed as chemopreventive agents, but in Euroscan, the largest-scale clinical trial till now, a 2-year supplementation of retinyl palmitate and/or N-acetylcysteine resulted in no benefit – in terms of survival, event-free survival or second primary tumors – to patients with HNSCC or lung cancer [62]. Folate supplementation has no known toxic effects, and it was reported to be effective in reducing the incidence of various malignancies and in inducing regression of precancerous lesions. So, a chemoprevention protocol with folate, in patients with leukoplakia of the oral cavity, oropharynx and larynx, under strict histological and clinical follow-up, is at present rational and ethically correct and is already in progress at our institution with encouraging preliminary results. As folate supplementation of initiated cells has been reported to promote neoplastic proliferation in experimental models, we excluded from such a trial patients with a previous diagnosis of malignancy.

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Reduced DNA Repair Capacity in Laryngeal Cancer Subjects

A Comparison of Phenotypic and Genotypic Results

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Abstract

The impact of genetic factors on laryngeal cancer risk was studied in relation to DNA repair capacity on the phenotypic and genotypic level. DNA lesions induced by bleomycin or S9-activated benzo[a]pyrene were determined in blood lymphocytes using the alkaline comet assay. Laryngeal cancer subjects (n = 52) were shown to have higher levels of spontaneous and mutagen-induced DNA damage as compared to healthy controls (n = 56). A level of spontaneous DNA damage tended to increase with tumour grading. A percentage of individuals with an efficient DNA repair was higher in controls than in cancer subjects for both used mutagens. The distribution of polymorphic variants of *XPD*, *XRCC1* and *XRCC3* DNA repair genes in the group of laryngeal cancer subjects (n = 293), subjects with second primary tumours (n = 84) and in the matched controls (n = 322) was estimated by PCR-based genotyping. Five polymorphisms were studied in 3 DNA repair genes. There were found only 2 *XPD* alleles significantly overrepresented in laryngeal cancer that could be interpreted as an increase in genetic risk. There were no significant differences in distribution of 'risk' and 'protective' genotypes between single primary and second primary tumours. Altogether, the established phenotypic deficit of DNA repair in laryngeal cancer subjects was only partly confirmed by overrepresentation of 'risk' genotypes of the studied DNA repair genes.

Laryngeal cancer is tightly associated with tobacco smoking and abuse of strong alcoholic beverages. However, independently of geographical factors

only a fraction of tobacco smokers and alcohol abusers develops laryngeal cancer. It is becoming evident that a genetic predisposition has to be taken into account [1]. Numerous studies provided a good background of genetically determined susceptibility to carcinogens but an application of this fact to a given individual remains still uncertain. Recently, a variability of DNA repair efficacy has been extensively studied assuming that DNA repair impairment is a primary genetic marker of predisposition to cancer [2]. Besides an association of interindividual differences with an individual risk to develop cancer it could also be considered in relation to a variable progression of cancer as well as in line with the clinician's claim to individualize cancer therapy.

Exposure of blood lymphocytes to benzo[a]pyrene (B[a]P) diol epoxide in vitro raised a suggestion that the individual sensitivity to the genotoxic activity of B[a]P diol epoxide may be associated with a suboptimal DNA repair capacity [3]. On the other hand, susceptibility to genotoxic and clastogenic agents could be connected with DNA and chromatic structure [3, 4]. Therefore, a concept of this study was to estimate together a susceptibility to B[a]P and bleomycin used as model carcinogens generating DNA lesions with DNA repair capacity. The latter was studied as a removal of carcinogen-induced DNA damage (phenotype) and distribution of polymorphic variants of *XRCC1*, *XRCC3* and *XPD* repair genes (genotype). The study was addressed to the genetic risk to develop laryngeal cancer that is much less studied than lung cancer, which is also associated with tobacco smoking [5, 6]. This type of cancer was chosen as (1) it is strongly associated with exogenous carcinogen exposure and (2) is lacking any known high-penetration genes that imply the studies of low-penetration genes. Another argument to deal with laryngeal cancer was connected with a frequent appearance of second (multiple) primary tumours considerably decreasing therapy outcome. Thus, a comparison of the distribution of polymorphic genes between subjects with single and multiple primary tumours was attempted. Finally, laryngeal cancer is a serious medical and social problem in Poland. Morbidity and mortality rates from laryngeal cancer in Hungary and Poland are among the highest in the world [7, 8].

Material and Methods

Study Subjects

For studies on susceptibility to DNA damage a case-control group of 52 male larynx cancer subjects (age: 59.9 $\pm$ 10.5 years) was compared with a matched healthy control group of 56 male volunteers (age: 48.3 $\pm$ 6.3 years). All the blood donors were moderate or heavy cigarette smokers. For genotyping, blood samples were derived from 293 laryngeal cancer subjects with single tumour (males, age range: 40–80 years), 84 subjects with second primary tumour (SPT,

larynx as primary site; 80 males, 4 females, age range: 40–85 years) and 322 healthy controls (all males, age range: 50–64 years). All blood donors were Caucasians from Poland.

Alkaline Comet Assay

DNA damage was induced by S9-activated B[a]P (1 μM, 10 min, 37°C) or bleomycin (30 μg/ml, 10 min, 37°C) in DMSO (final concentration 0.5%). For DNA repair, cells were placed in carcinogen-free medium and incubated at 37°C up to 30 min. DNA damage was estimated as the level of single-strand DNA (ssDNA) breaks by the alkaline comet assay. The protocol already described [9] was used without modifications. Image analysis was performed using an Axiophot fluorescence microscope (Opton, Germany). A length of 50 comets was measured for each experimental point.

Genotyping

Genomic DNA was isolated from peripheral blood samples collected into EDTA tubes. *XRCC1* at G28125A (exon 10) and C26304T (exon 6) polymorphic sites, *XRCC3* (C18067T) and *XPD* (A35931C, exon 23 and C22541A, exon 6) genotypes were determined by the RFLP-PCR technique. Restriction enzymes, primers and conditions of PCR for *XRCC1* and *XRCC3* analysis were taken from Butkiewicz et al. [5] and for *XPD* from Sturgis et al. [10].

Statistics

For DNA damage and repair, the data were rank transformed to estimate statistical significance by the Mann-Whitney U test using computer software. Results were considered significant when $p < 0.05$.

Fisher's exact and χ^2 tests were used to analyse the differences between groups in allelic or genotypic distribution. Logistic regression was carried out to calculate genotype-associated tumour risks (odds ratio and 95% confidence interval); analyses were performed using Prism (version 3.0) software.

Results

The average results of DNA sensitivity to mutagens and damage removal studied by the comet assay are shown in figures 1 and 2. The left-hand bars represent a level of spontaneous DNA damage that is higher in cancer subjects than in controls. An in vitro exposure of lymphocytes to bleomycin (fig. 1) or activated B[a]P (fig. 2) was followed by an increase in comet length due to DNA fragmentation, because an electrophoretical DNA migration is proportional to the number of ssDNA breaks. An average increase in comet length was significant both in larynx cancer subjects and in controls. The mutagen-induced DNA fragmentation was higher in larynx cancer subjects than in controls. When larynx subjects were divided into subgroups according to histological grading, it was established that a level of spontaneous DNA fragmentation tended to increase with parameter G that seems to reflect a genetic instability associated with tumour aggressiveness (fig. 3).

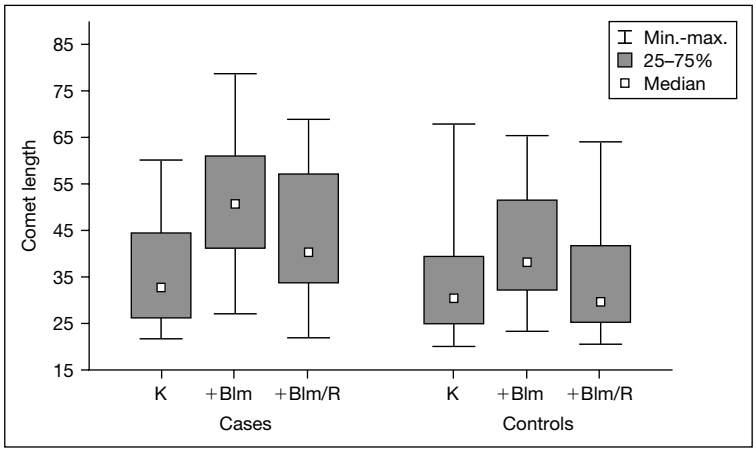

Fig. 1. Bleomycin (Blm)-induced DNA damage and its removal from lymphocytes proliferating in vitro shown as an average comet length calculated for larynx cancer subjects and healthy controls. K = control; R = repair.

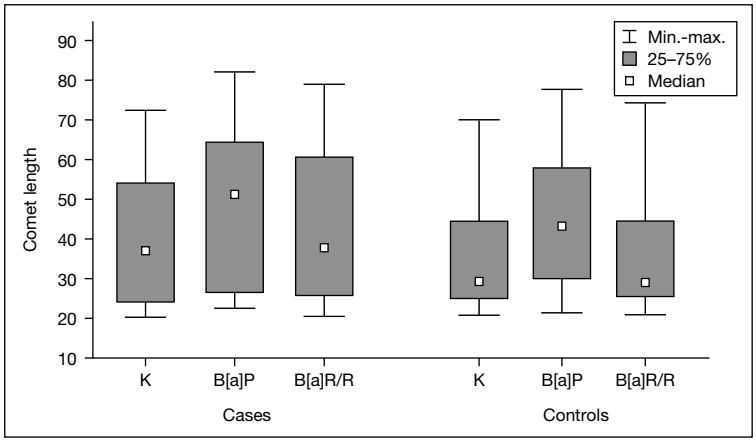

Fig. 2. B[a]P-induced DNA damage and its removal from lymphocytes proliferating in vitro shown as an average comet length calculated for larynx cancer subjects and healthy controls. B[a]P was activated by S9 microsomal fraction. K = control; R = repair.

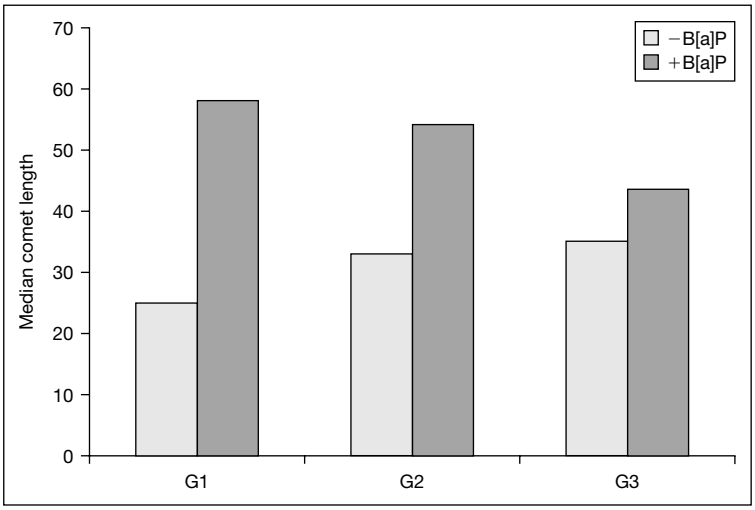

Fig. 3. Spontaneous and mutagen-induced DNA damage in human lymphocytes in vitro as a function of the histological classification of laryngeal tumour. G = Grade.

Table 1. Removal of mutagen-induced DNA lesions (%) from lymphocytes incubated in vitro for 30 min

Mutagen	Larynx cancer subjects	Controls
No mutagen	28.8	28.6
B[a]P	47.1	69.6
Bleomycin	39.4	58.2

The results are shown as a percentage of samples with arbitrarily chosen efficient DNA repair.

Then, a potential of DNA repair in vitro was estimated in lymphocytes exposed previously to bleomycin (fig. 1) or activated B[a]P (fig. 2). A number of samples with ssDNA breaks removed to the background level within 30 min (the arbitrary chosen repair time) was compared in the group of laryngeal cancer subjects and controls (table 1). An apparently more efficient removal of DNA lesions induced by both mutagens was observed for controls than for laryngeal cancer subjects. It was also noticed that repair of B[a]P-induced lesions was more effective than removal of bleomycin-induced DNA lesions. At

this point it must be stressed that the DNA-damaging effect of B[a]P is more relevant to tobacco smoke genotoxicity than that induced by bleomycin.

The genotype and allele frequencies of the studied DNA repair genes are shown in table 2. In principle, a distribution of genotypes of 5 studied polymorphisms in the group of subjects with a single tumour, SPT and controls was very much comparable. The only significant differences in risk estimates (shown in italics in table 2) were attributed to *XPG* genotypes in both studied polymorphic sites. There was also an increase in risk connected with the CT allele of *XRCC3* C18067T, but a difference between laryngeal subjects and the control group did not reach the level of significance.

The subjects with SPT (larynx as primary tumour site) were included into the study as another control group. Odds ratio values were not calculated for this group because of a relatively small group size. This group is under current analysis of genotype and allele distribution focused on the coincidence of risk-associated genotypes.

Finally, the influence of genotypes of DNA repair genes on removal of mutagen-induced DNA damage measured by the comet assay was examined. There were subgroups of subjects with a single primary tumour, and controls consisted of individuals with DNA lesion removal below the means. For each studied allele of DNA repair genes, frequency and risk estimates were calculated separately for removal of bleomycin- (table 3) and B[a]P-induced (table 4) ssDNA breaks. The highest risk estimates were attributed to the following alleles: *XPD* A35931C AC (for both mutagens), *XPD* C22541A CA (for bleomycin; for both mutagens), *XRCC1* G28152A AA (for both mutagens, particularly high for bleomycin) and *XRCC3* C18067T (for both mutagens). However, differences of frequency distribution between larynx cancer subjects and controls were far from significant.

Discussion

A relationship between mutagen sensitivity and cancer risk was described first on a chromosomal level. The bleomycin test established by Hsu et al. [11] has become a useful tool to identify individuals hypersensitive to environmental mutagens who are also at high risk to develop cancer [12]. A protocol of the bleomycin test provides a quantitative analysis of chromatid breaks but an extension of the method to identify particular breaking sites allows for a correlation of chromosome alterations with activation of oncogenes and inactivation of tumour suppressor genes. This attempt was also applied to estimate head and neck cancer risk [13–15].

The studies on a link between DNA lesions and cancer risk were more diverse because of the variety of measurable DNA lesions. The generation of

Table 2. Genotype and allele frequency of *XPD*, *XRCC1* and *XRCC3* repair genes in subjects with laryngeal tumour (single primary, second primary) and controls

Genotype	Controls, n	Single tumours, n	OR	95% CI	p	SPT, n
XPD A35931C (exon 23, Lys751Gln)						
AA	87 (27.2)	110 (37.5)	1.00			29 (35.4)
AC	175 (54.7)	135 (46.1)	*0.61*	*0.43–0.87*	*0.007*	41 (50.0)
CC	58 (18.1)	48 (16.4)	1.53	0.95–2.46	0.079	12 (14.6)
AA + AC	262 (81.9)	245 (83.6)	1.00			70 (85.4)
CC	58 (18.1)	48 (16.4)	1.13	0.74–1.72	0.569	12 (14.6)
A/C	349 (54.5)/291 (45.5)	355 (60.6)/231 (39.4)	*1.28*	*1.02–1.61*	*0.032*	99 (60.4)/65 (39.9)
XPD C22541A (exon 6, Arg156Arg)						
CC	105 (32.9)	127 (44.4)	1.00			27 (33.4)
CA	171 (53.6)	127 (44.4)	*0.61*	*0.43–0.87*	*0.005*	47 (58.0)
AA	43 (13.5)	32 (11.2)	0.62	0.36–1.04	0.069	7 (8.6)
CC + CA	276 (86.5)	254 (88.8)	1.00			74 (91.4)
AA	43 (13.5)	32 (11.2)	0.81	0.49–1.31	0.393	7 (8.6)
C/A	381 (59.7)/257 (40.3)	381 (66.6)/191 (33.4)	*1.35*	*1.06–1.70*	*0.013*	101 (62.3)/61 (37.7)
XRCC1 G28152A (exon 10, Arg399Gln)						
GG	124 (38.9)	106 (36.2)	1.00			34 (42.0)
GA	145 (45.5)	153 (52.2)	1.23	0.87–1.74	0.231	40 (49.4)
AA	50 (15.7)	34 (11.6)	0.79	0.47–1.32	0.376	7 (8.6)
GG + GA	269 (84.3)	259 (88.4)	1.00			74 (91.4)
AA	50 (11.7)	34 (11.6)	0.71	0.44–1.13	0.143	7 (8.6)
G/A	393 (61.6)/245 (38.4)	365 (62.3)/221 (37.7)	1.03	0.82–1.30	0.804	108/–

Table 2. (continued)

Genotype	Controls, n	Single tumours, n	OR	95% CI	p	SPT, n
XRCC1 C26304T (exon 6, Arg194Trp)						
CC	291 (89.5)	262 (90.4)	1.00		72 (87.8)	
CT	33 (10.2)	27 (9.3)	1.11	0.64–1.88	0.726	10 (12.2)
TT	1 (0.3)	1 (0.3)	1.11	0.06–17.8	1.000	0 (0)
CC + CT	324 (99.7)	289 (99.7)			1.00	82 (100)
TT	1 (0.3)	1 (0.3)	1.12	0.06–18.0	1.000	0
C/T	615 (94.6)/35 (5.4)	551 (95.0)/29 (5.0)	1.08	0.65–1.79	0.761	153 (93.9)/10 (6.0)
XRCC3 C18067T						
CC	144 (44.7)	135 (46.1)	1.00			9 (10.8)
CT	131 (40.7)	125 (42.7)	1.36	0.81–2.21	0.259	40 (48.2)
TT	47 (14.6)	33 (11.2)	0.75	0.45–1.24	0.236	34 (41.0)
CC + CT	275 (85.4)	260 (88.7)	1.00			49 (59.0)
TT	47 (14.6)	33 (11.2)	0.74	0.46–1.12	0.220	34 (41.0)
C/T	419 (65.1)/225 (34.9)	395 (67.4)/191 (32.6)	1.11	0.88–1.41	0.386	52 (32.5)/108 (67.5)

OR = Odds ratio; CI = confidence interval; figures in parentheses are percentages. Data in italics indicate significant differences in risk estimates.

Table 3. Allele distribution of DNA repair genes in subgroups with a reduced capacity of removal of bleomycin-induced DNA damage

Genotype	Controls, n	Cases, n	OR	95% CI	p
XPD A35931C (exon 23, Lys751Gln)					
AA	10 (20.8)	14 (29.2)	1.00		
AC	7 (14.6)	15 (31.3)	1.53	0.46–5.13	0.552
CC	6 (12.5)	3 (6.3)	0.36	0.07–1.78	0.259
XPD C22541A (exon 6, Arg156Arg)					
CC	5 (11.1)	9 (19.1)	1.00		
CA	11 (24.4)	17 (36.1)	1.16	0.31–4.41	1.000
AA	4 (8.9)	4 (8.5)	0.55	0.09–3.25	0.662
XRCC1 G28152A (exon 10, Arg399Gln)					
GG	9 (19.1)	10 (20.8)	1.00		
GA	11 (23.4)	16 (33.4)	1.31	0.4–4.28	0.766
AA	2 (4.3)	6 (12.5)	2.7	0.43–16.95	0.405
XRCC1 C26304T (exon 6, Arg194Trp)					
CC	24 (48.0)	29 (60.4)	1.00		
CT	2 (4.0)	2 (4.2)	1.21	0.16–9.23	1.000
TT	0	0	–	–	–
XRCC3 C18067T					
CC	13 (26.0)	17 (35.4)	1.00		
CT	10 (20.0)	16 (33.4)	1.22	0.42–3.57	0.789
TT	1 (2)	5 (10.5)	3.82	0.40–36.85	0.371

OR = Odds ratio; CI = confidence interval; figures in parentheses are percentages.

DNA adducts by polycyclic aromatic hydrocarbons was investigated to estimate breast cancer [16] and lung cancer [3] risks. With the same goal in mind, Schmetzer et al. [17] have successfully tested the usefulness of the comet assay in lymphocytes derived from lung cancer subjects. Many types of human cells taken from cancer subjects were shown to be very sensitive to mutagen-induced damage estimated by the comet assay [18, 19]. The paper by Kleinsasser et al. [20] describing the genotoxic sensitivity of lymphocytes and mucosa cells from the upper aerodigestive tract using the comet assay is closest to our work. The authors found a higher genotoxic sensitivity of cells derived from cancer subjects compared with non-cancer donors, but the main message of this article is that because of high intra- and interindividual differences peripheral blood lymphocytes are a poor predictor of sensitivity in target (mucosa) cells. The cancer group described in our article consisted exclusively of laryngeal cancer subjects, and in this way it is a novum when considering the high

Table 4. Allele distribution of DNA repair genes in subgroups with a reduced capacity of removal of activated B[a]P-induced DNA damage

Genotype	Controls, n	Cases, n	OR	95% CI	p
XPD A35931C (exon 23, Lys751Gln)					
AA	10 (20.8)	10 (33.3)	1.00		
AC	9 (18.7)	14 (45.2)	1.56	0.46–5.23	0.547
CC	5 (10.4)	4 (12.9)	1.25	0.26–6.07	1.000
XPD C22541A (exon 6, Arg156Arg)					
CC	3 (6.7)	7 (23.4)	1.00		
CA	13 (28.9)	17 (56.4)	1.78	0.39–8.27	0.711
AA	6 (13.3)	3 (10)	0.21	0.03–1.49	0.178
XRCC1 G28152A (exon 10, Arg399Gln)					
GG	9 (19.1)	6 (19.4)	1.00		
GA	12 (25.5)	15 (48.4)	1.87	0.52–6.76	0.502
AA	2 (4.3)	7 (22.6)	5.25	0.8–34.4	0.105
XRCC1 C26304T (exon 6, Arg194Trp)					
CC	24 (48.0)	27 (87.1)	1.00		
CT	2 (4.0)	1 (3.2)	0.44	0.04–5.22	0.604
TT	0	0	–	–	–
XRCC3 C18067T					
CC	13 (26.0)	17 (54.8)	1.00		
CT	10 (20.0)	6 (19.4)	0.46	0.13–1.59	0.353
TT	2 (4.0)	5 (16.1)	1.91	0.32–1.48	0.67

OR = Odds ratio; CI = confidence interval; figures in parentheses are percentages. Data in italics indicate significant differences in risk estimates.

sensitivity of lymphocytes to genotoxic agents followed by a reduced DNA repair capacity as described in this article.

An effect of DNA repair impairment on cancer incidence was recognized first in some human syndromes including defect(s) in DNA repair pathways [21]. Later on, it was established that a DNA repair deficit is common in cancer subjects [6, 22] including head and neck cancer [23].

To understand the impact of DNA repair genes in the estimation of genetic risk and in the development of laryngeal cancer we attempted to study the distribution of 3 polymorphic DNA repair genes. To choose the most representative genes it was decided to focus on *XPD*, *XRCC1* and *XRCC3*. The chosen genes take part in the 3 major mechanisms of DNA repair involved in the removal of DNA lesions induced by tobacco smoke carcinogens, namely nucleotide excision repair (*XPD*), base excision repair (*XRCC1*) and recombination repair

(*XRCC3*), but a cross-participation is not excluded [24]. The nucleotide excision repair pathway is responsible for the removal of 'bulky' aromatic DNA adducts, cross-links and other large lesions and therefore attracts studies on tobacco smoke genotoxicity [25]. Hence, polymorphism of *XPD*, mainly at position A35931C (Lys → Gln) seems to be associated with modulation of DNA repair followed at least by an increase in aromatic DNA adducts [26, 27] and supposedly also a risk of lung [26, 28] and head and neck [29] cancer. However, the increase in cancer risk connected with this particular polymorphism was small enough to claim an association of given polymorphisms with a real genetic risk [27]. Our results concerning the significance of *XPD* A35931C polymorphisms are in agreement with those of other papers dealing with tobacco-smoke-associated cancers [26–29].

The studied polymorphisms of *XRCC1* and *XRCC3* have not been proven to affect the laryngeal cancer risk in the studied group. The most relevant research by Sturgis et al. [10] concerned the same 2 *XRCC1* polymorphic sites studied in the group of head and neck cancer subjects of the same size. Although a general conclusion of the article is a statement on the contribution of *XRCC* to cancer risk, in the result section the authors wrote that 'no significantly elevated risk was associated with it in laryngeal cancer (data not shown'.

The data concerning the impact of DNA repair genotypes on DNA repair following mutagen-induced DNA fragmentation are calculated on a relatively small number of samples. Hence, these experiments have a preliminary character only.

Altogether our results seem to indicate a complex nature of the estimation of cancer risk. Without high-penetration genes one could expect that a risk results not only from an interaction of many polymorphisms, but also gene-environment interaction [30] has to be taken into account.

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Intratumoral Genomic Heterogeneity in Advanced Head and Neck Cancer Detected by Comparative Genomic Hybridization

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Abstract

Objectives: Little is known about the extent of intratumoral genetic heterogeneity in head and neck squamous cell carcinoma (HNSCC). **Material:** Therefore, we examined 79 stage III and IV primary HNSCCs and matched lymph node metastases for over- and underrepresentation of specific chromosome regions by comparative genomic hybridization. **Results:** The overall ratio of gains and losses was higher in metastases (M) than in primary (P) tumors (4/1 vs. 2.5/1). Gains of 3q (78.1% P vs. 87.5% M) and 11q (78.1% P vs. 62.5% M), and deletions of 3p (43.8% P vs. 34.4% M) and 9p (31.3% P vs. 15.6% M) were most frequently detected. The highest rate of intratumoral discordance was observed for primary tumors and corresponding metastases (32.8%) compared to matched pairs of 2 metastases (26.5%), and of 2 anatomically distinct sides of 1 primary tumor (24.3%). Furthermore, the discordance rate was dependent on the primary tumor site (oral cavity 49.2%, oropharynx 31%, hypopharynx 30.3% and larynx 27.3%). In some tumors, the extent of genomic discordance argues against a monoclonal origin. **Conclusion:** We demonstrate a high individual variation of intratumoral genomic heterogeneity depending on the localization and selection of matched pairs. These findings are of specific importance in view of establishing prognostic markers.

K.G. and S.C.T. contributed equally to this work and should be considered as first author.

Primary and advanced head and neck squamous cell carcinomas (HNSCCs) are characterized by complex karyotypes with multiple chromosome aberrations and cytogenetically different subclones [1–3]. This intratumoral heterogeneity can be explained by selective growth advantage of certain cells with slightly varying karyotypes (related subclones) reflecting ongoing clonal evolution [4, 5]. Furthermore, prolonged exposure to carcinogenic agents such as tobacco can lead to the development of multiple synchronous or metachronous tumors with karyotypically unrelated subclones due to the hypothesis of 'field cancerization' [6].

Several studies have addressed the complex process of tumor progression and metastatic formation in HNSCC. However, the detailed underlying genetic mechanisms are not completely understood. Using cytogenetic and fluorescence in situ hybridization (FISH) methods different groups found evidence that deletions of specific chromosomes or chromosomal subregions such as 3p, 5q, 8p and 9p are early events in head and neck carcinogenesis whereas tetraploidy followed by extensive chromosomal imbalances is a frequent feature in advanced carcinomas [3, 7, 8].

In the last decade, comparative genomic hybridization (CGH) has emerged as a powerful tool to analyze recurrent genomic imbalances in primary and advanced HNSCCs [9–18]. To date, two CGH studies have focused on genetic differences between primary tumors and their corresponding lymph node metastases [19, 20]. In one of these studies, a mean concordance rate of 68% was calculated between matched pairs of primary tumors and metastases [20].

To determine the clonality in advanced HNSCC and to investigate possible factors involving the discordance rate, we examined 79 HNSCC samples from 35 patients by CGH. Three groups of intratumoral constellations were investigated: (1) primary tumors (P) compared to 1 synchronous corresponding lymph node metastasis (M), (2) primary tumors compared to 2 synchronous corresponding lymph node metastases at different locations (M1 and M2) and (3) 2 anatomically distinct biopsies from 1 primary tumor (P1 and P2) compared to 1 corresponding lymph node metastasis. Additionally, we correlated the discordance rates with the primary tumor sites.

Materials and Methods

Patients and Tumor Specimens

Seventy-nine tumor samples from 35 patients (25 males and 10 females) with head and neck cancer were studied. Surgical resection was performed between 1998 and 2000 at the Department of Otolaryngology and Head and Neck Surgery (University Hospital Mannheim). The patients' age ranged between 47 and 74 years (average 58.5 years). Consumption of alcohol and tobacco was assessed in all patients. None of them received treatment (radiation therapy or chemotherapy) prior to surgery. All tumor specimens were

categorized according to the American Joint Committee on Cancer TNM classification system [21]. All tumors examined were stage III or IV, including primary tumors of the oral cavity (5 cases), oropharynx (7 cases), hypopharynx (15 cases), larynx (5 cases) and corresponding lymph node metastases.

Three groups of intratumoral constellations were investigated: (1) primary tumors (P) compared to 1 synchronous corresponding lymph node metastasis (M), (2) primary tumors compared to 2 synchronous corresponding ipsilateral and/or contralateral lymph node metastases (M1 and M2) and (3) 2 anatomically distinct biopsies from 1 primary tumor (P1 and P2) compared to 1 corresponding lymph node metastasis.

DNA Extraction and Degenerate-Oligonucleotide-Primed Polymerase Chain Reaction

Tumor specimens were snap-frozen immediately after surgery. Hematoxylin-eosin-stained sections (7.5 μm) were used to confirm the pathological diagnosis as well as to estimate the ratio of tumor cells in the specimens. DNA was prepared by phenol-chloroform extraction using standard protocols. Microdissection was performed on tumor specimens containing less than 80% neoplastic cells, and DNA was extracted using a DNA extraction kit (Roche Diagnostics, Mannheim, Germany). Tumor specimens that contained no sufficient DNA for CGH analysis (No. 21-P, 7-M and 5-P) were amplified by degenerate-oligonucleotide-primed polymerase chain reaction [22].

Comparative Genomic Hybridization

CGH analysis was performed as described previously [23]. Briefly, tumor DNA and genomic DNA of a healthy male donor (reference DNA) were labeled by standard nick translation procedures. Biotinylated tumor DNA and digoxigenin-labeled reference DNA were precipitated in the presence of Cot-1 DNA (Roche Diagnostics) and hybridized to metaphase spreads from a healthy male donor. After 3 days of hybridization, probe detection was carried out using fluorescein isothiocyanate for biotin- and Cy3 for digoxigenin-labeled probes. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole resulting in a G-banding-like pattern that was used for chromosome identification. Image acquisition, processing and evaluation were performed using a Leica DM RXA epifluorescence microscope, equipped with a Sensys CCD camera (Kodak KAF 1400 chip; Photometrics, Tucson, Ariz., USA) controlled by the Leica Q-FISH software (Leica Microsystem Imaging Solutions, Cambridge, UK). Three color images, green for tumor DNA, red for reference DNA and blue for 4,6-diamidino-2-phenylindole counterstaining, were acquired from 12–18 metaphase spreads per sample. Images were processed using the Leica Q-CGH software. Threshold values for detection of genomic imbalances were determined as 0.75 for losses and 1.25 for gains. For tumor specimens containing ≤50% neoplastic cells, threshold values were redefined as 0.85 for losses and 1.15 for gains. Overrepresentations were considered as high-level amplifications when the fluorescein isothiocyanate fluorescence showed a strong distinct signal detected by visual inspection and when the fluorescence ratio exceeded the value of 2.0.

Statistical Analysis

DNA copy number changes of primary tumors and corresponding lymph node metastases were compared using McNemar's test. For comparison of gains and losses detected in primary tumors and/or metastases from individual patients the discordance rates (in percent) were

calculated (modified after Waldman et al. [24]). Imbalances affecting more or less the same chromosomal region in two tumor samples of an individual patient were defined as a common event. Genetic changes were scored as one event if they affected entire chromosomes, all other changes were scored by chromosome arm. The Mann-Whitney U test was applied to compare average discordance rates between primary tumors and corresponding metastases dependent on the primary tumor site. The same test was used to compare average discordance rates between P-M matched pairs, M1-M2 matched pairs and P1-P2 matched pairs (2 anatomically distinct biopsies from 1 primary tumor). p values ≤ 0.05 were considered statistically significant.

Results

Genomic Imbalances in Primary versus Metastatic Tumors

For comparison of genetic changes only those patients who exhibited at least 1 primary tumor (n = 32) and 1 metastasis (n = 32) were selected. In the few cases presenting more than 1 biopsy of a primary tumor (P1 and P2, No. 25, 26, 27 and 33) or more than 1 metastasis (M1 and M2, No. 12, 22 and 23), the sample pairs were selected randomly. Chromosomal imbalances were detected in all but 2 tumor samples. The number of individual aberrations varied from 3 to 19 per primary tumor compared to 0–22 aberrations per metastasis. Sixty-nine percent of the primary tumors and 53% of the metastases showed more than 10 aberrations per tumor. In primary tumors, gains were 2.5 times more frequent (263 gains vs. 102 losses) whereas in metastases gains were 4 times more common (261 gains vs. 60 losses). Losses were observed more frequently in primary tumors than in metastases. Figure 1 summarizes all copy number changes observed in primary tumors and corresponding metastases. Gains affected the following entire chromosomes or chromosomal regions: 3q [78.1% P (25/32) vs. 87.5% M (28/32)], 11q [78.1% P (25/32) vs. 62.5% M (20/32)], X [43.8% P (14/32) vs. 43.8% M (14/32)], 19 [40.6% P (13/32) vs. 46.9% M (15/32)], 5p [37.5% P (12/32) vs. 34.4% M (11/32)], 8q [37.5% P (12/32) vs. 40.6% M (13/32)], 1q [34.4% P (11/32) vs. 40.6% M (13/329)], 7q [34.4% P (11/32) vs. 50% M (16/32)] and 17 [34.4% P (11/32) vs. 34.4% P (11/32)]. The most common losses affected 3p [43.8% P (14/32) vs. 34.4% M (11/32)], 9p [31.3% P (10/32) vs. 15.6% M (5/32)], Y [28.1% P (9/32) vs. 31.3% M (10/32)], 18q [25% P (8/32) vs. 18.8% M (6/32)] and 8p [18.8% P (6/32) vs. 12.5% M (4/32)]. High-level amplifications frequently affected the following chromosomal subregions: 11q13 (7/32 P vs. 9/32 M), 3q26.3–q27 (5/32 P vs. 5/32 M), 5p (2/32 P vs. 4/32 M), 8q24 (2/32 P vs. 2/32 M) and 12p (2/32 P vs. 3/23 M). Other regions were affected by high-level amplifications only once in primary or metastatic tumors (3q, 2q31, 4q12, 6p12, 7p11.2, 7q21–q22, 7q36, 8p11.2, 17q22, 18p, 18p11.3, 18q11.2, 20q13, and 21q21). None of the aberrations were significantly associated with only primary or

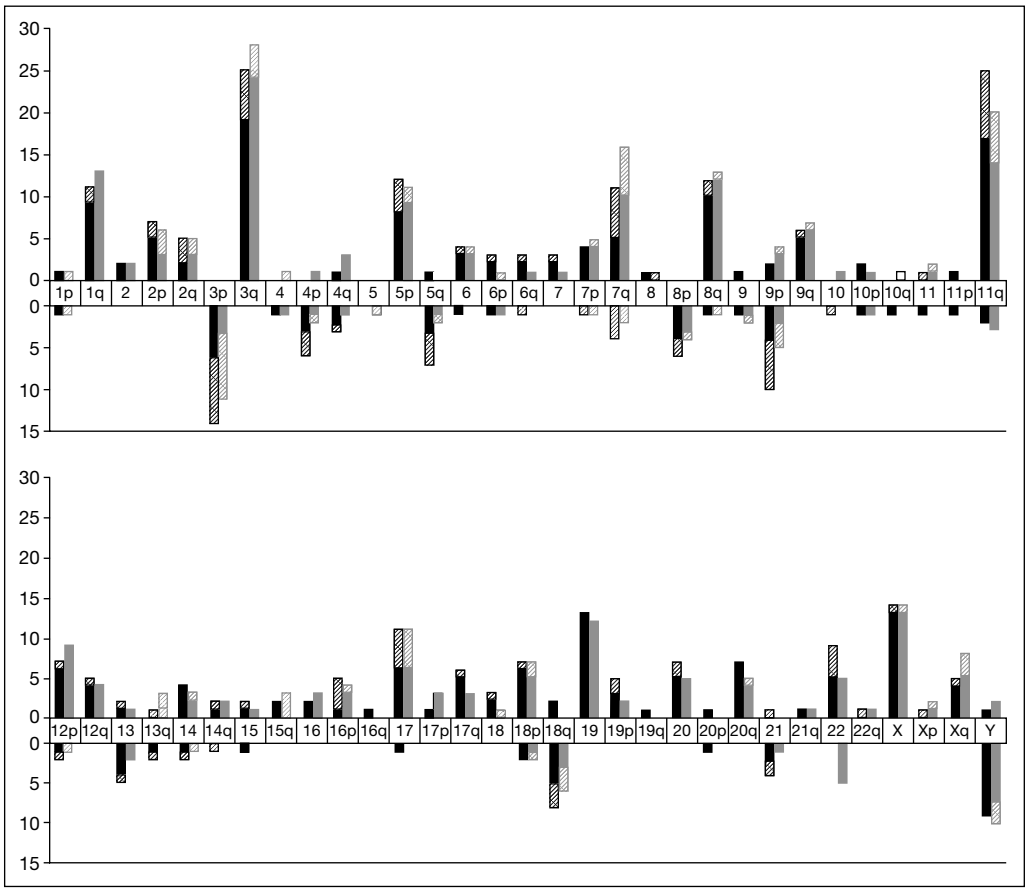

Fig. 1. Genomic imbalances detected in 32 primary tumors (black bars) and 32 corresponding lymph node metastases (gray bars) (threshold values for losses determined as 0.77 and 1.25 for gains). Hatched bars indicate genomic imbalances with threshold levels determined as 0.85 for losses and 1.15 for gains.

metastatic tumors. However, deletions of specific chromosome regions were more often observed in primary tumors, namely 4p (6/32 P vs. 2/32 M), 5q (7/32 P vs. 3/32 M) and 9p (10/32 P vs. 5/32 M). In addition, gains of distinct chromosome regions were differently distributed, namely 7q (11/32 P vs. 16/32 M) and 11q (25/32 P vs. 20/32 M).

Comparison of Discordance Rates

To determine the intratumoral genetic heterogeneity, the discordance rates of the following matched pairs were calculated for the individual patients: (1)

primary tumor compared to 1 synchronous corresponding lymph node metastasis, (2) primary tumor compared to 2 synchronous corresponding lymph node metastases at different locations and (3) 2 anatomically distinct biopsies from 1 primary tumor compared to 1 corresponding lymph node metastasis. A higher discordance rate was found for all P-M matched pairs (average discordance rate 32.8%, range 0–100%) compared to M1-M2 (average discordance rate 26.5%, range 3–50%) and P1-P2 matched pairs (average discordance rate 24.3%, range 0–53%). However, differences were statistically not significant.

Comparison of Anatomically Distinct Primary Tumor Sites

The discordance rates of the P-M matched pairs were correlated with the different anatomic sites of the primary tumors. P-M matched pairs of the oral cavity showed the highest average discordance rate (49.2%, range 10–100%) compared to P-M matched pairs of the oropharynx (31%, range 3–100%), P-M matched pairs of the hypopharynx (30.3%, range 0–87%) and P-M matched pairs of the larynx (27.3%, range 4–50%). However, differences were statistically not significant. Furthermore, there was no correlation between the average discordance rates of P-M matched pairs with regard to ipsi- or contralateral localization of the metastasis.

Discussion

Genetic aberrations in head and neck cancer are extensively studied using G-banding, interphase FISH, CGH analysis and molecular methods [for reviews, see 25–27]. However, fewer studies have analyzed the intratumoral heterogeneity by comparing genomic alterations of primary and corresponding metastatic tumors [19, 20, 28–32] and of different tumor sites [18, 33, 34]. In the present study, CGH identified recurrent chromosome aberrations in 77 of 79 HNSCC specimens. In general, the pattern of chromosomal imbalances in primary tumors and corresponding lymph node metastases was highly comparable with frequent gains of chromosomes 3q and 11q, and losses of chromosomes 3p and 9p. These results are in good agreement with other CGH analyses [9–13, 15–17, 20, 35–37]. Gains obviously dominated over losses in primary and metastatic tumors. However, more losses were observed in the primary tumors. These data support previous studies which showed that loss of specific chromosomal segments is an early event in HNSCC carcinogenesis. Later during progression tetraploidization frequently occurs followed by random loss of chromosome copies [3, 7].

The high incidence of 3q overrepresentation including amplification of 3q26–q27 observed in the present study supports the assumption that gain of

3q is an important transition event in the progression to invasive head and neck cancer [35, 38]. *PIK3CA*, which is located on 3q26, has recently been discussed as strong candidate gene in ovarian and cervical cancer [39, 40]. Furthermore, the *AIS* oncogene on 3q27–q29 was found to be amplified in lung cancer and HNSCC suggesting an important role in the progression of SCCs [41, 42]. The chromosome 11q13 region harbors several oncogenes whereby amplification of *Cyclin* D_1 was found to be associated with poor prognosis in head and neck tumors [43–45]. The *CDKN2A* gene, which encodes for p16, is localized on 9p21, a frequently underrepresented chromosome region. This gene is commonly inactivated in HNSCC [46, 47], and alterations have been related to a shorter overall survival in advanced laryngeal SCC [48]. Chromosome 3p contains several putative tumor suppressor genes such as *VHL, FHIT* and *MLH1* [49]. However, these genes are unlikely to be the targets in HNSCC [50–52]. According to a recent study, a new tumor suppressor gene, *RASSF1A* on 3p21.3, a regulator in the *ras* pathway, plays a role in the oncogenesis of HNSCC [53, 54].

Differences of more than 10% in genomic alterations between primary tumors and corresponding metastases were detected on 4p, 5q, 7q, 9p and 11q. It is noteworthy that we detected – in accordance with a recently published study [20] – a higher frequency (15%) of 7q gains in metastases compared to primary tumors. Bockmühl et al. [20] also postulated a particular relevance of 7q11.2 for metastatic spread in HNSCC. Along the same line, Petersen et al. [55] reported on 7q gains in carcinoma metastases to the brain.

The average discordance rate between primary tumors and metastases was 32.8% in our study, which is in agreement with the findings of Bockmühl et al. [20], who detected – dependent on the algorithm – concordance rates of 64 and 68%. Comparison of average discordance rates between matched pairs of primary tumor and metastasis (32.8%), 2 metastases on different sides (26.5%) and 2 biopsies of a primary tumor (24.3%) revealed the highest discordance rate in the group of primary tumors and corresponding lymph node metastases. The highest affinity was found in the group of anatomically distinct biopsies from 1 primary tumor. These findings are in line with previously published studies using microsatellite analyses [28, 31, 32, 34].

Besides the average discordance rates, it is worth considering the enormous genetic variation within individual tumors. While some tumors revealed almost complete concordance (e.g. tumors No. 17, 19, 21), others demonstrated nearly complete discordance (e.g. tumor No. 15). In this specific case, the discordance rate was 87%. Taking into account statistic chances of identical genetic aberrations, a common precursor of both the primary and the metastatic tumor is questionable. With the concept of 'field cancerization' for HNSCC in mind [6], this would not be surprising. Interestingly, in a recently published

cytogenetic study, Jin et al. [56] made a similar observation in 1 HNSCC with 2 highly complex cytogenetically unrelated clones indicating a multicellular origin. From a clinician's point of view, this could mean that in some rare cases one must suspect the coexistence of a hidden synchronous second primary tumor within the upper aerodigestive tract in the presence of a primary and a genetically unrelated metastatic tumor, which represents a 'CUP' syndrome ('carcinoma of unknown primary tumor').

Furthermore, we demonstrate that the average discordance rate is dependent on the primary tumor site (oral cavity 49.2%; oropharynx 31%; hypopharynx 30.3%; larynx 27.3%). There are no similar reports in the literature, although Huang et al. [18] have recently postulated a different CGH pattern for primary HNSCCs dependent on the tumor site. These results indicate that the analysis of a single biopsy from an oral cavity cancer may be least representative for the entire neoplasm. To date, it remains unknown whether subtle genetic differences between tumors of different localizations in the upper aerodigestive tract account for their different clinical behavior.

In conclusion, we have detected a high individual variation of intratumoral genomic heterogeneity depending on the localization and selection of matched pairs. These findings are of specific importance in view of establishing prognostic markers.

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Function and Importance of p63 in Normal Oral Mucosa and Squamous Cell Carcinoma of the Head and Neck

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Abstract

Background/Aims: Squamous cell carcinoma of the head and neck (HNSCC) is the 6th most common malignancy worldwide with a 5-year survival that has not improved over the last 20–25 years. Factors of prognostic significance for this tumour type include the presence of regional lymph node metastasis and amplification of chromosome 3q21–29, where the *p63* gene is located. This gene encodes 6 proteins and is crucial for formation of the oral mucosa, teeth, salivary glands and skin. Each of the 6 different p63 proteins has different characteristics and functions, where some resemble the tumour suppressor protein p53, whilst others have functions that oppose p53. **Methods:** To understand the function and importance of p63 in oral mucosa and tumour development we have studied protein as well as mRNA expression in normal oral mucosa and tumours. **Results/Conclusion:** Expression of p63 proteins differs between the cell layers in normal oral mucosa, and primary HNSCC has a high expression level of p63 isoforms normally expressed in basal cells. Data suggest that p63 expression in HNSCC influences tumour cell differentiation.

Background

Squamous Cell Carcinoma of the Head and Neck

Squamous cell carcinoma of the head and neck (HNSCC) is the 6th most common malignancy worldwide, and over recent years an increased number of these tumours has been seen [1]. The 5-year survival for this type of tumour is among the lowest for all cancer types and has not improved over the last 20–25 years. Both advanced disease as well as treatment cause the patient severe both functional and aesthetic problems, and reconstruction after major surgery can be very complicated. The majority of tumours are histologically highly or moderately differentiated but show striking dissimilarities in clinical course [2]. Until recently, the presence of regional lymph node metastases was the only single factor of prognostic significance for this tumour type. However, a few years ago a study of primary HNSCC showed amplification of chromosome 3q21–29 to have higher significance as a prognostic marker [3]. Within this region on chromosome 3, the *p63* gene is located.

p53 and p63

In accordance with other human solid tumours that have been studied, HNSCC has mutations within the tumour suppressor gene *TP53* in about half of the tumours, resulting in a non-functional p53 protein that lacks the protective function of the normal protein. Over the last few years, two relatives with strong homology to p53 have been identified, termed p63 and p73 [4, 5]. p63 comprises a group of (at least) 6 different proteins. Three of these possess an N-terminal sequence of amino acids that has high homology to the transcriptional activation (TA) domain of p53, a central domain and a C-terminal domain of varying length (TAp63α, TAp63β, TAp63γ). The other three p63 isoforms lack the N-terminal transactivation region but have the same common central domain and variable C-terminal sequences as the TA isoforms (ΔNp63α, ΔNp63β, ΔNp63γ) [6, 7] (fig. 1).

Because the ΔNp63 proteins lack the transactivation domain it was long thought that they also lacked the ability to transactivate, but still through the central DNA binding domain could inhibit p53 function by competing with p53 for binding to target genes. However, recent studies have shown the presence of a second transactivation domain within exons 11–12 of the *p63* gene, as well as a repressive domain within exons 13–14, mapping towards the C-terminal regions of the proteins [8, 9]. In this study, the transactivational capacity of all known p63 proteins was studied, showing the p63β proteins to be most efficient, which could be explained by their lack of exon 13 containing the repressive domain. The TAp63γ protein was not as efficient in transactivation as the p63β proteins, presumably due to its lack of the second transactivating domain

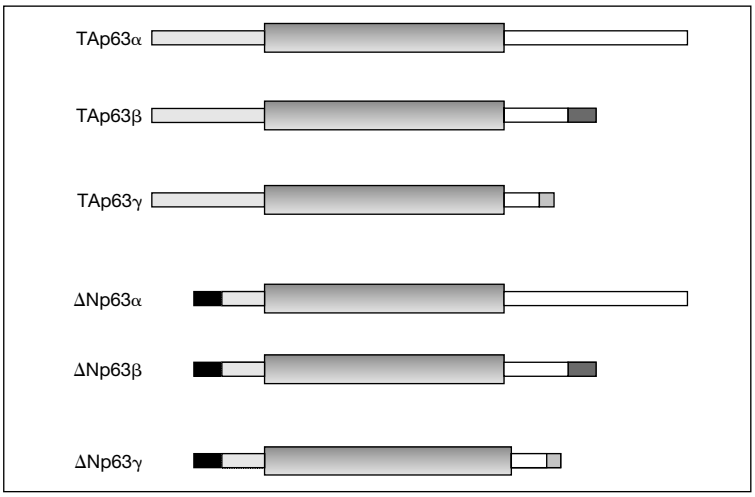

Fig. 1. Schematic drawing of the 6 p63 proteins showing common and divergent regions between the proteins. Modified after Nylander et al. [14].

within exons 11–12, and ΔNp63γ was as efficient in transactivation as the TAp63α protein [8].

The function of p53 is well established causing cell cycle arrest or apoptosis after genotoxic or other forms of stress. Clues into the function of p63 have come from a number of studies, including the production of transgenic mice in which the *p63* gene has been functionally deleted. These mice show severe developmental abnormalities of squamous epithelia and die rapidly due to dehydration, indicating that p63 expression is vital for the development of the skin as well as the oral mucosa [10, 11]. In addition, the mice also show abnormalities of other glandular epithelia. However, in these mice all 6 isoforms have been deleted, so it has remained unclear which particular isoform(s) is involved, and what the function of the other isoforms might be. Attempts at establishing 'single p63 knockout' mice, where each individual p63 protein has been inactivated, would clearly be extremely useful in this regard. However, achieving this goal is complicated by the fact that all isoforms are encoded by the same gene and arise by the use of two different promoters in combination with complex alternative splicing events. Thus, it may be impossible to prevent expression of a single isoform whilst allowing expression of the remainder.

Other clues to the function of p63 have come from studying the expression patterns of the protein in normal and pathological tissues. In contrast to the p53 protein, which is undetectable in normal adult tissues but is expressed in essentially all cell types following stress, expression of p63 can easily be detected

normally, but is limited to certain tissue types [12, 13]. Most studies of p63 expression reported to date have employed the 4A4 monoclonal antibody that does not discriminate between the different isoforms of p63. Results with this reagent suggested that p63 is most strongly expressed in epithelial stem/reserve cells in the basal layer of the skin and in other epithelial tissues [6]. More recently, we have begun to dissect out the isoform-specific expression patterns of p63 and have demonstrated that the N-terminal truncated proteins are expressed only in epithelial tissues, whilst the full-length proteins can also be detected in other types of tissue such as endothelium and lymphoid tissue [14].

In our most recent studies, we have been investigating the roles of individual p63 isoforms in the development of HNSCC by mapping changes in p63 status that have occurred in tumour tissue compared to normal corresponding tissue within each individual patient. For these studies we have used a multidisciplinary approach involving microdissection of specific anatomical regions, a highly sensitive quantitative RT-PCR method for analysing mRNA expression and immunocytochemistry to identify specific groups of p63 proteins.

Methods and Results

In order to distinguish between different p63 proteins and map their localization within the oral epithelium, we have developed antibodies specifically directed against the 2 N-terminal isoforms (TAp63 and ΔNp63) and against the C-terminal region of the p63α proteins. Using these antibodies in immunohistochemistry of formalin-fixed and wax-embedded tissue sections we could see strong staining for ΔNp63 in the basal layer, extending up to about halfway through the epithelium. The TAp63 proteins show a weaker but broader staining pattern [14] (fig. 2). Using an antibody specific for the p63α isoforms shows that these C-terminal variants are highly expressed in the oral epithelium. To date, there are no antisera available that can specifically identify p63β or p63γ due to the limited regions of uniqueness in these isoforms. These data indicate that the p63 isoforms switch from the ΔN to the TA forms during normal cellular differentiation, implying distinct roles for these isoforms in the proliferation and/or differentiation of the oral epithelium. To compare protein status with levels of mRNA for the respective proteins and to analyse the other C-terminal isoforms, we used laser dissection and divided frozen sections of normal buccal mucosa from non-smokers in 3 layers for analysis of RNA levels of the individual p63 forms. Data from this analysis confirmed results from the immunohistochemical study, showing mainly ΔNp63α in the basal cell layers and low levels of the full-length proteins, TAp63, within all levels of the epithelium. In addition, we were able to demonstrate low level expression of

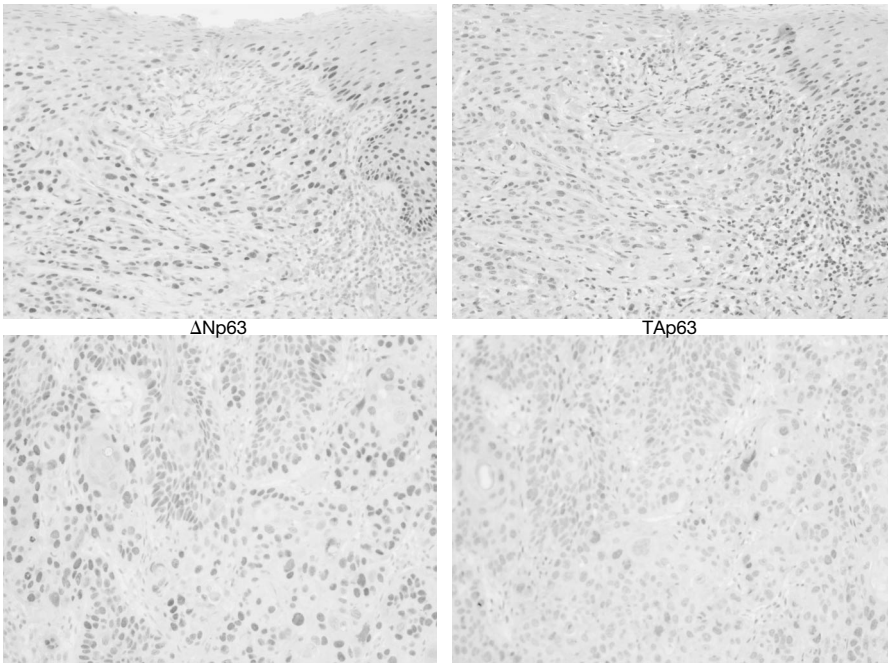

Fig. 2. Staining of parallel sections with antibodies directed against the N-terminal truncated ΔNp63 proteins (antibody KN-Δ) and the full-length TAp63 proteins (antibody KN-long). The N-terminal truncated proteins are located in basal and suprabasal layers up through approximately half of the epithelium, whereas the full-length proteins can be seen all the way through the epithelium. Furthermore, the N-terminal truncated proteins are more abundant (shown by a stronger staining reaction) and are also exclusively located within the epithelium, whereas the full-length proteins can also be seen in, for example, lymphocytes [14].

p63β mRNA [15]. These findings indicate that the transcriptionally inactive ΔNp63α [8] is the predominant isoform expressed, although transcriptionally active TAp63 and p63β isoforms are also present at low levels.

In our analyses of HNSCC, the ΔNp63α isoform was again the most widely expressed and the level of the full-length TAp63 mRNA was, as in normal mucosa, lower than the corresponding levels of ΔNp63. Some tumours also contained mRNA for p63β at low levels (table 1). An interesting finding in both normal and tumour tissue was the individual variation found in levels of the different p63 isoforms, emphasizing the importance of using normal mucosa from each patient as his/her own control in studies of p63 [15]. To further elucidate the role of p63 in proliferation, inhibition of wild-type p53 activity and/or extension of cellular life-span in HNSCC, levels of Ki-67, p53 and hTERT were also measured. Ki-67 and p53 were assessed at the protein level

Table 1. p63 mRNA levels in tumour samples

	p63α	ΔNp63	p63β
Tumour	69%	85%	77%
Statistical significance (Wilcoxon signed rank test)	–	0.016	0.046

In the analysis of p63 mRNA levels in paired normal and tumour samples from the same individual, tumours in general showed higher levels for all isoforms. For the ΔNp63 and p63β isoforms, results were statistically significant [15].

by immunohistochemistry, and hTERT levels were determined at the mRNA level. There was no obvious correlation between any of these factors and p63 [15]. Immunocytochemical analysis of p63 expression revealed an association between the differentiation state of the tumour cells and the intensity of staining for p63. In particular, tumours with nests of keratinizing cells showed only weak staining or were negative for p63 in these areas.

As smoking is a well-known risk factor for developing HNSCC, we have also collected clinically normal mucosa from smokers and age- and sex-matched non-smokers. An individual variation in levels of p63 mRNAs was seen in these 2 groups, and in accordance with the other tissues studied, the ΔN and α-isoforms predominated. Although p63 is considered as a homologue to p53, which is induced by genotoxic agents such as those found in tobacco smoke, there were no statistically significant differences in the p63 expression profiles of smokers versus non-smokers. These data indicate that p63 is unlikely to play a role in defending the oral mucosa from the damaging effects of tobacco-derived carcinogens.

Conclusions

The 5-year survival for patients with HNSCC has not improved over the last 20–25 years. As disease as well as treatment can cause patients severe aesthetic and functional problems, it is of the utmost importance to map tumour development as well as characterize the tumour cells if we are to develop more effective treatments. Therefore, as part of this process, we are studying a group of proteins called p63, which are crucial for the formation of a normal oral mucosa. These 6 proteins have, despite the fact that they belong to the same family, different characteristics, for example concerning their ability to transactivate certain downstream target genes. In this aspect, although p63 shares

some target genes in common with p53, some of the p63 proteins are more efficient than p53, and p63 can also transactivate certain genes that are apparently not regulated by p53.

To understand tumour development in the oral epithelium (as well as other tissues), one first has to map and understand conditions in the normal oral epithelium. We have developed antibodies directed towards different subgroups of the p63 family and found differences in distribution between the full length (TAp63α, TAp63β, TAp63γ) and N-terminal truncated p63 proteins (ΔNp63α, ΔNp63β and ΔNp63γ). In order to refine this mapping of location we then took biopsies from normal buccal mucosa of non-smokers and divided them into different layers to analyse mRNA. This analysis confirmed data from immunohistochemical analysis and also confirmed the ΔNp63α isoform to be the most prevalent in the basal part of the epithelium. We could further detect mRNA for p63β, mainly in the basal layer but also within the spinosum layer of normal mucosa and also in HNSCC [15]. The statistically significant up-regulation of p63β in HNSCC is interesting, as these isoforms (TAp63β and ΔNp63β) have been shown to be very efficient transactivators, in fact the most potent transactivators of all p63 isoforms [8]. This could suggest that even low levels of p63β mRNA could have a high impact on the biology of the tumours in which it is expressed [15].

Smoking is a well-known risk factor for developing HNSCC, and we therefore also looked at clinically normal oral mucosa from smokers (defined as persons who had smoked for at least 10 years and currently smoked at least 10 cigarettes per day). When comparing mucosa from these smokers to age- and sex- matched non-smokers, no difference in levels of the different p63 isoforms could be seen, and thus no indications that p63 is involved in the response to tobacco-derived genotoxic agents [15]. One striking finding was, however, the interindividual variation in p63 mRNA levels, which could also be seen in normal mucosae from HNSCC patients [15]. The fact that levels of p63 normally vary between individuals emphasizes the importance of using each individual tumour patient as his/her own control.

In HNSCC, mRNA levels of the N-terminal truncated ΔNp63 and the p63β isoforms showed a statistically significant up-regulation. Accordingly, as these isoforms are mainly found in the basal cell layers of normal epithelium, what we see in HNSCC could represent a maintenance of a high expression level of p63 isoforms normally expressed in basal cells, rather than an up-regulation of certain p63 isoforms in the tumorigenic process [15].

In all HNSCC we have studied so far, an up-regulation of hTERT could be seen in most tumours, but without correlation to expression of a particular p63 isoform. No correlation could either be seen between quantitation of p63 isoforms and the percentage of Ki-67-positive cells, as a measure of the proliferative index of the tumour [15]. Nor did we find any correlation between any p63

isoform and p53 status. Taken together, these findings do not support a role for any of the p63 isoforms in the enhancement of proliferation, inactivation of wild-type p53 or extension of the cellular lifespan [15]. However, there is a noticeable correlation between p63 expression and differentiation in both normal epithelium and in tumours, suggesting that p63 has an important role in the development of squamous epithelia and in the degree of differentiation of HNSCC.

As with all science, these findings should not be considered in isolation, but must be taken in the broader context of the physiology of the oral mucosa, where multiple proteins act in concert to regulate proliferation and differentiation. One surprising result from the human genome mapping project is that there are fewer genes than had been thought, raising the question as to how a complex organism can be formed and maintained by the action of relatively few proteins. One part of the answer to this question is exemplified by p63: a single gene gives rise to multiple different proteins with different activities, and individual cells can express different isoforms and at different levels, giving in itself a high degree of flexibility. Furthermore, p63 can interact with p53 and also with a third member of the family, termed p73 [16]. The latter also undergoes extensive splicing to produce a series of proteins from a single gene (currently at least 10 proteins). Undoubtedly, it will be an extremely difficult and laborious process to identify the true roles of each of these proteins, but we are already able to draw some conclusions concerning their functions. Our data so far point to a primary role for p63 in regulating the differentiation status of epithelial cells. In the future, we need to further define the role of p63 in neoplasia and assess whether intervening in the expression of p63 can be therapeutically beneficial. We have previously mapped the p63 status of HNSCC cell lines and seen overrepresentation of chiefly ΔNp63α, in accordance with HNSCC tumours *in vivo* [14]. Some lines also express low levels of p63β, again mirroring the situation in primary tumours. As these cell lines were established from human squamous tumours, they provide a good model system for these types of studies into the roles of the various p63 isoforms in tumour development and growth. It is likely that the next few years will provide us with answers to many of the questions that remain unanswered concerning the role of p63 in normal and pathological conditions of the oral mucosa.

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p53, p63 and p73 Expression in Squamous Cell Carcinomas of the Head and Neck and Their Response to Cisplatin Exposure

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Abstract

p63 and *p73* share significant structural and functional homologies with the tumour suppressor *p53*. Unlike the *p53* gene, both encode for several isoforms which vary in their NH_2 and COOH termini with variable and, in part, opposed biological functions. The objective of the present study was to analyse the expression profiles of p53 family members in squamous cell carcinomas of the head and neck (HNSCC) and their alterations caused by exposure to the clinically active drug cisplatin. Using multiplex RT-PCR combined with the Southern technique, we determined transcription of p53 family members in 10 established HNSCC cell lines. In the majority of HNSCC, p53 and different p63/p73 isoforms were expressed with cell-line-specific patterns for composition and intensity of transcript expression. Exposure to cisplatin caused multiple alterations in the p63 and p73 profiles suggesting a complex regulation which may influence the sensitivity to chemotherapy.

p53 plays a decisive role in counteracting tumorigenesis, and the majority of human cancers shows evidence for a profound impairment of normal p53 function [1]. In particular, it has been shown that the abrogation of p53 tumour suppressor activity appears to be a mandatory event in the development of squamous cell carcinomas of the head and neck (HNSCC) [2]. p53 is activated in response to cellular stress, such as DNA damage or oncogene activation, and functions to inhibit cell proliferation by causing cell cycle arrest and apoptosis. Several observations have suggested the presence of homologs for *p53*, including the activation of *p53* targets independent of *p53* regulation [3]. Eventually,

two homologs of *p53*, *p63* and *p73*, were identified [4–7]. Both genes have remarkable structural similarity with *p53*, and they encode for a set of proteins exhibiting both p53-agonistic and p53-antagonistic functions as well as entirely novel properties. The major reason for this functional diversity lies in the gene architecture of *p63* and *p73* (fig. 1).

p53 produces a single protein, including transactivation, proline-rich, DNA-binding and oligomerization domains (TAD, PXXP, DBD and OD, respectively). In contrast, the products of *p63* and *p73* are much more complex and yield two functionally opposed protein classes via alternative promoters and exon splicing at the NH_2 terminus: p53-like proteins retain the TAD (TA isoforms), and p53-inhibitory proteins lack the TAD (ΔTA isoforms). Furthermore, p63 and p73 undergo extensive alternative COOH-terminal splicing, which results in 5 and 7 different variants, respectively. Some of the full-length TAD isoforms were described to support or even substitute p53 activity; they induce cell cycle arrest and apoptosis by activation of p53-dependent genes, at least when being overexpressed [6–10]. On the other hand, isoforms encoded by transcripts derived from the second promoter (ΔN isoforms) and alternative NH_2-terminal splice variants are believed to be transcriptionally inactive and to have a dominant-negative effect on p53 and its homologs by blocking their transactivation [9, 11–13]. Furthermore, apoptosis induced by DNA damage has been shown to require the cooperation of p53, p63 and p73 [14]. Therefore, these 3 family members are considered to play a significant role in both the multifactorial process of carcinogenesis and the response of cancers to antineoplastic measures (fig. 2).

In contrast to p53-deficient mice, which are highly tumour prone but lack developmental malfunction, p63- and p73-deficient mice bear specific developmental defects and a non-cancerous phenotype [9, 15, 16]. p63 null mice are born alive but display severe deformations of the limbs and cranium as well as altered epithelial tissues, including skin, breast, urothelia and prostate. p63 appears to be involved in the ordered differentiation of stratified epithelia, because basal cells strongly express ΔNp63 as most prominent isoform [9] and gradually lose it when they withdraw from the stem cell compartment [17]. In accordance with its role in the development and maintenance of epithelia, and consistent with its frequently amplified locus at the chromosomal region 3q27–29, p63 levels are significantly increased in squamous cell carcinomas [18–20]. In squamous cell carcinomas of the larynx, p63 protein expression was detected by immunohistochemistry in all 150 specimens analyzed [21]. Moreover, anti-apoptotic ΔNp63 is the isoform transcript mainly expressed in HNSCC, whereas TAp63 transcripts are frequently down-regulated [21, 22]. Loss of *p73* expression in p73 null mice leads to defects in neurogenesis, pheromonal signalling causing abnormal reproductive and social behaviour,

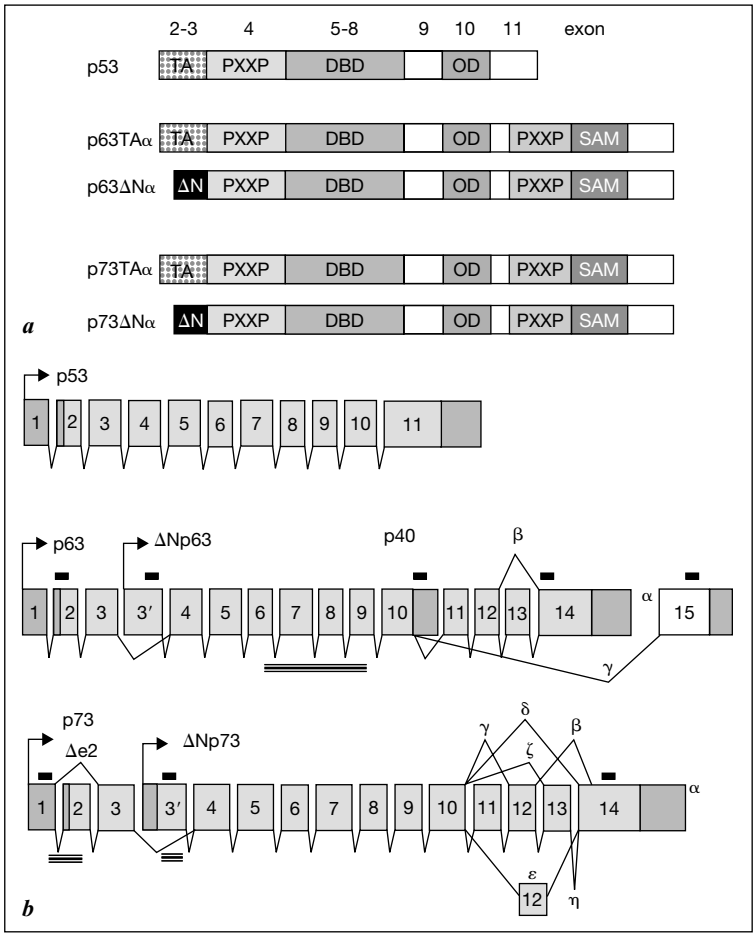

Fig. 1. Gene architecture and functional organization of the p53 family members. ***a*** In contrast to the single p53 protein comprising TAD, PXXP, DBD and OD, the p63 and p73 products are much more complex. Exemplarily shown are the α-isoforms of p63TA/ΔN and p73TA/ΔN, which additionally contain a PXXP and a sterile alpha motive (SAM) at the COOH terminus. ***b*** Genomic organization of *p53*, *p63* and *p73*. In comparison to *p53* – here a single promoter generates 1 transcript – both *p63* and *p73* feature 2 promoters. In addition, p63 and p73 undergo alternative splicing at the COOH terminus, giving rise to 5 and 7 different variants, respectively. In case of p73, there are additional splice variants lacking exon 2, exon 2 and 3, or inserting parts of exon 3′. The dark boxes show the 5′and 3′ untranslated regions. Black bars above the genes display the localization of primers used for multiplex RT-PCR, multiple lined bars beneath the genes indicate hybridization probes.

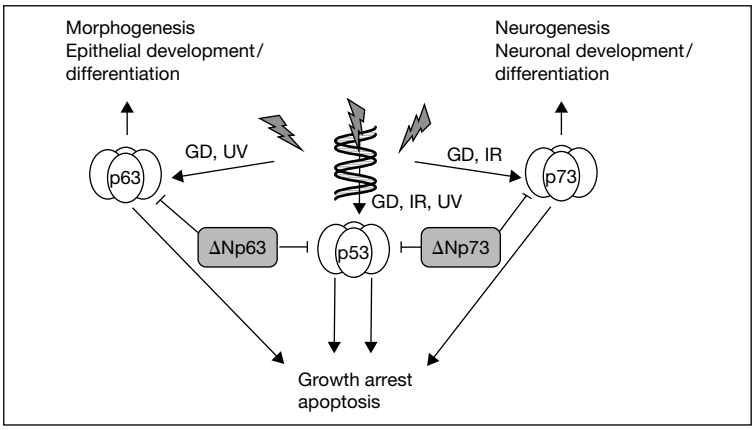

Fig. 2. Schematic p53 family pathways. Besides specific developmental and physiological functions, p63 and p73 participate in genomic guardian functions. Upon genotoxic stress by ultraviolet irradiation (UV), γ-irradiation (IR) or genotoxic drugs (GD), such as cisplatin, the two homologs interplay with p53 to induce growth arrest and apoptosis.

cerebrospinal fluid circulation and immunity of the respiratory mucosa [16]. ΔNp73 counteracts p53-mediated neuronal death and is mandatory for central nervous system development [11]. Like p63, p73 also plays a role in the differentiation of stratified epithelia. p73α was described to be necessary for the differentiation of stratified squamous epithelia and transitional epithelia [23].

Although p63 and p73 null mice do not spontaneously develop tumours, the network formed by the p53 family members is indicative of their likely impact on carcinogenesis. However, in contrast to *p53*, *p63* and *p73* lack a well-established tumour suppressor feature, i.e. the high frequency of inactivating mutations [24]. Rather overexpression of p73 has been described in hepatocellular [25], colorectal [26] and breast carcinoma [27], and this overexpression is supposed to be correlated with poor prognosis. In HNSCC, the observed patterns of p73 down-regulation and p53 inactivation support the concept of independent functions fulfilled by these two family members [28].

p53 serves as a molecular stress response device. This activity is characterized by the integration of signals emanating from a wide range of cellular insults and the response to these insults by activating a set of genes to induce protective and adaptive measures, e.g. cell cycle arrest, DNA repair and apoptosis (fig. 2). Upon genotoxic stress resulting in DNA damage, such as γ-irradiation, UV radiation and exposure to antineoplastic agents, p53 accumulates in the cell nucleus. This is mainly achieved by phosphorylation and thus stabilization of the p53 peptide. Interestingly, p73 is also activated by phosphorylation in

response to antitumour drugs and γ-irradiation (but not UV radiation) and is capable of triggering apoptosis [29–32]. Responses of p63 to DNA damage appear to be cell type and insult specific. UV radiation of human keratinocytes causes up-regulation of TAp63, whereas ΔNp63 isoforms are down-regulated [33]. The down-regulation of ΔNp63 parallels p53 stabilization, which in turn mediates apoptosis in keratinocytes damaged by UV radiation. On the other hand, expression of TAp63γ in mouse erythroleukaemia cells is stabilized after γ-irradiation, UV radiation or treatment with actinomycin D, and induces erythroid differentiation instead of apoptosis [9].

So far, well-defined integration of *p53*, *p63* and *p73* into a conclusive concept of the development and progression of the malignant phenotype remains notably complicated. As a major obstacle, oncogenic and tumour-suppressive activities of the various p63 and p73 isoforms are difficult to define as well as to distinguish. In order to improve our understanding of the multifunctional p53 family members, two issues should be addressed: the identification of predominant isoforms in normal and malignant tissue and the determination of altered isoform patterns after exposure to antineoplastic agents. The aim of the present study, therefore, was to characterize the expression of p53 family members in established cell lines of HNSCC before and after treatment with the clinically active antineoplastic agent cisplatin.

Methods

Cell Culture and Chemosensitivity Assay

The analysis included 10 cell lines established from HNSCC at the Universities of Düsseldorf, Germany (UD-SCC 1, 2, 7A and 7B), Ann Arbor, Mich., USA (UM-SCC 10B, 17A, 17B and 22B), and Turku, Finland (UT-SCC 9, 24B and 33). All cell lines were grown under standard culture conditions. The 50% inhibitory cisplatin concentration (IC_{50}) for each cell line was determined with the dimethylthiazol diphenyltetrazolium (MTT) test as described previously [34]. Briefly, on day 0, exponentially growing cells were harvested and plated at 6×10^3 cells/well in 96-well flat bottom microtitre plates. On day 3, cells were treated with cisplatin, and on day 6, MTT was added; the tetrazolium salt MTT is cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the respiratory chain of the mitochondria. Colorimetric readouts at 570 nm reflect the number of metabolically active mitochondria in viable cells. This experimental procedure has been shown to produce a linear correlation between absorbance and number of live tumor cells [34, 35]. The IC_{50} was determined by logarithmic regression from 6-fold replicates in at least 3 independent experiments (table 1).

RT-PCR Analysis and Southern Blot

Total RNA was isolated from untreated cells and from cells exposed to cisplatin (IC_{50}/4 or 24 h) with the QIAshredder/RNeasy kit (Qiagen, Hilden, Germany), including on-column DNAse treatment. Three micrograms RNA were converted to cDNA using anchored oligo-dT

Table 1. p53 status and cisplatin sensitivity of HNSCC cell lines

Cell line	p53 status in transcript	Cisplatin sensitivity (IC_{50}) μM
UD-SCC 1	Wild type/AA25 Del 22 Nt	4.1
UD-SCC 2	Wild type	11.7
UD-SCC 7A	AA248 Arg → Leu	0.7
UD-SCC 7B	AA248 Arg → Leu	0.5
UM-SCC 10B	AA245 Gly → Cys	8.3
UM-SCC 17A	Wild type	8.3
UM-SCC 17B	Wild type	3.3
UT-SCC 9	Del exon 2–9	6.6
UT-SCC 24B	Wild type/AA225 Ins 48 Nt	5.7
UT-SCC 33	AA282 Arg → Trp	8.3

The p53 status was determined by sequence analysis of the entire coding region of p53 transcripts [44]. The colorimetric MTT assay was used to define the cisplatin IC_{50}. AA = Amino acid; Del = deletion; Ins = Insertion; Nt = nucleotide.

primers and reverse transcriptase (Sigma, Eggenstedt, Germany), and aliquots were subjected to semiquantitative RT-PCR analysis for p53. For analysis of p63 and p73, RT-PCR primers were designed to distinguish individual isoforms by amplificate length. For semi-quantitative analysis, we performed multiplex RT-PCRs to amplify all known isoforms in a single reaction. As control for cDNA quality as well as for normalization purposes, RT-PCR analysis for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. RT-PCR products were separated by gel electrophoresis and transferred to a nylon membrane (Applied Biosystems, Weiterstadt, Germany). Specific hybridization probes were synthesized by RT-PCR using the cDNA of cell line UD-SCC 7A, which expresses the isoforms of all p53 family members. Amplificates were labelled with alkaline phosphatase (AlkPhos labelling kit, Amersham, Freiburg, Germany), hybridized and visualized with CDP-Star (NEN, Cologne, Germany). Finally, size determination of the fragments and normalization to GAPDH expression was performed using a one-dimensional gel electrophoresis software (Aida Image Analyzer, Schwelm, Germany). This novel approach enables the simultaneous determination of all known p63 and p73 isoforms. The primers for RT-PCR reactions and generation of hybridization probes as well as RT-PCR conditions are listed in table 2. The localization of primers and hybridization probes is shown in figure 1.

Results

The sensitivity to cisplatin (IC_{50}) ranged between 0.5 and 11.7 μM, and this appeared to be independent of the p53 mutation status (table 1). The panel comprises 3 wild-type (UD-SCC 2, UM-SCC 17A and UM-SCC 17B) and

Table 2. Primer and PCR conditions for amplification of p53, p63, p73 and GAPDH transcripts and hybridization probes

Sense primer	Antisense primer	PCR conditions
p53 blot fragment		
p53 1-1: CCGGATCCGCGTGCTTTCCACGACG	p53 3-2: CGGAATTCCTGACGCACACCTATTGCAA	3 min 95 °C/5 × (30 s 95 °C, 30 s 70 °C, 70 s 72 °C)/5 × (30 s 95 °C, 30 s 64 °C, 70 s 72 °C)/15 × (30 s 95 °C, 30 s 66 °C, 70 s 72 °C)/5 min 72 °C
p53 hybridization probe		
p53 C1: CCGGATCCTTGCATTCTGGGACAGCCAA	p53 2-4: CGGAATTCTCAAAGCTGTTCCGTCCCAG	3 min 95 °C/35 × (30 s 95 °C, 30 s 66 °C, 45 s 72 °C)/5 min 72 °C
p63 blot fragments (multiplex RT-PCR)		
p63TAs: ATGTCCCAGAGCACACAG p63DNs: CAGACTCAATTTAGTGAG	p63αβas: CTCAGGGATTTTCAGACTTG p63γas: ACACTTGAGACCTTCGTTTC p63I10as: GAAAGAGGGTTGCCATACCA	3 min 95 °C/5 × (30 s 95 °C, 30 s 63 °C, 70 s 72 °C)/5 × (30 s 95 °C, 30 s 61 °C, 70 s 72 °C)/20 × (30 s 95 °C, 30 s 59 °C, 70 s 72 °C)/5 min 72°C
p63 hybridization probe		
Hybp63s: TTCGAGTAGAGGGGAACAGC	Hybp63as: CATCATCTGGGGATCTTCGT	3 min 95 °C/35 × (20 s 95 °C, 20 s 62 °C, 20 s 72 °C)/5 min 72 °C
p73 blot fragments (multiplex RT-PCR)		
p73TA3: ACGCAGCGAAACCGGGGC p73ΔN1: CCTCACTAGCTGCGGAGCCTCTC	p73as3: CTGCTGCGCGGTGCTGTAGTC	3 min 95 °C/5 × (30 s 95 °C, 70 s 72 °C)/5 × (30 s 95 °C, 30 s 70 °C, 70 s 72 °C)/26 × (30 s 95 °C, 30 s 68 °C, 70 s 72 °C)/5 min 72 °C
p73 hybridization probe (TA isoforms)		
Hybp73TAs: GCTGCGACGGCTGCAGAG	Hybp73TAas: AGAGAGCTCCAGAGGTGCTCAAAC	3 min 95 °C/35 × (20 s 95 °C, 20 s 57 °C, 20 s 72 °C)/5 min 72 °C

p73 hybridization probe (ΔN isoforms)		
Hybp73ΔNs: AAGCGAAAATGCCAACAAAC	Hybp73Δnas: CGACGTACAGCATGGTAG	3 min 95 °C/35 × (20 s 95 °C, 20 s 52 °C, 20 s 72 °C)/5 min 72 °C
p73 promoter analysis (multiplex RT-PCR)		
p73TAvars: GGACGGACGCCGATGCC p73ΔN1: CCTCACTAGCTGCGGAGCCTCTC	p73allas: GGAAAGTGACCTCAAAGTG	3 min 95 °C/29 × (15 s 95 °C, 15 s 56 °C, 45 s 72 °C)/7 min 72 °C
GAPDH blot fragment		
GAPDH Ges s: ACAGTCAGCCGCATCTTCTT	GAPDH Ges as: TTCCTCTTGTGCTCTTGCTG	3 min 95 °C/5 × (30 s 95 °C, 30 s 65 °C, 60 s 72 °C)/5 × (30 s 95 °C, 30 s 63 °C, 60 s 72 °C)/4 × (30 s 95 °C, 30 s 61 °C, 60 s 72 °C)/5 min 72 °C
GAPDH hybridization probe		
GAPDH-1: ACCACAGTCCATGCCATCAC	GAPDH-2: TCCACCACCCTGTTGCTGTA	3 min 95 °C/30 × (20 s 95 °C, 20 s 66 °C, 20 s 72 °C)/5 min 72 °C

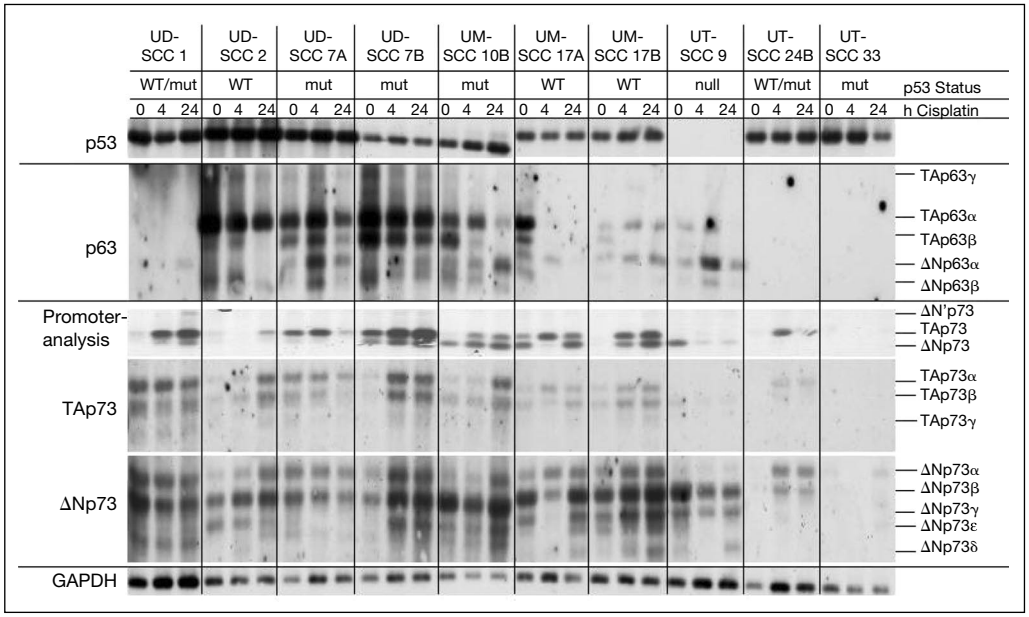

Fig. 3. Differential response of p53, p63 and p73 to cisplatin in 10 established cell lines of HNSCC. RNA was extracted from untreated cells (0 h) and cells exposed to cisplatin at the respective IC_{50} for 4 and 24 h. RT-PCR products of p53, p63 and p73 were separated in agarose gels, blotted on nylon membranes and hybridized with specific probes. While p53 and p63 expression was analysed using a single hybridization probe, analysis of p73 isoforms was subdivided into three steps: first, analysis of promoter activity and NH_2-terminal splice variants was performed with a multiplex RT-PCR covering the NH_2-terminal region of p73; second, a single multiplex RT-PCR produced amplification products which served for 2 identical nylon membranes; finally, the membranes were hybridized with TA-specific and ΔN-specific probes. RT-PCR amplificates for GAPDH served as loading control and for normalization purposes. WT = Wild-type p53 transcript; mut = mutated p53 transcript.

4 mutated cell lines (UD-SCC 7A, UD-SCC 7B, UM-SCC 10B and UT-SCC 33); 2 cell lines harbour mutated as well as wild-type p53 (UD-SCC 1 and UT-SCC 24B), and 1 cell line (UT-SCC 9) carries a homozygous deletion of p53 exons 2–9, resulting in loss of protein expression. Irrespective of the p53 status, the expression of p53 transcript was abundant except for the p53 null cell line UT-SCC 9 (fig. 3). Upon treatment with cisplatin, the levels of transcript remained unaffected in most cell lines. Normalization to GAPDH expression revealed minor up-regulation of p53 in cell lines expressing wild-type p53.

Expression of p63 was less uniform: 3 of the 10 HNSCC showed no p63 (UD-SCC 1, UT-SCC 24B and UT-SCC 33), and profiles of the remaining 7 cell lines showed considerable variations with regard to isoform composition

and intensity of expression. In most cases, the main isoforms were TAp63α and TAp63β (UD-SCC 2, UD-SCC 7A, UD-SCC 7B, UM-SCC 10B and UM-SCC 17A), whereas ΔNp63α and ΔNp63β were expressed to a much lesser extent. Cell lines UM-SCC 17B and UT-SCC 9 expressed only the α-isoforms of both TAp63 and ΔNp63.

Treatment with cisplatin (4 and 24 h) altered the expression levels but did not influence the characteristic isoform patterns in the majority of HNSCC. While expression of all isoforms was down-regulated after 4 h of exposure in UD-SCC 2, UD-SCC 7B, UM-SCC 10B and UM-SCC 17A, expression was initially up-regulated and subsequently down-regulated in UD-SCC 7A and UT-SCC 9. There was no correlation between p53 status and p63 expression; cell lines with mutated p53 behave quite differently with regard to p63 up- and down-regulation (e.g. UD-SCC 7A and UM-SCC 10B), and the same phenomenon was true for p53 wild-type HNSCC.

For p73, we determined the active promoters and NH_2-terminal splice variants prior to the analysis of alternative COOH-terminal isoforms. All cell lines with the exception of UT-SCC 33 expressed p73 transcripts. The majority of transcripts derived from TA- and ΔN-promoters encoding either full-length or ΔN isoforms. Only in 1 cell line (UD-SCC 1) ΔN′ transcripts were found, and neither Δexon 2 nor Δexon 2/3 transcripts were present. Closer analysis of transcripts derived from the TA promoter revealed expression of 2 major transcripts in most cell lines, TAp73α and TAp73β. In case of ΔN transcripts, the main isoforms were ΔNp73β and ΔNp73α. Occasionally, ΔNp73γ, ΔNp73δ and ΔNp73ε were also expressed.

Transcripts of both promoters were up-regulated by cisplatin in cell lines UD-SCC 1, UD-SCC 2, UD-SCC 7A, UD-SCC 7B, UM-SCC 10B and UM-SCC 17B, with a more pronounced effect for TA transcripts (UD-SCC 1, UD-SCC 7B and UM-SCC 17B). In UD-SCC 7A and UT-SCC 9, expression of p73 was down-regulated after cisplatin exposure. However, in cell lines showing either up- or down-regulation of p73 transcripts, the composition of COOH-terminal variants did not change. As for p63, the p53 status showed no correlation with p73 expression. Finally, the expression profiles of p63 and p73 appeared to be unrelated to cisplatin sensitivity and displayed independent drug responses.

Discussion

In order to define the complex expression pattern of p53, p63 and p73 in established cell lines of HNSCC, we performed an isoform-specific multiplex RT-PCR and identified resulting amplicons with the Southern technique. Regardless of the mutation status, p53 was found to be abundantly transcribed

in all cell lines but p53 null UT-SCC 9, and transcript levels remained more or less the same after exposure to cisplatin. This was to be expected, because drug-induced DNA damage causes activation and accumulation of p53 protein but not up-regulation of transcription [36].

Besides p53, various isoforms of p63 and p73 are constitutively expressed in these tumor cell lines. Coexistent expression of p63 and p73 in HNSCC was already demonstrated by immunohistochemistry employing pan-p63 and pan-73 antibodies [28, 37]. A more detailed RT-PCR analysis of p63 expression in carcinomas of the larynx looked for TA and ΔN transcripts: ΔNp63 mRNA was present in all 23 analysed specimens, and TAp63 transcripts were absent in 5 cases [21]. In the present tumour panel, 3 cell lines showed no detectable p63 expression, and 7 HNSCC exhibited considerable variations for both composition and intensity of p63 isoform expression. However, we generally found surplus TAp63, and TAp63α was the most prominent isoform in 5 HNSCC.

Sensitivity to cisplatin appeared to be independent of p63 expression. For example, UD-SCC 2 and UD-SCC 7B showed similar basal and stimulated expression patterns but differed substantially in their IC_{50} (11.7 vs. 0.5 μ*M*), and HNSCC lacking p63 expression featured average sensitivity (UD-SCC 1: 4.1 μ*M*, UT-SCC 24B: 5.7 μ*M*, and UT-SCC 33: 8.3 μ*M*). Studies in an immortalized mammary epithelial cell line revealed a dramatic decline in ΔNp63α transcript and protein upon cisplatin exposure, and this effect required p53 wild-type expression [38]. In our experimental system of HNSCC, drug exposure also affected p63 expression levels but neither altered the individual isoform pattern nor induced uniform up- or down-regulation. Moreover, we found no correlation with the p53 status.

Expression of p73 was regularly observed in HNSCC cell lines, although the level of expression was lower in comparison to p53 or p63, thus confirming earlier immunohistochemical studies in tumour specimens [28]. Several reports on p73 splice variants have shown that p73α and p73β are ubiquitously present in both normal tissues and cancers. The smaller splice variants, p73γ, δ, ε, ς and η, are expressed in some normal tissues and mostly derive from the TA promoter [39, 40]. They were reported to be up-regulated in ovarian cancer and haematopoietic malignancies [40, 41]. Our analysis showed the constitutive expression of α- and β-isoforms transcribed from both TA and ΔN promoters in the majority of HNSCC. Occasionally, ΔNp73γ and ΔNp73δ were present, though at lower levels. With regard to the NH_2 terminus, transcripts of TA and ΔN promoters were present in 9 of 10 cell lines; only 1 cell line also showed ΔN′ transcripts. Computer-assisted analysis of *p63* and *p73* promoters disclosed potential p53-binding sites in ΔN but not in TA promoter regions [42]. However, in reporter assays with exogenously expressed p53, only ΔNp73 was shown to be up-regulated by p53, whereas ΔNp63 transcription was repressed

[38, 42]. An up-regulation of ΔNp73 could indeed be demonstrated for cell lines UD-SCC 2 and UM-SCC 17B. In contrast, p53 wild-type UM-SCC 17A showed ΔNp73 down-regulation after 4 h and subsequent up-regulation, which may indicate a defect in p53 target activation. Transcripts of TAp73 promoters were up-regulated in most cell lines regardless of their p53 mutation status, suggesting a p53 independent response to cisplatin-induced DNA damage [30, 43]. As for p63, the individual p73 isoform composition was not markedly changed, and sensitivity to cisplatin did not appear to depend on the constitutive level of p73 expression.

In conclusion, we encountered a variety of expression patterns for the p53 family members p63 and p73 in a panel of 10 HNSCC cell lines. This may reflect a more or less stringent selection pressure for cancer cells to survive pro-apoptotic alterations during malignant transformation or is just the consequence of genetic instability. Exposure to the DNA-damaging agent cisplatin caused complex and difficult to interpret alterations of the p63 and p73 expression profiles. The differential and possibly independent regulation of the p53 family members suggests different biological roles as well as redundant pathways, which may be involved in the response of cancer cells to antineoplastic treatment.

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Expression of p16 Protein Is Associated with Human Papillomavirus Status in Tonsillar Carcinomas and Has Implications on Survival

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Abstract

Our recent analysis of papillomavirus (HPV) DNA in different malignant head and neck tumors revealed that HPV infections occurred most frequently in tonsillar carcinomas (58%) and that 84% of positive cases contained the highly oncogenic HPV type 16. We could also present data in favor of the hypothesis that in view of their clinical behavior and the involved risk factors HPV-positive and HPV-negative tonsillar carcinomas may represent two separate tumor entities. Looking for a surrogate marker, which in further epidemiological studies could replace the laborious and expensive HPV detection/typing we analyzed p16 protein expression in 34 tonsillar carcinomas for their correlation with HPV status. p16 is an inhibitor of cyclin-dependent kinases 4 and 6 which activate the negative cell cycle regulator protein pRB which in turn downregulates p16 expression. It could be shown that in neoplastic cells of the cervix uteri E7 protein of the high-risk HPVs can interfere with this regulatory circuit by its virtue to inactivate pRB and thus lead to the overexpession of p16. We found 53% of the tested tonsillar carcinomas to be HPV positive. 56% of all tumors tested were immunohistochemically positive for the p16 protein. In 16 of 18 of the HPV-positive carcinomas diffuse p16 expression was observed. In contrast, only 1 of the HPV-negative carcinomas showed focal p16 staining ($p < 0.001$). Clinical outcome analysis revealed a significant correlation of p16 expression with increased disease-free survival ($p = 0.02$). These data indicate that p16 is a technically simple immunohistological marker, applicable for routine pathological histology, and its prognostic value for survival is fully equivalent to HPV DNA detection.

p16 is a cyclin-dependent kinase inhibitor regulating the activity of CDK4 and CDK6. p16 is inactivated in many cancers by mutation, deletion or hypermethylation of the gene [1]. Loss of p16 protein leads to cell proliferation by indirectly suppressing hyperphosphorylation and functional inactivation of pRB [2, 3]. Cells with reduced or absent pRB proliferate even in the presence of very high levels of p16 [4]. In squamous cell carcinoma (SCC) of the cervix, mutations or homozygous deletions of p16 have been shown to be absent; however, immunohistochemical analysis revealed strong p16 overexpression in human-papillomavirus (HPV)-positive cervical carcinomas. Moreover, p16 was found to be a specific biomarker to identify dysplastic cervical keratinocytes in sections of cervical biopsy samples or cervical smears [5, 6]. These observations are in line with the hypothesis that the viral oncoprotein E7 blocks the RB protein [7].

For head and neck SCC (HNSCC), a high frequency of p16 inactivation has been reported. Abnormalities at the p16 gene locus on chromosome 9p21 were reported as early events in the development of HNSCC [8, 9]. Other reports favor transcriptional silencing of the p16 promoter by hypermethylation as dominant mechanism of inactivation of this tumor suppressor in HNSCC [10]. Loss of p16 expression determined by immunohistochemical analysis could be noted in 54% [11] to 82% [12] of the tumors and was associated with decreased survival [13].

Cancer of the oropharynx has emerged as the second type of mucosal neoplasia to be associated with HPV infection [14, 15]. We have recently reported on the prevalence of HPV DNA in a series of carefully stratified HNSCC [16]. These and prior results have shown that oncogenic HPVs (especially HPV-16) are present in about 20% of all HNSSCs and in nearly 60% of tonsillar cancers. We could demonstrate by laser-assisted microdissection and quantification of HPV-DNA that HPV DNA is consistently located in the tumor cells of tonsillar carcinomas and that HPV-16 loads are comparable to other HPV-associated SCCs [16]. First evaluations revealed the value to differentiate these tumors from the HPV-negative HNSCC as this has strong implications for prognosis [17] and risk factors [18]. It has been shown that the expression and function of cell cycle proteins in HPV-positive HNSCC is influenced by the HPV-related proteins [18–20]. There is accumulating evidence that HPV-positive tonsillar SCC may represent a separate tumor entity [21].

In the present study, we demonstrate the relationship between the expression of p16 and infection with HPV-16 in a series of tonsillar carcinomas. Moreover, our data suggest that p16/HPV-positive tumors have a favourable prognosis compared to the p16/HPV-negative cancers.

Materials and Methods

We investigated 34 patients with newly diagnosed SCC of the tonsils. Tumor specimens were obtained during surgery and were shock frozen in liquid nitrogen and stored at –80°C until further investigation. Tumor staging was performed according to the 2002 American Joint Committee on Cancer staging criteria [22]. After confirming integrity of DNA by β-globin gene PCR, HPV sequences were detected by nested PCR protocols with degenerate primers A10/A5-A6/A8 for group A (genital/mucosal) HPVs and CP62/70-CP65/69a for group B1 (cutaneous/EV) HPVs. PCR products were separated in 2% agarose gels and visualized by ethidium bromide staining. HPV typing was performed as previously described [16].

Five-micrometer sections of formalin-fixed and paraffin-embedded samples were dewaxed and rehydrated by a graded series of ethanol, followed by microwave treatment in 10 m*M* citrate buffer (pH 6.0). Endogenous peroxidase was inactivated by 3% H_2O_2 in phosphate-buffered saline (PBS, pH 7.4) for 10 min. After rinsing with PBS, nonspecific binding was blocked with 10% rabbit serum (Dako, Hamburg, Germany; 1:10 dilution) in PBS for 20 min. Sections were incubated at room temperature for 90 min with two different anti-p16 mouse monoclonal antibodies (16P04 and 16P07; NeoMarkers, Fremont, Calif., USA). After rinsing thoroughly with PBS, the slides were incubated with biotinylated goat antimouse antibody (Dako; 1:200 dilution) for 30 min. After washing with PBS, sections were incubated with avidin-biotin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, Calif., USA; 1:50) for 30 min. Visualization was performed with diaminobenzidine tetrahydrochloride (Vectastain ABC Kit, Vector Laboratories), and sections were counterstained with hematoxylin.

Strong nuclear staining as well as strong cytoplasmic staining were considered positive for p16 expression. Immunostaining was graded and scored on whole sections according to Klaes et al. [5] as follows: negative (<1% of the cells were positive), sporadic (isolated cells were positive, but <5%), focal (small cell clusters, but <25% of the cells were positive) and diffuse positive (>25% of the cells were stained). For survival analyses, immunoreacivity of more than 25% of the tumor cells were scored as p16 positive, less than 25% as p16 negative.

p16 and HPV status were analyzed using cross-tabulations and Fisher's exact test with the SPSS Base System, version 10.0.7 (SPSS, Chicago, Ill., USA). Disease-free survival and overall survival rates were estimated using the Kaplan-Meyer algorithm for incomplete observations. The disease-free survival was measured as the period of time between the date of diagnosis and the date of the last follow-up examination, where the patient was disease-free (censored) or the date of first recurrence independently if it was a local, regional or distant recurrence (uncensored). All patients were treated according to the same protocol and were included in disease-free survival analysis. The log rank test was used to test for differences between subgroups. All p values were considered statistically significant if less than or equal to 0.05.

Results

HPV sequences were detected in 18 out of 34 (53%) patients; 17/18 (94%) patients carried HPV-16 and 1 (6%) HPV-33 sequences. According to the criteria of Klaes et al. [5], 16/18 (89%) of the HPV-positive carcinomas showed diffuse p16 expression (table 1). In contrast, 15/16 (94%) of the HPV-negative cancers lacked any p16 immunoreactivity (table 1). The correlation of p16

Table 1. Distribution of p16 expression in relation to the HPV status

	Cases, n	Diffuse	Focal	Sporadic	Negative
HPV-positive	18	16 (89)	1 (6)	1 (6)	0 (0)
HPV-negative	16	0 (0)	1 (6)	0 (0)	15 (94)
Total	34	16 (47)	2 (6)	1 (3)	15 (44)

Figures in parentheses indicate percentages. Diffuse = More than 25% of the cells were stained; focal = less than 25% or small clusters were positive; sporadic = isolated cells, but less than 5% were positive; negative = less than 1% of the cells were positive, an overall percentage of more than 100 results from rounding.

expression and HPV status was highly significant ($p < 0.001$). Of the HPV-positive patients, 1 showed diffuse and 1 only sporadic p16 expression, respectively. However, these patients were shown to carry low amounts of viral DNA [19]. Notably, 1 single patient showed focal p16 expression, and no HPV DNA could be detected (table 1).

In sections graded as diffuse positive (table 1), p16 expression was seen in almost all neoplastic cells in the nuclei and also in the cytoplasm (fig. 1a). This pattern of p16 immunoreactivity could only be seen in neoplastic cells of HPV-positive tonsillar cancers. In general, surrounding stromal cells showed no p16 immunoreactivity. The pattern of p16 immunoreactivity of the HPV-positive tonsillar carcinomas was similar to that observed in HPV-positive cervical carcinomas, which were used as positive controls. In contrast, the HPV-negative tonsillar cancers showed by far no reactivity to p16 antibody (fig. 1b).

To determine the predictive value of the HPV status and p16 immunoreactivity for prognosis, we analyzed our 34 cases for disease-free survival. The median follow-up period was 33 months with a maximum of 57 months. The tumor stages of all cases in relation to HPV/p16 status and events are given in table 2. The distribution of tumor stages was similar between HPV/p16-positive and HPV/p16-negative cases. Nine of 34 (26%) of all patients died, and 15/34 patients (44%) had a tumor recurrence. Deaths were observed in equal proportions in the HPV/p16-positive and HPV/p16-negative groups. However, recurrences were observed significantly more frequently in HPV/p16-negative patients (3/16 patients) than in the HPV/p16-positive patients (10/16 patients; see table 2, $p = 0.028$, Fisher's exact test).

The 2 patients with HPV-positive/p16-negative tumors suffered from a tumor recurrence. Using the Kaplan-Meyer algorithm for the complete study sample, the 4-year disease-free survival was 33%. Using HPV/p16 positivity

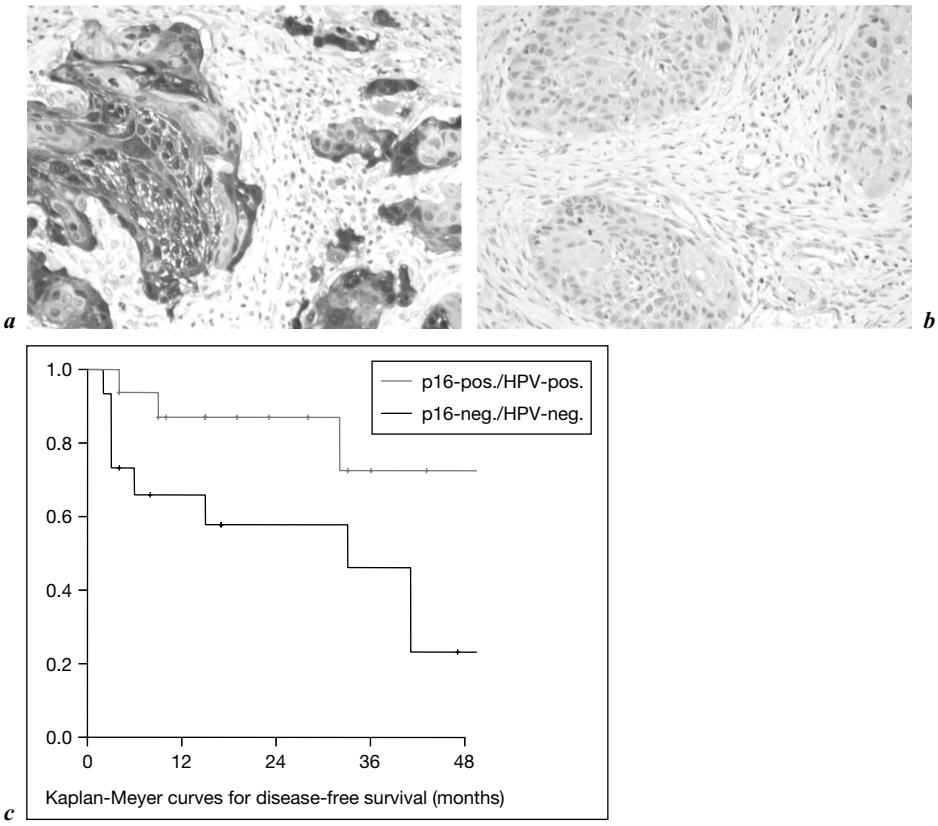

Fig. 1. ***a*** Immunohistochemical staining for p16. Positive staining for p16 is seen in nuclei and cytoplasm of tumor cells of an HPV-positive tonsillar carcinoma. ***b*** In an HPV-negative tonsillar carcinoma, all tumor cells are p16 negative. ***c*** Kaplan-Meyer curves for disease-free survival for p16-positive tonsillar carcinoma cases (gray) and p16-negative tonsillar carcinoma cases (black).

for stratification revealed a significant difference for disease-free survival curves between HPV/p16-positive and HPV/p16-negative patients (log rank test, $p = 0.02$, fig. 1c). The 4-year disease-free survival rates were 72% for HPV/p16-positive and 23% for HPV/p16-negative cases.

Discussion

The biological behavior and prognosis of HNSCC is very heterogeneous despite TMN staging [23]. Although many molecular predictors have been

Table 2. Tumor stage according to HPV/p16 status and events

Stage	HPV/p16-positive			HPV/p16-negative		
	total	R	D	total	R	D
	16 (100)	3/16 (19)	4/16 (25)	16 (100)	10/16 (63)	4/16 (25)
I	0 (0)	0	0	1 (6)	1	0
II	3 (19)	1	1	4 (25)	2	0
III	3 (19)	1	1	3 (19)	1	0
IVa	8 (50)	0	2	6 (38)	4	3
IVb	2 (13)	1	0	2 (13)	2	1

Figures in parentheses indicate percentages. R = Recurrence; D = deaths. There were no HPV-negative/p16-positive tumors. No patient had stage IVc disease. Immunoreactivity of more than 25% of the tumor cells was scored as p16 positive, less than 25% as p16 negative.

discussed to predict prognosis, no clear marker could be defined so far. Infection with oncogenic HPVs has recently been identified as risk factor for HNSCC [14, 16, 24, 25]. Patients with HPV-positive carcinomas tend to have significantly lower exposure to known risk factors for HNSCC [18]. Also serological studies have shown that HPV-16-seropositive subjects have an increased risk for the development of a tonsillar carcinoma [15, 26]. There is now increasing evidence that HPV-associated tonsillar carcinomas represent a different tumor entity [21], distinct from other HNSCCs in regard to risk factors and tumor biology [18]. Remarkably, improved survival of HPV-positive HNSCC patients has been shown [17], which might be the result of a higher tumor sensitivity to radiation therapy [27]. This is in accordance with the observation that HPV status and p53 mutations are inversely correlated [28, 29] since tumors with intact p53 are more susceptible to radiation-induced apoptosis [30].

For cancer of the cervix uteri it could be demonstrated that p16 is a specific biomarker to identify dysplastic cervical epithelia. These observations are in line with the hypothesis that the viral oncoprotein E7 blocks the RB protein [7] which leads to a loss of p16 inhibition by the RB protein. Using previously described grading criteria to evaluate p16 immunoreactivity [5] we were able to show a highly significant correlation of p16 and HPV DNA detection. Herewith we pointed out that p16 immunohistochemical staining of HNSCC may be used as a surrogate marker for HPV status also of carcinomas of tonsillar origin.

In this study, HPV/p16-positive tumors had a significantly lower recurrence rate than the HPV/p16-negative cases. In view of the rather small number of cases and the relatively short follow-up time, the true predictive power of

p16 immunostaining will have to be elucidated for larger cohorts of patients with a longer clinical follow-up.

In conclusion, p16 expression is highly correlated with HPV status in tonsillar cancers. p16 immunoreactivity is likely to result from transcriptionally active HPV infection, thus underlining the hypothesis that HPV-positive tonsillar carcinomas represent a biologically different tumor entity. The immunohistochemical detection of p16 expression might be a surrogate marker for transcriptionally active HPV infection. The analysis of tumor recurrence rates could depict a distinctly better prognosis for those patients showing p16 expression. If our results are confirmed in future studies, p16 could be a discriminator towards a better prognosis which will be easily applicable in routine pathology.

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EGFR-Antibody-Supplemented TPF Chemotherapy

Preclinical Investigations to a Novel Approach for Head and Neck Cancer Induction Treatment

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Abstract

Recent studies on polychemotherapy of head and neck cancer showed an improved remission rate on adding taxanes to the standard cytotoxic drugs cisplatin and 5-fluorouracil (5-FU). Moreover, for enhancing the response rate of chemotherapy today, a series of biological response modifiers are of interest, including modulators of the epidermal growth factor receptor (EGFR). Therefore we investigated whether the addition of monoclonal antibodies against the EGFR could enhance the response rate of cisplatin, 5-FU and docetaxel. Squamous cell cancer lines were transplanted into nude mice. After tumors had begun to grow, they were treated either with cisplatin, 5-FU or docetaxel alone or in combination with escalating doses of a humanized monoclonal anti-EGFR antibody.

Compared with controls, docetaxel alone as well as the combination of docetaxel, cisplatin and 5-fluorouracil (5-FU) resulted in a significant tumor growth delay. The antibody alone also slowed down the tumor growth significantly at each concentration. Nevertheless, neither chemotherapy agents nor antibody alone yielded complete tumor remissions over an observation period of up to 6 weeks. Only the combination of cisplatin/5-FU/docetaxel (TPF) and the antibody resulted in highly significant complete tumor remissions. Therefore we can show for the first time that the effect of TPF, which is now used as novel phase II protocol for induction chemotherapy in head and neck cancer, could be

highly significantly enhanced by the addition of antibodies against epidermal growth factor receptor (EGFR). Because we did not observe an increased toxicity in animal experiments, TPF/anti-EGFR therapy may define a new strategy in the induction treatment of head and neck carcinomas.

Patients with locally advanced squamous cell carcinoma of the head and neck (HNSCC) are usually treated by surgery and irradiation or by a combination of chemotherapy and radiotherapy. The latter therapeutic modality is especially used in patients with carcinoma of the larynx and hypopharynx who are treated for organ preservation. In this case, cisplatin/5-fluorouracil (PF)-based induction chemotherapy followed by radiation is highly active and can preserve the larynx in about 50% of patients and more [1].The original PF chemotherapy regimen results in major response rates of 69–90% and complete responses in the range of 20–50%. Recently published data show that the addition of taxanes to the PF regimen can raise the complete response up to 60% [2, 3]. More aggressive regimens adding leucovorin to the taxane/PF polychemotherapy result in a significantly higher toxicity [2]. Therefore physicians aspire to combine an effective and tolerable chemotherapy with a biological-response-modifying therapy with minor side effects.

Monoclonal antibodies against the EGFR have shown good tolerability in preclinical and clinical trials [4, 5]. However, as single agents they have not shown to date that effectiveness which we normally observe with standard therapies [6]. Therefore we combined, in a treatment protocol for nude mice, the most effective chemotherapeutic agents in head and neck cancer with EGFR antibodies. We wanted to elucidate if the additional application of the monoclonal antibody results in an altered tumor remission rate. Combining these therapies, we can demonstrate for the first time that the TPF combination with the anti-EGFR antibody EMD 72000 results not only in the normally observed tumor growth delay [4, 7], but in complete durable remissions. This may define a new strategy for induction chemotherapy of head and neck cancer.

Materials and Methods

Antibody

The murine antibody EMD 55900 and its humanized version EMD 72000 were generously provided by E. Merck KGaA (Darmstadt, Germany). The monoclonal antibody EMD 55900 was induced by immunization of BALB/c mice with cells of the human carcinoma cell line A431. Phase I clinical trials revealed that the immune systems of patients receiving EMD 55900 were inducing antibodies (human antimouse antibodies) against the drug because of its murine origin. For creating EMD 72000, mouse-complementary-determining regions were grafted from EMD 55900 into a human IgG1 framework. The monoclonal antibodies are highly specific for the human EGFR and recognize the deglycosylated region of

the EGFR Mr 110,000 EGF-binding domain, but not the other peptides of the EGFR. Binding of both antibodies correlates with the number of EGF-binding sites and is strongest with A431 carcinoma cell membranes. Scatchard's analyses of ^{125}I-labeled EMD 55900 binding to A431 membranes revealed the presence of 2 binding components: (a) a high-affinity, low-capacity component (K_d '10 p*M*, 5 3 108 sites/mg membrane protein) and (b) a relatively low-affinity, high-capacity component (K_d '1 n*M*, 2 3 1,010 sites/mg membrane protein). Plasma elimination half-lives of the murine (EMD 55900) and reshaped (EMD 72000) versions were similar: short in the *Cynomolgus* monkey (26 h for EMD 55900 and 31 h for EMD 72000) and long in rats (240 h for EMD 55900 and 225 h for EMD 72000). Biodistribution studies of ^{125}I-EMD 72000 in xenografted nude mice revealed a tumor:blood ratio of 1:2 on day 1 and 5:1 on day 18, respectively.

Chemotherapeutics

The chemotherapeutics were Taxotere (docetaxel) 20 mg (Aventis Pharma, Germany), Cisplatin 50 Hexal® PI and 5-FU Hexal 250 mg (Hexal AG, Germany). The dose of chemotherapeutics chosen for subcutaneous application in nude mice has been defined by the maximum tolerated dose (MTD) evaluated in prestudy investigations (docetaxel 20 mg/kg body weight, 5-FU 17 mg/kg body weight and cisplatin 6 mg/kg body weight).

Cell Lines, Culture Conditions, EGFR Western Blotting

The following cell lines were used for experiments: Detroit 562 (squamous cell carcinoma of the pharynx; American Type Culture Collection No. CRL-138), SCC 1623 (SCC of the tongue; American Type Culture Collection No. CRL-1623) and SCC 1624 (SCC of the tongue; American Type Culture Collection No. CRL-1624). The cell lines were cultured in Eagle's minimal essential medium (catalogue No. 4655, Sigma Aldrich). Cultures were maintained at 37 °C and 5% CO_2 in a humidified incubator.

Evaluation of the in vivo Antitumor Activity of Chemotherapeutics and Anti-EGFR Monoclonal Antibodies

For establishing cell lines as tumors on NMRI *nu/nu* mice (6–8 weeks of age), 2–3 × 10^7 cells of each cell line in 200 µl minimal essential medium were inoculated subcutaneously into both flanks of NMRI *nu/nu* mice (average body weight 25 g). After 7–14 days, when the tumors reached a size of 25 mm^2, the animals were randomly separated into 8 groups (10 animals per group) per experiment according to table 1.

Referring to the literature, dose escalation studies were undertaken for each chemotherapeutic drug to evaluate the LD_5/ED_{95} doses. Thereby the chemotherapeutic treatment schedule could be optimized to the MTD level. The dose for the antibody EMD 72000 was chosen according to our previous studies [7].

Treatment toxicity was evaluated by the number of animals who died during the observation period. Because with increasing tumor size animals in the control group die more and more from tumor burden, the experiments were closed on day 42 after treatment, and the animals were killed.

To determine the amount of proliferating cells in the transplanted tumors at the beginning of therapy, the Ki-67 index was determined in 2 tumors of each group by immunohistochemistry (Ki-67 antibody, catalogue No. 7187, Dako, diluted 1:25 in 5′ TBS buffer). In these tumors, also the EGFR content was measured by Western blotting to assure that EGFR density was not altered after transplantation.

Table 1. Treatment regimen of chemotherapeutics and EMD 72000 used in this investigation

After randomization	Treatment (i.p. on days 1 and 8)
Antibody dose 1	0.005 mg EMD 72000
Antibody dose 2	0.05 mg EMD 72000
Antibody dose 3	0.5 mg EMD 72000
Cisplatinum	6 mg/kg body weight
5-FU	17 mg/kg body weight
Docetaxel	20 mg/kg body weight
Antibody 1/CIS	0.005 mg EMD 72000 + 6 mg/kg body weight cisplatin
Antibody 2/CIS	0.05 mg EMD 72000 + 6 mg/kg body weight cisplatin
Antibody 3/CIS	0.5 mg EMD 72000 + 6 mg/kg body weight cisplatin
Antibody 1/5-FU	0.005 mg EMD 72000 + 17 mg/kg body weight 5-FU
Antibody 2/5-FU	0.05 mg EMD 72000 + 17 mg/kg body weight 5-FU
Antibody 3/5-FU	0.5 mg EMD 72000 + 17 mg/kg body weight 5-FU
Antibody 1/docetaxel	0.005 mg EMD 72000 + 2 mg/kg body weight
Antibody 2/docetaxel	0.05 mg EMD 72000 + 20 mg/kg body weight
Antibody 3/docetaxel	0.5 mg EMD 72000 + 20 mg/kg body weight
TPF	20 mg/kg body weight docetaxel + 6 mg/kg body weight cisplatin + 17 mg/kg body weight 5-FU
Antibody 1/TPF	0.005 mg EMD 72000 + TPF dose
Antibody 2/TPF	0.05 mg EMD 72000 + TPF dose
Antibody 3/TPF	0.5 mg EMD 72000 + TPF dose

All TPF drugs were administered subcutaneously.

The animals' condition and tumor development were registered daily according to a fixed schedule. Tumor sizes were measured with Vernier calipers as the product of the 2 longest right-angled diameters.

Statistics

Statistical analysis was performed using the SPSS 10.1 software. For statistical analysis, the Kruskal-Wallis test for *k*-independent samples as well as the Mann-Whitney U test were used because of nonparametric data; p values were considered to be significant at <0.05.

Results

EGFR Concentration of Tumors

EGFR concentrations of the cell lines were 115.6 fmol/mg (Detroit 562), 87.8 fmol/mg (SCC 1623) and 362.46 fmol/mg (SCC 1624). These concentrations are representative of HNSCC. After transplantation, measurements of EGFR concentrations in the developing tumors showed values within the normal variability of each cell line. Thus, transplantation did not alter the EGFR content of the tumors at the start of treatment.

Tumor Growth Rate at the Start of Treatment

At the start of treatment, tumors had a Ki-67 estimated median growth fraction of 69% with a range from 25 to 82%. But the majority were not in an exponential growth phase, which reflects the normal growth situation at treatment start in patients.

Tumor Growth Inhibition with EMD 72000 Is Dose Dependent

Dose escalation studies showed that tumor growth delay is dependent on the antibody concentration. Whereas a concentration of 0.005 mg/g body weight has only a slight, nonsignificant effect on tumor development, a concentration of 0.5 mg/g body weight leads to a highly significant growth delay. Tumor sizes of control versus treated groups 7 weeks after treatment were 300 and 50 mm^2 (fig. 1–3). Within this dose range we did not observe a treatment-related death quite in contrast to doses >1 mg antibody. Therefore we did not increase the antibody dose. These observations were made in all experiments.

Tumor Growth Inhibition by Chemotherapy Is Most Effective with a Combination of Docetaxel, Cisplatin and 5-FU

Monotherapy with docetaxel, cisplatin and 5-FU at the MTD did not yield a significant tumor growth retardation during the observation period of 7 weeks. In contrast, the combination of the drugs, TPF, led to a significant growth delay at the MTD (fig. 4). These results could be verified with all 3 transplanted cell lines. Moreover complete tumor eradication was only observed in the TPF-treated animals (fig. 5).

Antibody-Supplemented Polychemotherapy Results

When EMD 72000 treatment was combined with single chemotherapeutic agents, we observed, mainly at dose levels of 0.05 and 0.5 mg/g, a significant tumor growth retardation throughout, which is in the range of the EMD 72000 effect alone (fig. 1–3). In contrast, only EMD 72000 in combination with TPF polychemotherapy resulted in a significant additional growth inhibition up to a complete tumor eradication in the high-dose group (0.5 mg/g; fig. 4).

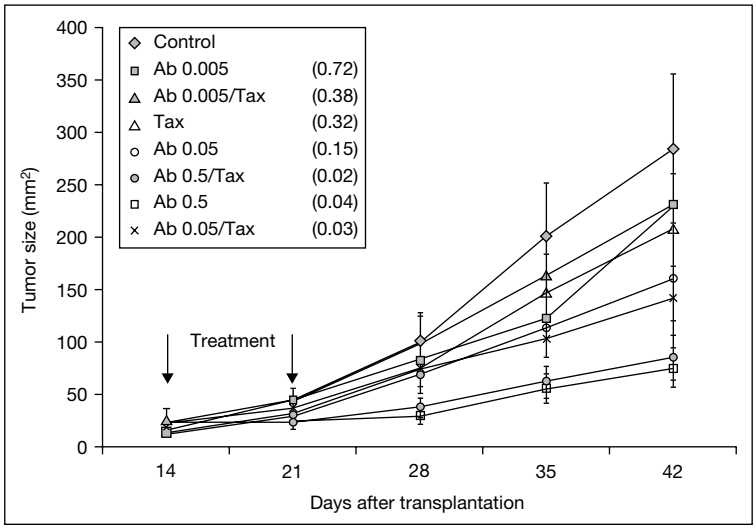

Fig. 1. The effect of EMD 72000 (Ab) in combination with docetaxel (Tax) on the growth of a human tumor xenograft (Detroit 562) with high EGFR concentration (300 fmol/mg). Tumors of 2 mm in diameter were transplanted subcutaneously into nude mice (n = 10/treatment group). Treatment was started when tumors reached a mean size of 25 mm^2. EMD 72000 was given intraperitoneally at 3 dose levels (0.5, 0.05, 0.005 mg). Docetaxel was given at an MTD level (see text). The figure shows tumor growth of each treatment group over 42 days after transplantation. The p values for differences between control and treatment groups are listed in parentheses. Control mice received the vehicle. The data represent the mean tumor size ± SE.

The effects of antibody-combined polychemotherapy in the tumors derived from different cell lines are illustrated in figure 5. It shows that a complete tumor response according to the WHO criteria was mainly observed in the EMD 72000/TPF treatment groups.

MTD Is Not Reduced when EMD 72000 Is Added to TPF

Comparing death rates of animals in the TPF group versus TPF/antibody group, we did not observe a significantly higher mortality in the latter. The death rate was at maximum 2 animals in each group.

Discussion

While surgery and/or radiotherapy remain cornerstones in the treatment of head and neck cancer, induction chemotherapy and chemoradiotherapy are now

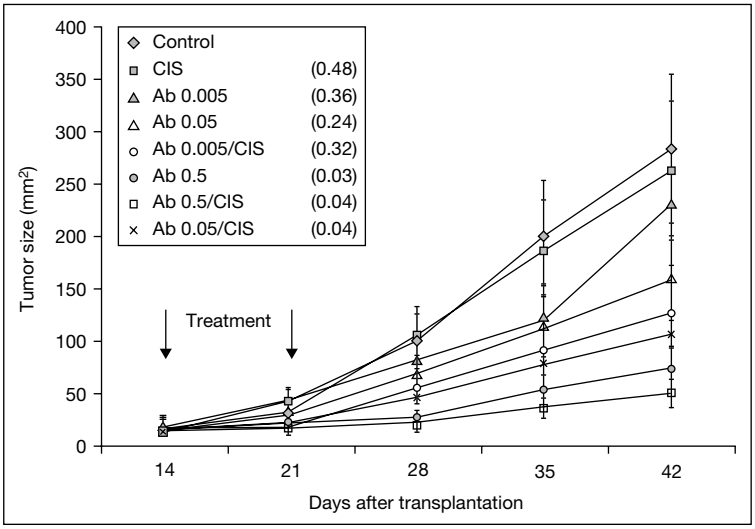

Fig. 2. The effect of EMD 72000 (Ab) in combination with cisplatin (CIS) on the growth of a human tumor xenograft (Detroit 562) with high EGFR concentration (300 fmol/mg). Tumors of 2 mm in diameter were transplanted subcutaneously into nude mice (n = 10/treatment group). Treatment was started when tumors reached a mean size of 25 mm^2. EMD 72000 was given intraperitoneally at 3 dose levels (0.5, 0.05, 0.005 mg). Cisplatin was given at an MTD level (see text). The figure shows tumor growth of each treatment group over 42 days after transplantation. The p values for differences between control and treatment groups are listed in parentheses. Control mice received the vehicle. The data represent the mean tumor size ± SE.

firmly integrated in the treatment plans. Induction chemotherapy is mainly used in organ preservation protocols for locally advanced HNSCC [8] normally followed by radiotherapy. Cisplatin and 5-FU (PF regimen) are the standard therapeutics for induction therapy yielding up to 40% complete and up to 85% overall responses in chemonaive advanced cancer [9]. Recently taxanes have been added to the cisplatin/5-FU regimen (TPF) in phase II protocols resulting in an improved rate of complete responses and overall response rates over 90% [10]. Regarding the excellent 3-year survival rates in combination with radiotherapy, some authors suggest that TPF-based regimens could lead to an improvement over PF regimens [2]. However, as with PF induction therapy, locoregional failure remains the major problem [11]. The need for further improvements in HNSCC care has stimulated intensive investigations into new agents with potential in induction chemotherapy and chemoradiotherapy regimens. New agents modifying EGFR signaling are of interest because EGFR plays a key role in the development of head and neck cancer and is overexpressed

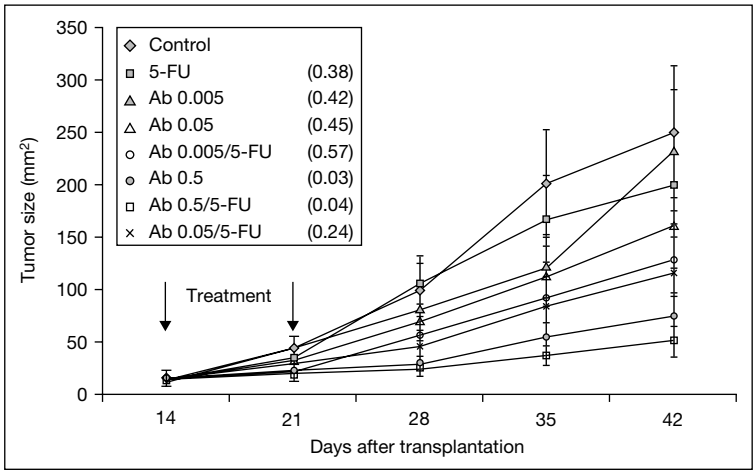

Fig. 3. The effect of EMD 72000 (Ab) in combination with 5-FU on the growth of a human tumor xenograft (Detroit 562) with high EGFR concentration (300 fmol/mg). Tumors of 2 mm in diameter were transplanted subcutaneously into nude mice (n = 10/treatment group). Treatment was started when tumors reached a mean size of 25 mm^2. EMD 72000 was given intraperitoneally at 3 dose levels (0.5, 0.05, 0.005 mg). 5-FU was given at an MTD level (see text). The figure shows tumor growth of each treatment group over 42 days after transplantation. The p values for differences between control and treatment groups are listed in parentheses. Control mice received the vehicle. The data represent the mean tumor size ± SE.

over a long time in the tumor evolution. There have been preclinical studies showing that targeting the overexpressed EGFR with monoclonal antibodies leads to a reduction of tumor growth in vivo [7, 12–17]. Moreover, a few phase I/II clinical studies in head and neck cancer have been completed or are under way, which demonstrate tumor remissions during anti-EGFR therapy [5, 18]. Because anti-EGFR treatment is thereby mostly conducted in the form of a second- or third-line therapy and not as part of an induction treatment protocol, the full potential in therapy-naive tumors cannot be evaluated. Moreover combinations of chemotherapy and anti-EGFR therapy in preclinical and clinical studies were mainly conducted with single-agent chemotherapeutics [19, 20]. Therefore the aim of our study was to combine a highly effective poly-chemotherapy with an EGFR antibody therapy in chemonaive tumors.

We chose cell lines with representative EGFR concentrations for head and neck cancer which are stable after transplantation. The doses of the chemotherapeutics and the monoclonal antibody were adapted to the tolerability observed in nude mice and cannot be transferred to the human system. Nevertheless, the

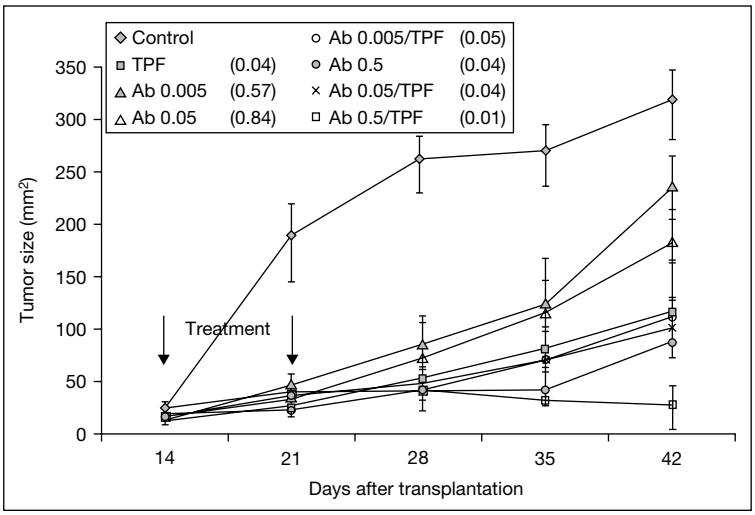

Fig. 4. The effect of treatment with EMD 72000 (Ab) added to chemotherapy (TPF) on the growth of a human tumor xenograft (Detroit 562) with high EGFR concentration (300 fmol/mg). Tumors of 2 mm in diameter were transplanted subcutaneously into nude mice (n = 10/treatment group). Treatment was started when tumors reached a mean size of 25 mm^2. EMD 72000 was given intraperitoneally at 3 dose levels (0.5, 0.05, 0.005 mg). TPF was given at an MTD level (see text). The figure shows tumor growth of each treatment group over 42 days after transplantation. The p values for differences between control and treatment groups are listed in parentheses. Control mice received the vehicle. The data represent the mean tumor size ± SE.

MTDs were given and distributed on a 2-day schedule (day 1 and day 8), which is more tolerable than a single-day schedule. In comparison with the human system, these schedules are the equivalent of one cycle of chemotherapy at the MTD level. We did not repeat the cycles during the observation period because the intention of the study was that of a proof of principle. Moreover, the sample (animals) size in each group must be greatly increased to repeat cycles because of the dropout rate of this species under repeated cytotoxic or cytostatic therapies. This choice of only one treatment cycle may be the reason for the seldom seen complete tumor eradication under single chemotherapy, polychemotherapy (TPF) or antibody therapy alone. But the combination of TPF polychemotherapy with the EMD 72000 antibody therapy clearly demonstrates that one treatment cycle can result in a complete tumor eradication in a large number of animals without a significant elevation of life-threatening toxicities. Translating these results to the human system, we therefore expect that the addition of EMD 72000 to the TPF schedule used in phase II studies further

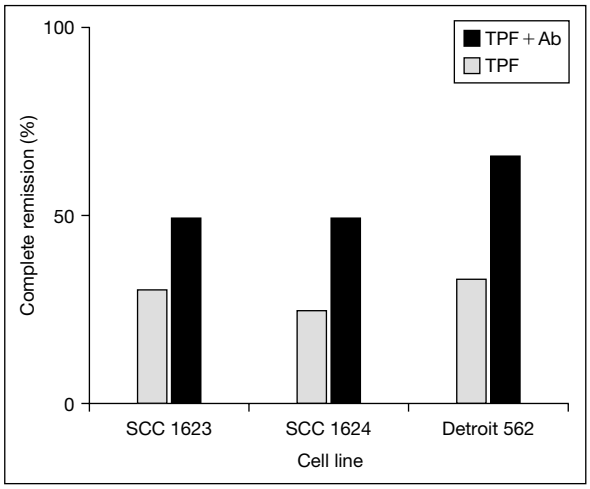

Fig. 5. The effect of additional treatment with EMD 72000 (Ab) on the response rate of 3 different human tumor xenografts with high EGFR concentration. Tumors of 2 mm in diameter were transplanted subcutaneously into nude mice (n = 10/treatment group). Treatment was started when tumors reached a mean size of 25 mm^2. EMD 72000 was given intraperitoneally at 3 dose levels (0.5, 0.05, 0.005 mg). TPF chemotherapy was given at an MTD level (see text). Control mice received the vehicle. The data represent percentages of mice having complete remissions.

enhances the high response rate in chemonaive tumors. For this reason, we propose this EGFR antibody-supplemented TPF polychemotherapy as the basis for the development of a new induction treatment protocol in head and neck cancer.

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Nuclear Factor-κB as a Common Target and Activator of Oncogenes in Head and Neck Squamous Cell Carcinoma

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Abstract

Head and neck squamous cell carcinomas exhibit alterations in cell proliferation, survival (apoptosis), migration, angiogenesis and inflammation. The transcription factor nuclear factor-κB integrates multiple signals and regulates expression of multiple genes invovled in these phenotypic responses, suggesting the hypothesis that nuclear factor-κB is an important molecular switch for development of head and neck squamous cell carcinoma. Nuclear factor-κB has been found to be constitutively activated, and a common target and activator of oncogenes in cancer. Because of its important role, activation of nuclear factor-κB by the proteasome and other signal molecules may provide targets for molecular therapy of squamous cell carcinoma and other cancers.

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and accounts for 90% of cancers arising in the upper aerodigestive tract. Approximately 363,000 new cases of oral and pharyngeal cancer are diagnosed annually worldwide, and almost 200,000 deaths occur each year [1]. Despite progress in refining surgical, radio- and chemotherapeutic approaches, long-term survival rates in patients with HNSCC have not improved over the past 25 years [2].

The transcription factor nuclear factor κB (NF-κB) is well recognized as a regulator of genes encoding cytokines, chemokines and cell adhesion molecules important in immune and inflammatory responses [3], as well as genes critical in the control of cellular proliferation and apoptosis [4]. Identification of multiple molecules related to NF-κB in HNSCC led to the discovery that NF-κB is a

common target and activator of oncogenes important in the development and progression of HNSCC. Activation of NF-κB has been associated with several aspects of oncogenesis, including the control of apoptosis, the cell cycle, differentiation and cell migration [4]. NF-κB activation in tumor cells by chemotherapy or radiation has been shown to blunt the ability of cancer therapy to induce cell death [5].

Understanding the molecular mechanisms involved in tumor development and progression has enabled the design of new biological approaches that may help diversify the standard treatments of cancer. Potential therapeutic agents are now being developed that target NF-κB and its downstream genes. Such agents include the proteasome inhibitor bortezomib (Velcade™; formerly PS-341), which is currently being studied in a phase I clinical trial for patients with recurrent and/or metastatic HNSCC.

NF-κB and Oncogenesis

NF-κB was originally identified as a nuclear factor that activated transcription of antibody κ-chain genes in B lymphocytes, but has since been found to be expressed in all cells, where it plays an essential role in coordinating cellular and host defenses. NF-κB was first implicated in oncogenesis when a retrovirus that causes avian lymphoid malignancies was found to encode a transforming NF-κB-related gene, designated v-Rel [6]. Aberrant activation of NF-κB has now been found in a variety of malignancies, including HNSCC [7], pancreatic cancer [8], colon cancer [9], breast cancer [10, 11], T cell leukemia [12] and Hodgkin's and B cell lymphomas [13, 14].

The common activation of NF-κB in cancer is likely to be related to its central role in integrating protective responses to molecular damage by injury and pathogens (fig. 1). The NF-κB family of transcription factors in mammalian cells is comprised of 5 members: p50/p105 (NF-κB1), p52/p100 (NF-κB2), c-Rel, c-RelB and p65 (RelA), which are assembled into homo- and heterodimers. The p50/p65 heterodimer has been found to play an important role in cellular responses. In normal cells, NF-κB complexes are chiefly cytoplasmic and are transcriptionally inactive until a cell receives an appropriate stimulus. NF-κB may be activated by the molecular signatures of a variety of pathogens, including bacterial lipopolysaccharides, fungi and viruses, and may be directly activated by genes encoded by Epstein-Barr virus and human immunodeficiency virus. It may also be activated as a result of damage to the cell or DNA by trauma, chemical toxins or radiation. In addition, NF-κB activation may be elicited adjacent or distant to the inciting injury by proinflammatory cytokines such as tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) as well as growth factors including epidermal growth factor (EGF).

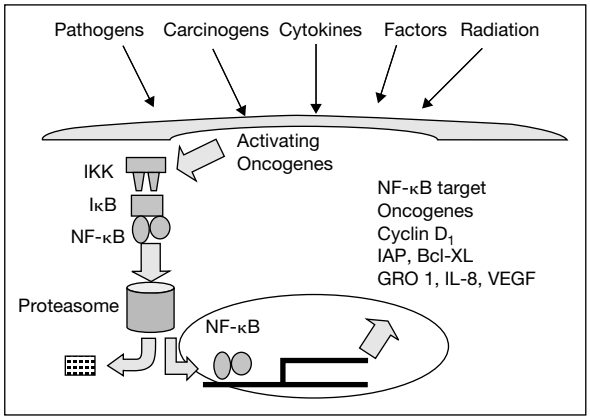

Fig. 1. NF-κB in response to injury and as a target and activator of oncogenes in cancer. NF-κB may be stimulated by pathogens, carcinogens and chemical toxins, cytokines, growth factors and radiation. It may be activated constitutively by stimulating oncogenes expressed in transformed cells. NF-κB activation results from signals involving inhibitor-κB kinase (IKK), which phosphorylates inhibitor-κB (IκB), marking it for ubiquitination and degradation by the proteasome. IκB degradation liberates NF-κB to localize to the nucleus, resulting in the transcription of a variety of target genes or oncogenes involved in proliferation (cyclin D_1), decreased cell death (IAP, Bcl-XL), inflammation and angiogenesis (GRO-1, IL-8, VEGF).

Among these stimuli, Epstein-Barr virus, IL-1 and EGF have been linked to the oncogenesis and activation of NF-κB in HNSCC [15–17]. Thus, a variety of viruses, chemicals, cytokines and growth factors are implicated in NF-κB activation and the development of HNSCC and other cancers.

The activation of NF-κB as classically described is illustrated in figure 1. The various signals converge on and activate the inhibitor-κB kinase (IKK) complex, consisting of IKKα, IKKβ, IKKγ and IKK-complex-associated protein. Inhibitor-κB (IκB), which binds with and inhibits NF-κB nuclear localization and DNA binding, is phosphorylated by IKK, thereby resulting in rapid ubiquitination and subsequent proteolysis by the 26S proteasome. Proteasome-dependent degradation of the IκB proteins results in the liberation of NF-κB and its accumulation in the nucleus where it binds DNA and activates the promoter of specific genes involved in cell growth control, apoptosis and immune and inflammatory responses.

NF-κB promotes the expression of over 150 target genes, the majority of which are involved in growth control, cell survival and resistance, and the host immune response [18] (table 1). It acts in cell cycle control, which is critical in determining the degree of cellular proliferation in response to injury or in

Table 1. Genes regulated by NF-κB

Category	Gene	Function
Cell cycle and apoptosis	*Cyclin* D_1 *IAP1* and *IAP2* *TRAF1* and *TRAF2* *Bcl-2* family *Bcl-2* *Bcl-XL* *Bfl-1/A1*	Cellular proliferation and apoptosis
Proinflammatory and proangiogenic cytokines and growth factors	*TNF-α* *IL-1*, *-2*, *-3*, *-6*, *-8*, *-12* *VEGF* *GRO-1*	Inflammation and angiogenesis
Adhesion molecules and proteases	*ICAM-1* *MMP* *Plasminogen activator* *Heparanase*	Cell adhesion and migration

VEGF = Vascular endothelial growth factor; GRO-1 = growth-regulated oncogene 1; ICAM-1 = intercellular adhesion molecule 1; MMP = matrix metalloproteinase.

carcinogenesis. NF-κB activates the expression of cyclin D_1 – a positive regulator of G_1-to-S phase progression, by directly binding to multiple sites in its promoter. Inhibitors of NF-κB activation decrease cyclin D_1 protein and subsequent phosphorylation of the retinoblastoma (Rb) protein, thereby resulting in delayed cell cycle progression [19]. Importantly, CGNDS D_1 is commonly overexpressed and plays a significant role as an oncogene in HNSCC, as well as other cancers [20].

NF-κB is also an essential regulator of genes involved in the control of cellular death (apoptosis), another important feature that is altered in HNSCC and other cancers. Antiapoptotic genes that are directly activated by NF-κB include the cellular inhibitors of apoptosis (*c-IAP1*, *c-IAP2*, *IXAP*), the TNF-receptor-associated factors (*TRAF1* and *TRAF2*), the *Bcl-2* homologues *A1*/*Bfl-1*, *IEX-IL* and *Bcl-XL*. *Bcl-XL* and *IAP1* have been shown to be expressed by human HNSCC and murine squamous cell carcinoma (SCC) [21, 22]. One of the best-studied mechanisms in which NF-κB can inhibit apoptosis involves NF-κB activation following TNF-α treatment in cells. Treatment with TNF-α induces NF-κB and *TRAF1*, *TRAF2*, *c-IAP1* and *c-IAP2* expression. Upregulating these proteins can protect NF-κB-deficient cells, which are highly sensitive to TNF-α-induced apoptosis, from cell death. These antiapoptotic proteins inhibit activation of caspase 8, an initiator protease involved in mediating apoptosis [23–26].

Moreover, NF-κB activity may provide additional selective benefits in locally advanced and metastatic tumors through expression of several NF-κB-dependent cytokines and angiogenic factors. Cytokines that are stimulated by NF-κB, such as IL-1β and TNF-α, can also directly activate the NF-κB pathway, thereby promoting a positive autoregulatory loop that can amplify NF-κB activation. Extensive angiogenesis, which ensures an adequate supply of nutrients and growth stimuli to tumor cells, is crucial to invasive tumors and metastases. Vascular endothelial growth factor (VEGF), a dominant regulator of angiogenesis, is partially regulated by NF-κB [27]. Likewise, IL-8 and growth-regulated oncogene 1 (GRO-1), which promote angiogenesis and metastasis, are also regulated by NF-κB [28, 29].

Invasion and metastasis in advanced tumors are also promoted by NF-κB through the activation of matrix metalloproteinases, plasminogen activator and heparanase [30]. Additionally, NF-κB-induced secreted factors, including cytokines, may facilitate stimulation of prometastatic proteases in surrounding tumor stroma [31]. Adhesion molecules also mediate local and distant invasiveness and participate in transducing growth and proliferation signals. NF-κB controls numerous adhesion molecules, among them intercellular adhesion molecule 1, which is overexpressed in some tumors and linked with poor prognosis [32].

Role of NF-κB in Malignant Behavior and Gene Expression by HNSCC

HNSCCs have been noted to induce strong host inflammatory and angiogenic responses, which are important in tumorigenesis and metastasis. Investigation of the molecular factors expressed by SCC that promote inflammation and angiogenesis resulted in the identification of a repertoire of proinflammatory cytokines and angiogenic factors secreted and detected in the serum and tumor specimens of patients [33, 34]. These include the proinflammatory cytokines IL-1α, IL-6, IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF) and proangiogenic cytokines VEGF and basic fibroblast growth factor. In a murine model of SCC, tumors which expressed IL-1α, IL-6, the IL-8 homologue GRO-1 and GM-CSF at increased levels exhibited more aggressive growth and metastasis in vivo [35, 36]. GRO-1 was shown to be an important factor in the promotion of tumor growth and the development of metastases in vivo [37]. A search for a common regulatory mechanism to explain the coexpression of these factors revealed that expression of several of these factors is regulated by NF-κB p50/p65 RelA [7].

The 5′ promoter regions of the *IL-1α*, *IL-6*, *IL-8*, *GRO-1* and *GM-CSF* genes expressed by HNSCC contain binding sites for the NF-κB family of transcription factors, and autonomous expression of these cytokines in HNSCC and murine SCC was found to correlate with constitutive activation of NF-κB p50/p65 [7, 38]. Blockade of NF-κB activation by transfection of phosphorylation and ubiquitination site mutants of IκB resulted in inhibition of cytokine expression and tumorigenesis [39].

In a murine SCC model that shows many similarities in behavior and expression of cytokines to human HNSCC, broader profiling of gene expression by microarray revealed that up to 40% of genes expressed with metastatic tumor progression are related to the NF-κB pathway [40]. These included the proliferative and antiapoptotic genes *cyclin* D_1 and *IAP1*, and angiogenic and inflammatory factors such as *GRO-1*.

Consistent with its role in cell survival, most HNSCC cells transfected with a dominant-negative inhibitor of NF-κB showed a 70–90% reduction in cell viability, providing evidence for the role of NF-κB in proliferation and antiapoptotic activity in HNSCC [39]. Constitutive NF-κB activity was also found to have influence on the sensitivity of HNSCC to TNF-α and radioresistance of HNSCC cell lines. TNF-α resistance of HNSCC cell lines was attenuated by NF-κB inhibition [39], and radiation resistance of in vitro HNSCC cell lines correlated with increased constitutive activation of NF-κB [41]. Moreover, HNSCC could be sensitized to radiation by the dominant-negative inhibitor of NF-κB [41]. Didelot et al. [42] also showed that inhibition of NF-κB activity by dexamethasone led to a significant increase in radiosensitivity in HNSCC cell lines. These results provide evidence that NF-κB may be an important target for therapy of HNSCC.

Although the exact mechanism by which HNSCC cells acquire constitutive NF-κB activity has yet to be completely delineated, autocrine expression of IL-1 and EGF receptor have been shown to contribute to activation of NF-κB in HNSCC [16, 17]. Several laboratories have also reported that SCC of the oral cavity express higher levels of the NF-κB p65 subunit and its upstream kinase IKKα when compared to that of normal squamous epithelia and epithelial dysplasia [43, 44]. Moreover, the invasiveness and metastasis of SCC were observed to correlate with the degree of immunohistochemical staining of these molecules, suggesting that high expression of NF-κB and IKKα may contribute to the malignant behavior and antiapoptotic activity of SCC [43]. Others have also shown that HNSCC cell lines have significantly higher NF-κB binding activity and IKK activity compared to normal oral epithelial and salivary gland cells, implying that enhanced NF-κB activity could be a result of increased IKK activity [44]. As a result of their central role in NF-κB activation, the proteasome and IKK complex have been initial targets of drug discovery efforts for therapeutics directed at NF-κB in cancer and inflammation.

NF-κB as a Therapeutic Target in Cancer and HNSCC

The breadth of NF-κB activity has led to intense interest in the potential role of NF-κB in tumorigenesis, metastasis, drug and radiation resistance, and as a therapeutic target. The activation of NF-κB can lead to resistance to cell death ordinarily induced by radiation and a variety of chemotherapeutic agents. Increased understanding of the mechanism of NF-κB activation has led to several trials using gene therapy, peptides, small molecules and proteasome inhibitors. Several antioxidants, natural products, salicylates and nonsteroidal anti-inflammatory drugs have been shown to inhibit NF-κB [45].

One approach to blocking NF-κB activation is through proteasome inhibition. Proteasome inhibitors are known to inhibit degradation of IκB, halt cell cycle progression and induce apoptosis. In addition to its effects on NF-κB, these inhibitors are also likely to exert additional effects via stabilizing cell-cycle-inhibitory proteins like cyclin-dependent kinase inhibitors and p53 [46]. The proteasome inhibitor bortezomib is a novel dipeptide boronic acid small molecule that has shown antitumor activity in preclinical studies and is the first such agent to have progressed to clinical trials. In a study of HNSCC, bortezomib inhibited the activation of NF-κB DNA binding and functional reporter activity at concentrations between 10^{-8} and 10^{-7} *M* in vitro. Bortezomib inhibited tumor growth of murine SCC and human HNSCC xenografts in mice at doses of 1–2 mg/kg administered 3 times per week for 25 days. Bortezomib also exhibited antiangiogenic properties: tumor growth inhibition was correlated with decreased blood vessel density, and the proangiogenic cytokines GRO-1 and VEGF were decreased in a dose-dependent manner [47]. Moreover, exposure to bortezomib can induce cytotoxicity and further sensitize HNSCC to cytotoxic effects of radiation in vitro in association with cell cycle block in the G_2/M phase and inhibition of the NF-κB prosurvival pathway [48]. Based on these cytotoxic, antiangiogenic and radiation-sensitizing activities, there is currently a phase I trial under way using bortezomib with concurrent radiation therapy in patients with recurrent and/or metastatic HNSCC.

Conclusion

NF-κB is an extensively studied transcription factor shown to be constitutively activated in HNSCC. Traditional therapies for advanced HNSCC currently include surgery, radiation and combined chemoradiotherapy, with little improvement in the outcome over the last few decades. Emerging evidence

demonstrates that inhibition of the NF-κB pathway and its downstream gene expression may offer new therapeutic options. In addition, inhibitors of NF-κB may be synergistic with chemotherapy and/or radiation. As NF-κB inhibition significantly increases cancer cell death in response to cytotoxic drugs and radiation, this may allow for a reduction in the current recommended dosages, thus potentially providing for more effective, less toxic therapies. Further, the ability to suppress NF-κB activity could render tumors sensitive to nontoxic agents, leading to the development and use of new drugs against cancer. Additional studies are required to determine the role of NF-κB targeting in the treatment of HNSCC. Finally, a better understanding of the regulation of the NF-κB signaling pathway may provide opportunities for the development of new chemopreventive approaches to inhibit prolonged activation of this pathway.

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Antiangiogenic Therapy of Head and Neck Squamous Cell Carcinoma by Vascular Endothelial Growth Factor Antisense Therapy

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Abstract

Angiogenesis is increased in various human cancers, including head and neck squamous cell carcinoma (HNSCC), and correlates with tumor progression and metastasis. Vascular endothelial growth factor (VEGF) has been shown to be a key regulator of angiogenesis. We determined whether VEGF antisense oligonucleotide treatment can decrease the angiogenic activity of HNSCC cell lines in vitro and of HNSCC xenografts in vivo. Established human HNSCC cell lines were screened for VEGF expression at both mRNA and protein levels. By using a 21-mer antisense phosphorothioate oligonucleotide targeting the translation start site of human VEGF mRNA, we examined the modulation of VEGF expression in cell line supernatants by capture ELISA and in cell lysates by Western blotting. Human endothelial cells were grown in conditioned medium produced from the treated tumor cells. Endothelial cell proliferation was determined by cell count, and endothelial cell migration was measured using a modified Boyden chamber. Mice with HNSCC xenografts were treated with PBS, VEGF antisense or sense oligonucleotides (10 mg/kg i.p. injection, 3 times/week), respectively, and tumor volumes were measured for 5 weeks. VEGF antisense oligonucleotide treatment resulted in a significant reduction of VEGF protein expression compared to treatment with the sense control. Although the growth rate of the tumor cell lines was not affected, the addition of conditioned medium from VEGF antisense-treated tumor cells resulted in decreased endothelial cell proliferation and migration. VEGF antisense oligonucleotide treatment of HNSCC xenografts resulted in a significant tumor growth suppression. These results suggest that downmodulation of VEGF using antisense oligonucleotides may be a potential therapy for the inhibition of angiogenesis in HNSCC.

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is the most common neoplasm arising in the upper aerodigestive tract. At current rates, approximately 50,000 new cases of HNSCC are reported annually in the USA, and more than 500,000 cases worldwide are diagnosed every year [1]. The prevalence of HNSCC is increasing worldwide [2]. Improved techniques in surgery, radiation and chemotherapy have increased the local control of HNSCC, but the overall survival rates have not changed significantly in 25 years [3]. Despite optimal therapy, the overall survival rate is poor, with a 40% 5-year survival for those with resectable and a 20% survival rate for patients whose tumors are not amenable to excision [3]. More than two thirds of the patients with HNSCC present with advanced disease (stage III and stage IV) where half of those patients will go on to develop local recurrences and 30% or more will develop distant metastasis [4]. Chemotherapy is generally employed in advanced cases, but the response rates average only 25–40% with overall survival being measured in months [3]. Thus, it is imperative that new treatment modalities are developed to increase the long-term survival of patients with HNSCC.

Angiogenesis, the process leading to the formation of new blood vessels from preexisting ones, plays a key role in the survival of cancer cells, in local tumor growth and in the development of distant metastasis [5]. Angiogenesis enables tumors to grow larger than 1–2 mm in diameter, invade surrounding tissue and travel to distant sites in the body [5]. The intensity of angiogenesis, as assessed by counting of microvessels in neoplastic tissue, acts as a prognostic factor for many solid tumors. Indeed, the intensity of angiogenesis has been shown to be increased in various human tumors, including HNSCC [6]. A malignant tumor can rely on a variety of molecular weapons that promote the switch to the angiogenic phenotype. These include an array of angiogenic growth factors, among others the vascular endothelial growth factor family (VEGF, VEGF-B. VEGF-C, VEGF-D), acidic and basic fibroblast growth factors, hepatocyte growth factor/scatter factor, transforming growth factors α and β, tumor necrosis factor α, interleukin 8 and angiogenin. The induction of tumor vascularization is regulated by the release of these angiogenic peptides from tumor cells, macrophages and extracellular matrix [7]. These factors are required for endothelial cell migration and proliferation and capillary tubule formation [8]. Among the angiogenic factors mentioned, VEGF is thought to be the most important [9]. VEGF is a potent endothelial mitogen that activates the angiogenic switch in vivo and enhances vascular permeability [10]. VEGF has been shown to increase tumor growth and angiogenesis in vivo in a nude mouse model [11]. Enhanced expression of VEGF has been detected in a large variety of malignant human tumors including HNSCC [12–14]. We have previously demonstrated extended VEGF serum concentration in HNSCC patients compared with healthy controls

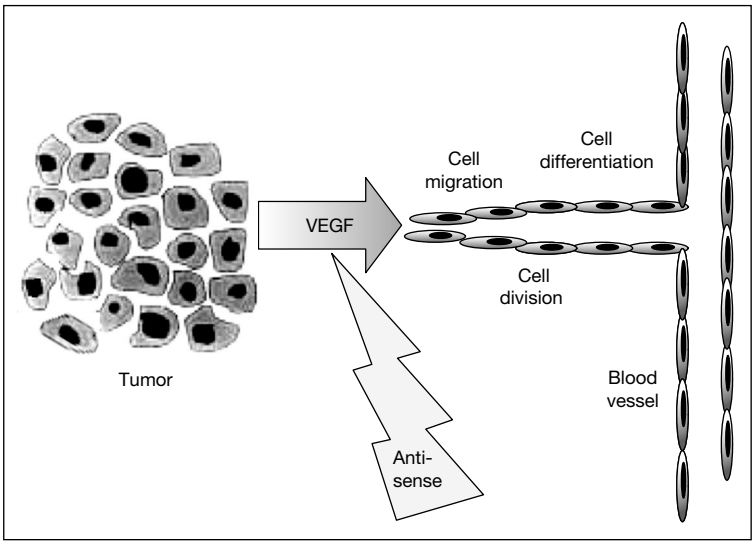


Fig. 1. Basic principle of this study: decrease in VEGF expression in head and neck cancer cells by VEGF antisense treatment may affect endothelial cell proliferation and migration in vitro and HNSCC tumor progression in vivo.

[15]. VEGF expression in HNSCC tumors strongly correlated with angiogenesis [16] and was inversely correlated with apoptosis [17]. The recognized importance of tumor-induced angiogenesis has stimulated the development of agents able to interfere with the molecules involved in this process [5]. The role of VEGF as a potential target for antineoplastic therapy has been demonstrated in several studies in which neutralizing antibodies against VEGF inhibited tumor growth and vascularization in vivo [18].

In this study, we sought to determine whether decreased VEGF expression in head and neck cancer cell lines may affect endothelial cell proliferation and migration in vitro and HNSCC tumor progression in vivo (fig. 1).

Material and Methods

Cell Culture

The different UMSCC cell lines are well-described human HNSCC cell lines obtained from T. Carey (University of Michigan, Ann Arbor, Mich., USA). The human carcinoma cell line MDA1483 is also a well-described HNSCC cell line derived from a tumor of the retromolar trigone region of the oropharynx (University of Texas, M.D. Anderson Cancer Center, Houston, Tex., USA). Cell cultures were carried out in Falcon Petri dishes at 37°C in a 5% CO_2 fully humidified atmosphere using Dulbecco's modified minimum essential medium

(DMEM; Fisher Scientific Co., Pittsburgh, Pa., USA) supplemented with 10% fetal calf serum (FCS) and antibiotics (Life Technologies Inc., Gibco BRL, Gainthersburg, Md., USA). For antisense treatment, the medium from the cultures was aspirated and replaced with DMEM containing 5% FCS and antibiotics followed by the addition of oligodeoxynucleotides. Human umbilical vein endothelial cells (HUVEC-p, PromoCell, Heidelberg, Germany) and human microvascular endothelial cells (HMVEC, PromoCell) were used for in vitro angiogenesis analysis. Cells were grown in Endothel Cell Growth Medium (PromoCell) supplemented with 2% fetal bovine serum.

Oligodeoxynucleotides

Phosphorothioated 21-mer oligodeoxynucleotides were synthesized on an Applied Biosystem 394 DNA synthesizer my means of B-cyanothylphosphoramidite chemistry to minimize degradation by endogenous nucleases. The antisense oligonucleotide (5′-CAGAAAGTTCATGGTTTCGGA-3′) was directed against the translation start site (AUG codon) and surrounding nucleotides of the human VEGF cDNA. The corresponding sense oligonucleotide was 5′-TCCGAAACCATGAACTTTCTG-3′. All experiments were performed with 12.5 μM oligodeoxynucleotides, unless otherwise stated.

Characterization of the Cell Lines

VEGF expression by the HNSCC cell lines was demonstrated immunohistochemically by anti-VEGF monoclonal antibody (VEGF, monoclonal mouse antihuman antibody, Calbiochem, Hamburg, Germany). Secreted VEGF was measured in the supernatant of the cell lines using an ELISA technique (R&D Systems, Wiesbaden, Germany) as described below. The VEGF mRNA level was measured in 7 HNSCC cell lines using RT-PCR (VEGF-CytoXpress Multiplex PCR Kit, BioSource).

Flow Cytometry

For uptake analysis, cells were grown in 6-well plates in DMEM. Confluent cultures were incubated in oligo medium with or without fluorescein-isothiocyanate-labeled oligodeoxynucleotides (FITC-ODN) for 24 h. Cells were trypsinized, washed with phosphate-buffered saline (PBS) and fixed in 0.5% paraformaldehyde. Analysis was carried out using a flow cytometer (Becton Dickinson), gating on live cells. Fluorescence was monitored at 488 nm with a 525-nm band pass filter.

Fluorescence Microscopy

For nucleus uptake analysis, cells were grown in 6-well plates in DMEM. Confluent cultures were incubated in oligo medium with or without FITC-ODN for 24 h. Cells were trypsinized, washed with PBS and incubated in Hoechst dye for 15 min. After washing with PBS, cells were fixed in 0.5% paraformaldehyde. Fluorescence staining was analyzed using a fluorescence microscope.

Human VEGF ELISA

Cell culture supernatants were collected in sterile test tubes and stored at –20°C until used. Then, VEGF concentrations were determined by an ELISA technique (R&D Systems). The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant VEGF165. The specificities of antihuman VEGF antibodies

used in the ELISA kit were examined by sodium dodecylsulfate polyacrylamide gel electrophoresis followed by Western blotting [15]. According to the manufacturer's directions, each ELISA assay measured 100 μl of supernatant. All analyses and calibrations were carried out in duplicate. The calibrations on each microtiter plate included recombinant human VEGF standards provided in the kit. Optical density was determined using a microplate reader at a wavelength of 450 nm. Wavelength correction was set to 540 nm, and concentrations were reported as picograms per milliliter. The coefficient of variation of interassay determinations reported by the manufacturer varied from 6.2 to 8.8% when VEGF concentrations ranged between 50 and 1,000 pg/ml.

Cell Counts

To determine the effect of oligonucleotides on cell growth rates of tumor cells, HNSCC cell lines were plated in DMEM at a density of 10^5 cells/microtiter well in 24-well polystrene plates (Falcon). After 24 h, the cells were rinsed twice with medium, and then fresh oligo medium containing sense or antisense oligodeoxynucleotides was added. To determine the effect of oligonucleotide treatment of tumor cells on cell growth rates of endothelial cells, UMSCC22b cells were plated in Endothel Cell Growth Medium (PromoCell) followed by the addition of oligodeoxynucleotides and incubated for 48 h. Endothelial cells (HUVEC) were then incubated in this conditioned medium transferred from the UMSCC culture. Cell counts were determined using a hemocytometer in duplicate samples at each time point. Viability of the cells was analyzed by trypan blue exclusion.

Migration Assay

Human endothelial cells were grown on gelatin-coated dishes until confluence in Endothel Cell Growth Medium (PromoCell) supplemented with 2% fetal bovine serum. Migration assays were performed in transwell chambers (Corning-Costar Corp., Cambridge, Mass., USA). Conditioned medium from the tumor cell lines was placed in the lower chambers, which were covered with polycarbonate filters (8 μm pore size). Then, 0.5 ml of 1×10^5 cells/ml of endothelial cells were placed in the upper chamber. After 4 h of incubation at 37°C, medium in the upper chamber was aspirated, and cells on the upper surface of the filter were removed with a cotton swab. Cells on the lower surface were fixed, stained with Diff Quick (Dade International Inc., Miami, Fla., USA), placed on a microscope slide and covered with a coverslip as previously described [19]. The number of stained nuclei were counted in 5 high-power fields per each chamber.

In vivo Tumor Xenograft Studies

The animal experiments were approved by the Regierungspräsidium, Karlsruhe, Germany. The MDA1483 cell line reportedly grows well as xenografts in nude mice [20]. Cells in log phase were harvested by trypsination, resuspended in DMEM media supplemented with 10% FCS, centrifuged at 1,000 rpm for 10 min and resuspended in culture media at a concentration of 1×10^7 cells/ml prior to subcutaneous implantation into mice. Female athymic nude *nu/nu* mice (4–6 weeks old) were acclimated to the University animal facility for 2 weeks prior to injection of cancer cells. Mice were implanted with 1×10^6 cells into the right flank with a 26-gauge needle/1-ml tuberculin syringe. Approximately 10 days later when the tumor nodules were palpable, mice were randomly assigned to treatment groups of 8 mice each. PBS, VEGF antisense or sense oligonucleotides (10 mg/kg;

3 times/week) were injected intraperitoneally, respectively. Tumors were measured with calipers, and tumor volumes were calculated (tumor volume = length × width^2 × 0.52). Student's t test was used to calculate p values. Mice were sacrificed when tumors became ulcerated or reached a maximum diameter of 2 cm.

Results

Characterization of the Cell Lines

Immunohistochemical investigation using an anti-VEGF monoclonal antibody demonstrated expression of VEGF protein in all carcinoma cell lines. Representative examples of VEGF staining are shown in figure 2. VEGF protein was also detectable in the supernatant of all carcinoma cell lines. These results are summarized in figure 3a. Values are reported as the means and standard deviation per 10^6 cells of duplicate experiments. Accordingly, RT-PCR for VEGF mRNA exhibited VEGF expression in all 7 HNSCC cell lines (fig. 3b). Among the head and neck cancer cell lines, a relatively high level was noted in UMSCC22b cells (2,532 ± 265 pg/ml/10^6 cells). This cell line was chosen for further study. The human endothelial cell lines showed strong expression of von Willebrand factor and VEGF receptor Flt-1. VEGF receptor Flk-1 was weakly expressed in HUVEC (fig. 4). This cell line showed a dose-dependent growth rate depending on the concentration of VEGF supplemented in the endothelial cell medium (data not shown).

Oligonucleotide Uptake Analysis

To determine the uptake of oligonucleotides into UMSCC22b cells, we performed flow cytometry using FITC-ODN after 8 h of incubation. Figure 5a shows the logarithmic fluorescence for untreated cells and cells treated with FITC-ODN. More than 90% of the cells incorporated the oligonucleotides. Fluorescence microscopy exhibited the uptake of FITC-ODN into the nucleus of UMSCC22b cells. A representative result is shown in figure 5b.

Effect of Antisense Oligonucleotides on HNSCC Cell Lines

To quantitate VEGF secretion to the supernatant of HNSCC cell lines treated with medium (control) or medium containing VEGF antisense or sense oligonucleotides, ELISA was performed after 48 h. VEGF levels were significantly decreased by antisense VEGF oligonucleotide treatment in all HNSCC cell lines tested (fig. 6a). Endogenous VEGF expression in UMSCC22b cells treated with medium (control) or medium containing VEGF antisense or sense oligonucleotides for 48 h was measured by Western blot analysis. The VEGF level is significantly decreased by VEGF antisense

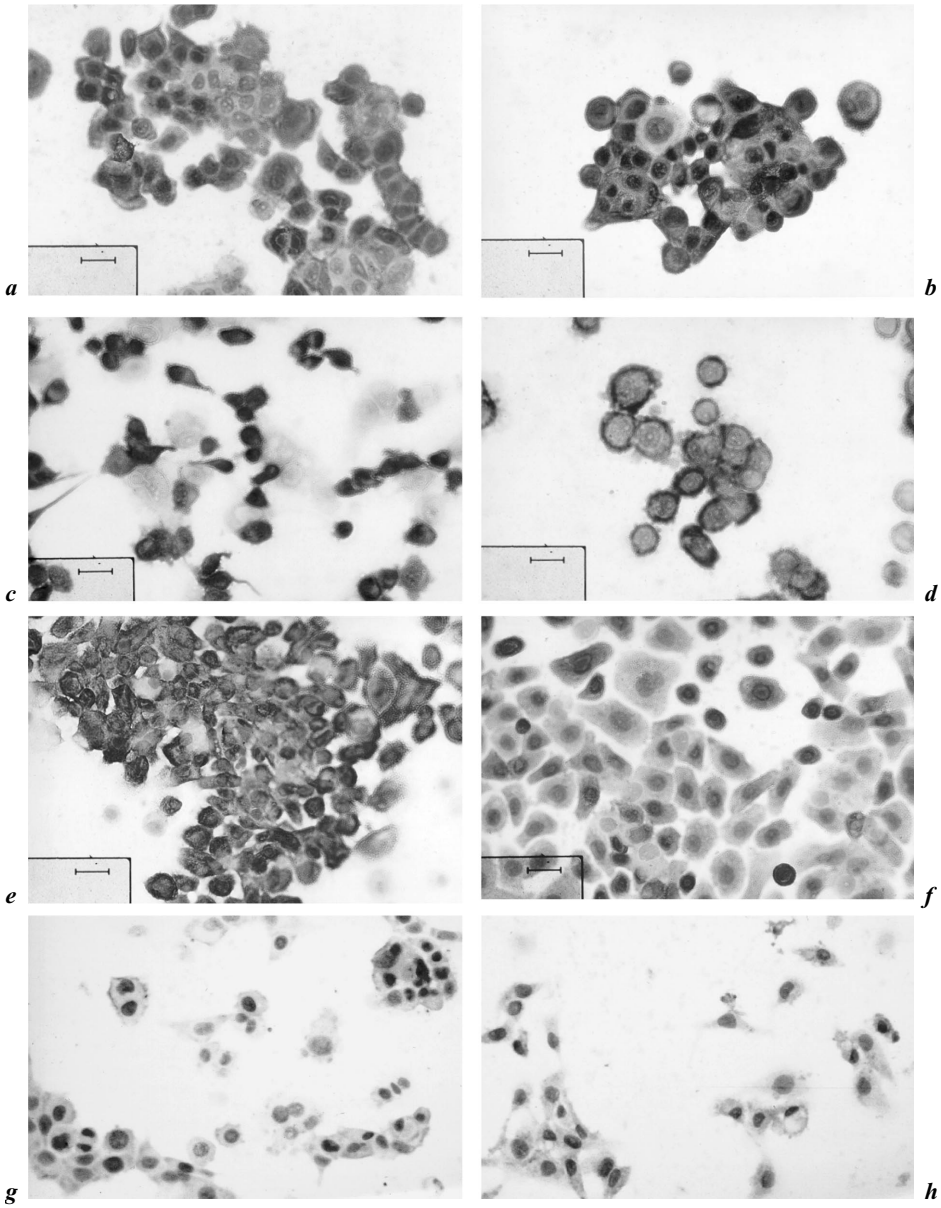


Fig. 2. Representative examples of VEGF immunostaining in HNSCC cell lines. Magnification ×400. ***a*** UMSCC22b. ***b*** UMSCC11b. ***c*** UMSCC74b. ***d*** UMSCC10a. ***e*** UMSCC17a. ***f*** UMSCC14c. ***g*** MDA1483. ***h*** A431.

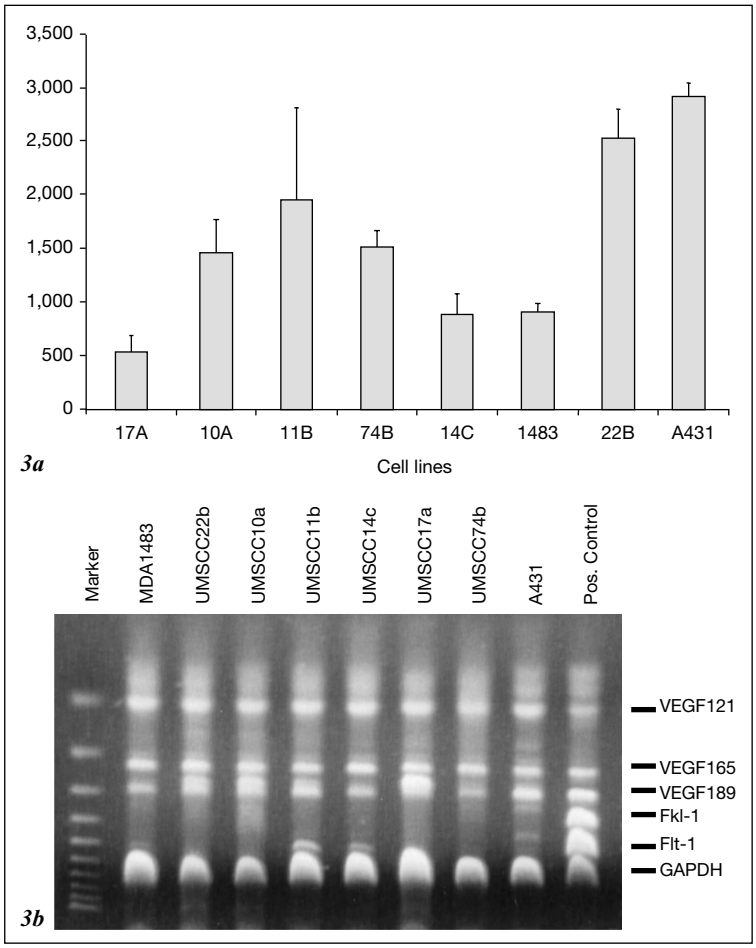
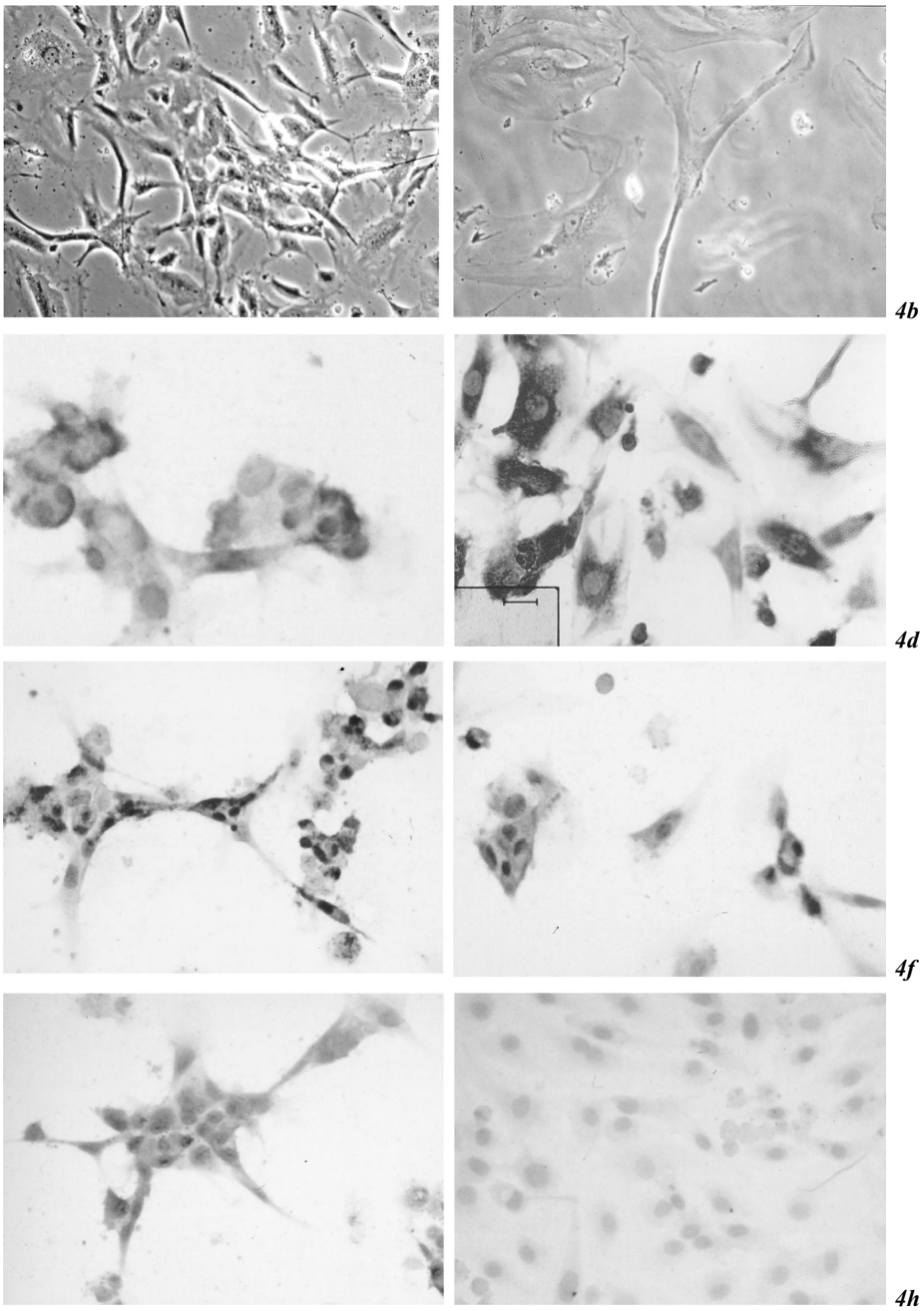


Fig. 3. ***a*** VEGF production by 7 HNSCC cell lines measured in the supernatant by ELISA after the cells had been cultured for 48 h. ***b*** VEGF mRNA in 7 HNSCC cell lines measured using an mRNA analysis kit (VEGF-CytoXpress Multiplex PCR Kit, BioSource). Secreted VEGF forms (VEGF121 and VEGF165) are detectable in all cell lines.

Fig. 4. Representative examples of immunostaining in the two different endothelial cell lines (HUVEC left; human microvascular endothelial cells, HMVEC, right). Magnification ×400. ***a, b*** Light microscopy. ***c, d*** Von Willebrand factor. ***e, f*** Flt-1. ***g, h*** Flk-1.

oligonucleotide treatment (fig. 6b). Although VEGF protein secretion was decreased in tumor cells treated wit h antisense VEGF oligonucleotides, we did not observe any reduction of the growth rate of the tumor cells as demonstrated (fig. 7).


4a
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4c
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4h
HUVEC
HMVEC

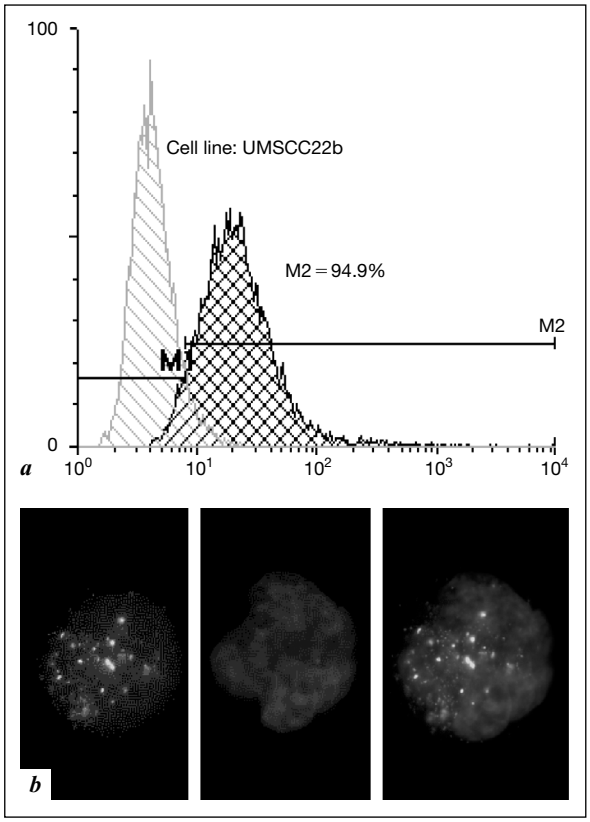

Fig. 5. ***a*** Uptake of FITC-ODN into UMSCC22b cells after 8 h of incubation. More than 90% of the cells incorporate the oligonucleotides. Values shown represent the mean of logarithmic fluorescence values in the count versus logarithmic channel histogram. ***b*** Uptake of FITC-ODN (green) into the nucleus (blue) of UMSCC22b cells after 8 h of incubation.

Effect on Endothelial Cells

The growth pattern of endothelial cells in response to conditioned medium from UMSCC22b cells treated with VEGF antisense or sense oligonucleotides was significantly affected showing a decrease in tubule formation in the presence of conditioned medium taken from the VEGF antisense-treated tumor cells (fig. 8). The growth rate of human endothelial cells (HUVEC) growing in conditioned medium from UMSCC22b cells treated with VEGF antisense or sense oligonucleotides was measured via cell counts. Endothelial cell growth was significantly decreased in the presence of conditioned medium taken from the VEGF antisense-treated tumor cells, as shown in figure 9a. The effect of antisense VEGF oligonucleotides on endothelial cell migration was examined using

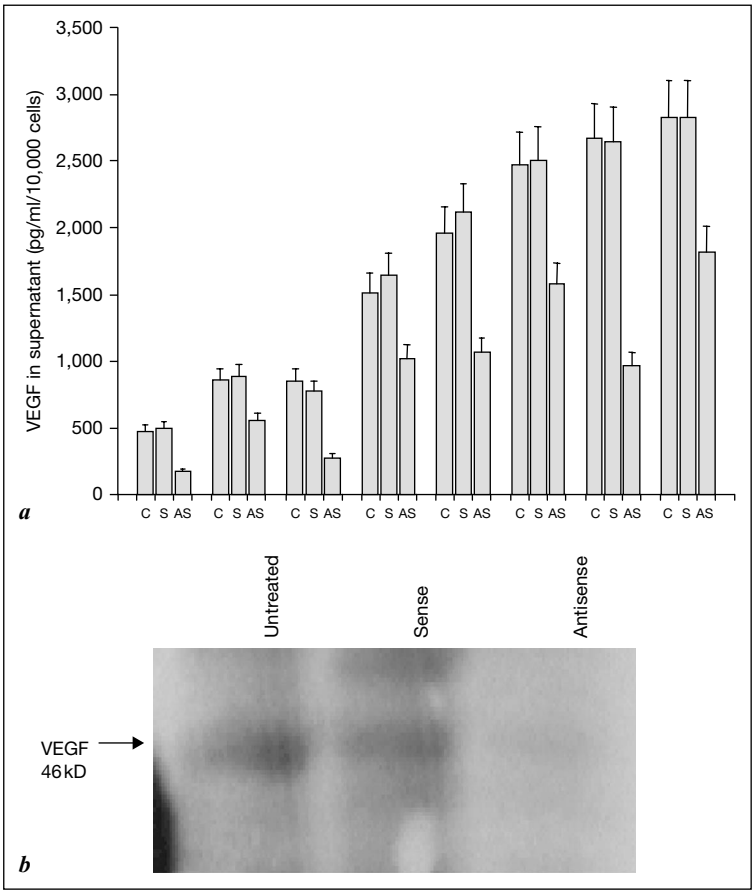

Fig. 6. ***a*** VEGF concentration in the supernatant of different HNSCC cell lines treated with medium (C = control) or medium containing VEGF antisense (AS) or sense (S) oligonucleotides for 48 h, measured by ELISA. ***b*** VEGF expression in UMSCC22b cells treated with medium (control) or medium containing VEGF antisense or sense oligonucleotides for 48 h, measured by Western blot.

transwell chambers. The addition of nonconcentrated conditioned medium from UMSCC22b cells treated with VEGF antisense oligonucleotides resulted in a significant decrease in HUVEC migration compared with the effect of conditioned medium of the untreated or sense-oligonucleotide-treated cells (fig. 9b).

Effect on HNSCC Xenografts

One million of MDA1483 cells were inoculated into each nude mouse. PBS, VEGF antisense or sense oligonucleotides (10 mg/kg; 3 times/week) were

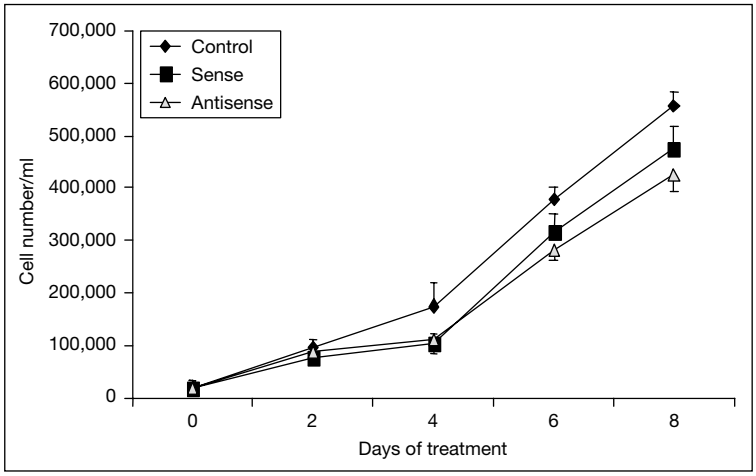

Fig. 7. Treatment of UMSCC22b cells with medium (control) or medium containing VEGF antisense or sense oligonucleotides shows no reduction of growth rate.

injected intraperitoneally, respectively. Eight mice were used per group. Student's t test was used to calculate p values. For the last 2 time points (32nd and 34th days), the differences between VEGF antisense and PBS control were significant ($p < 0.01$). The results are demonstrated in figure 10. It is also of note that VEGF sense treatment did not alter the growth behavior of the MDA1483 xenografted tumors, indicating that the VEGF antisense therapy was specifically directed against VEGF activity.

Discussion

The inhibition of angiogenesis of malignant tumors has become an important strategy to be considered in novel approaches to cancer therapy. It is widely accepted that an increase in the tumor cell population must be preceded by an increase in microvessels supplying the neoplasm [5, 7]. It was demonstrated that the supernatant from HNSCC cell lines induced an angiogenic response in vivo [21]. Dray et al. [22] demonstrated a strong correlation between high microvessel counts and recurrent or metastatic disease in HNSCC. Their data also suggested that microvessel counts were associated not only with an increased incidence of early recurrence or metastasis, but also with more aggressive forms of recurrent or metastatic disease [22]. One of the angiogenic stimulators is the VEGF, a directly acting endothelial cell mitogen. A significant body of evidence is accumulating that favors the notion that VEGF plays an important role in the

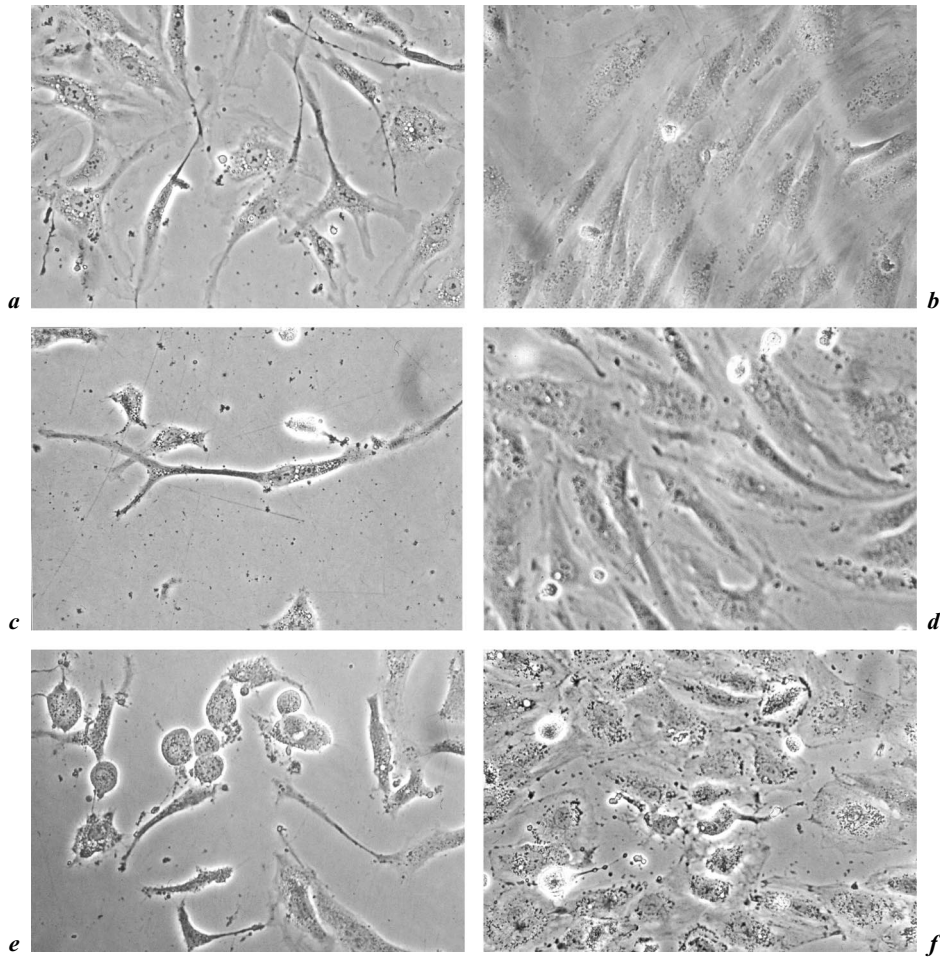

Fig. 8. Endothelial cell tubule formation in response to conditioned medium from UMSCC22b cells treated with medium (control; ***a, b***) or medium containing VEGF sense (***c, d***) or antisense (***e, f***) oligonucleotides.

development of solid tumors, such as head and neck cancer [10]. VEGF expression has been detected in a large number of malignant human tumors including HNSCC [12–14]. This prompted us to investigate the effect of VEGF antisense oligonucleotides. The objective of antisense treatment is to specifically inhibit expression of a particular gene product. The possible mechanism of action of antisense oligonucleotides includes inhibition of transcription or translation and mRNA degradation through an RNase H cleavage mechanism [23]. This study

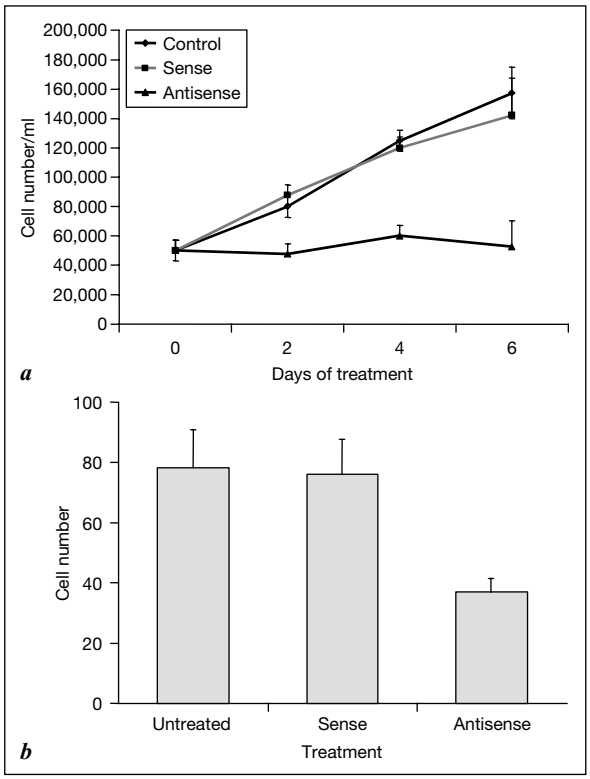

Fig. 9. ***a*** Growth rate of HUVEC growing in conditioned medium from UMSCC22b cells treated with VEGF antisense or sense oligonucleotides. ***b*** Endothelial cell migration assay in response to conditioned medium from UMSCC22b cells treated with VEGF antisense or sense oligonucleotides. In vitro migration was measured in Boyden chambers.

demonstrated that the treatment of HNSCC cells with VEGF antisense oligonucleotides in vitro efficiently downregulated VEGF expression. The cell growth rate of the tumor cells themselves was not affected by the VEGF antisense oligonucleotides. However, human endothelial cells grown in conditioned medium from the VEGF antisense-treated tumor cells exhibited a significant reduction of capillary tubule formation as well as cell proliferation and migration. Our results are in line with other recent in vitro studies showing downregulation of VEGF expression by VEGF antisense oligonucleotides in normal human keratinocytes [24] and in human glioma cells [25]. Accordingly, antisense oligonucleotide treatment of HNSCC xenografts resulted in a significant tumor growth suppression.

Our report suggests that antisense VEGF oligonucleotides may have a potential therapeutic role in the treatment of head and neck cancer. Antisense

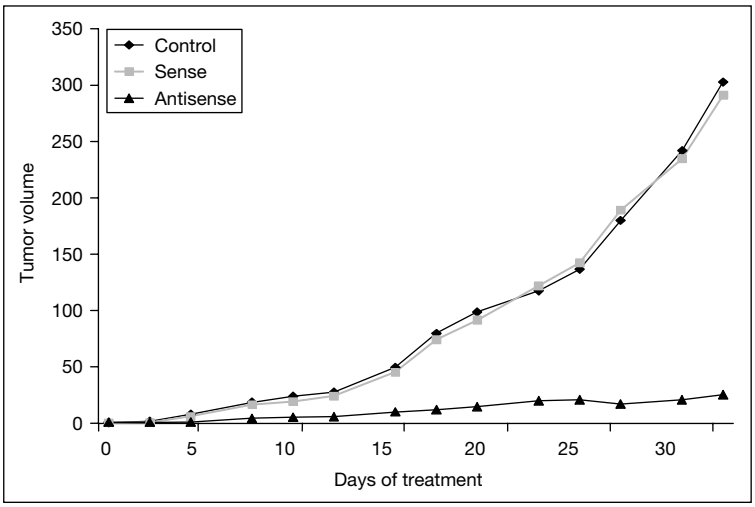

Fig. 10. Effect of VEGF antisense oligonucleotides on MDA1483 human HNSCC xenograft in nude mice. One million cells were inoculated into each nude mouse. PBS, VEGF antisense or sense oligonucleotides (10 mg/kg; 3 times/week) was injected intraperitoneally, respectively. Tumor volumes are presented as mean and plotted against the number of days of treatment. Eight mice were used per group. Student's t test was used to calculate p values. For the last 2 time points (32nd and 34th days), the differences between VEGF antisense and PBS control were significant ($p < 0.01$).

therapeutics have already been suggested for many clinical applications such as viral infections (human immunodeficiency virus, human papillomavirus, hepatitis C virus and human cytomegalovirus) and acute myelogenous leukemia or myelodysplastic syndrome [26]. In recent studies, antisense oligonucleotides have been tested for toxicity and clinical activity in a phase I evaluation in patients with advanced cancer [27].

Several other approaches have been proposed for blocking VEGF-induced endothelial cell proliferation and subsequent tumor angiogenesis. An anti-VEGF monoclonal antibody that inhibits the growth of a variety of human cancer xenografts in nude mice has been generated [18]. This monoclonal antibody has recently been humanized and is under clinical development [28]. Another promising approach is the development of selective inhibitors of the VEGF-specific Flk-1/KDR receptor tyrosine kinase [29]. In addition, experimental evidence has been provided for the potential therapeutic effect of blocking VEGF production by expression vectors containing VEGF antisense mRNA sequences [30, 31]. It has been demonstrated that antisense VEGF transfection effectively downregulated VEGF secretion from HNSCC cells and reduced endothelial cell migration [19]. Accordingly, VEGF downregulation in human colon carcinoma

Table 1. Angiogenesis inhibitors in clinical trials for cancer therapy

Drug	Mechanism
Phase I	
EMD121974	Small-molecule integrin antagonist
Combretastatin A-4 prodrug	Apoptosis in proliferating endothelium
PTK787/ZK2284	Blocks VEGF receptor signaling
Endostatin	Induces endothelial cell apoptosis in vivo
BMS-275291	Synthetic MMP inhibitor
SU6668	Blocks VEGF, FGF and PDGF receptor
Phase II	
CAI	Inhibitor of calcium influx
Squalamine	Inhibits Na/H exchanger
COL-3	Synthetic MMP inhibitor
CGS-27023A	Synthetic MMP inhibitor
TNP-470	Fumagilin analogue, inhibits endothelial
Vitaxin	Antibody to integrin on endothelial sur- face
Interleukin 12	Induces interferon γ and IP-10
Anti-VEGF antibody	Monoclonal antibody to VEGF
Phase III	
SU5416	Blocks VEGF receptor signaling
Thalidomide	Unknown
Marimastat	Synthetic MMP inhibitor
AG3340	Synthetic MMP inhibitor
Neovastat	Natural MMP inhibitor
Interferon α	Inhibition of bFGF and VEGF production
IM862	Unknown

From Carmeliet and Jain [34] and the NCI database (http://www.cancer.gov/clinical_trials/). MMP = Matrix metalloproteinase; FGF = fibroblast growth factor; PDGF = platelet-derived growth factor; bFGF = basic fibroblast growth factor.

cell lines by antisense transfection decreased endothelial cell proliferation [32]. In addition, ribozymes designed to target the VEGF sequence downregulated VEGF expression [33]. Tumors of glioma cells, transfected with a eukaryotic vector expressing VEGF in an antisense orientation, demonstrated a reduced number of microvessels, a higher degree of necrosis and inhibited growth rates [31]. Adenovirus vector transfer of an antisense VEGF sequence into glioma cells in an animal model resulted in inhibition of tumor growth [30].

Antiangiogenic therapy is an attractive modality for treating malignant neoplasms. Based on successful preclinical data, several antiangiogenic agents

alone or in combination with conventional therapies are now in clinical trials as demonstrated in table 1 [34]. We have demonstrated that antisense VEGF oligonucleotide treatment downregulates VEGF secretion and biological activity in vitro. Moreover, our results represent the first demonstration that abrogation of VEGF suppressed the growth of HNSCC tumors in vivo. Therefore, antisense oligonucleotide technology may be a potential therapy for the inhibition of angiogenesis in head and neck cancer.

Acknowledgement

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Chemokine Receptors 6 and 7 Identify a Metastatic Expression Pattern in Squamous Cell Carcinoma of the Head and Neck

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Abstract

Squamous cell carcinoma of the head and neck (HNSCC) metastasizes predictably to locoregional, cervical lymph nodes. Tumor cells can express various receptors that facilitate metastatic spread to lymph nodes and other nonlymphoid organs. Chemokine receptors (CCRs), normally expressed on lymphocytes, control immune and inflammatory cell migration, providing a link between innate and adaptive immunity. CCR expression was evaluated in HNSCC, and we showed a consistent pattern of CCR6 downregulation and upregulation of CCR7 in metastatic cells and tissues. Functional assays indicate that these surface receptors were functional on metastatic tumor cells. CCR6 downregulation is consistent with its decreased expression in cells emigrating from peripheral mucosal sites, while CCR7, important for homing of immune cells to secondary lymphoid organs, was significantly upregulated. Thus, CCR6, CCR7 and their ligands, normally important in controlling immune cell trafficking in response to inflammatory stimuli, may have an important role in determining the metastasis of HNSCC cells in vivo. Our data indicate that inhibition of CCR signaling may provide a targeted molecular therapy to prevent HNSCC metastasis.

Head and neck cancer is the 6th most common tumor in the USA, with 45,000 new cases annually and 500,000 new cases worldwide [1]. The 5-year survival is only 30–40%, mainly due to the frequent presence of invasive spread

and metastasis at diagnosis, and the pattern of regional cervical metastasis (as opposed to distant organs) in this disease is remarkably consistent [2]. Because tumor invasion and metastasis are the best predictors of prognosis, an urgent goal in head and neck oncology is to develop improved therapeutic agents for clinical use. A better understanding of these processes is necessary to enable the development of therapies designed to prevent tumor dissemination.

Selection of aggressive variants in malignant tumors may facilitate loss of attachment and subsequent emigration of cells to tissues via lymphatics and/or blood vessels. Both tumor angiogenesis and expression of stromal degradative enzymes, such as metalloproteinases, by tumor cells are known to play a role in their vascular dissemination. The predilection of metastases for specific organs may depend on a variety of factors, and three theories have been put forward [1]. According to the first theory, all migrating tumor cells can enter any tissue, but form a metastasis only if all requirements for their growth are met. The second suggests that tissue-specific adhesion molecules on endothelial cells select migrating cells able to attach and form a premetastatic nucleus of cells. The most recent theory proposes that chemoattractants, produced by stromal or immune cells, lead invasive cancer cells to the tissue of their potential secondary growth [2].

Chemokine Receptor and Ligand Interactions Mediate Inflammatory and Immunologic Functions

Chemokines are small molecules involved in lymphocyte migration, trafficking and homing throughout the lymphatic system. These molecules constitute a superfamily of inducible, secreted, proinflammatory proteins involved in a variety of immune responses, acting primarily as chemoattractants and activators of specific types of leukocytes. Four classes of chemokines have been defined based on the spatial arrangement of the conserved cysteine (C) residues in the mature protein sequences (fig. 1): the CXC chemokines that have 1 amino acid (aa) residue separating the first 2 conserved cysteine residues; the CC chemokines in which the first 2 conserved cysteine residues are adjacent; the C chemokines that lack 2 (the first and third) of the 4 conserved cysteine residues, and the CX_3C chemokines which have 3 intervening aa residues between the first 2 conserved cysteine residues [2–5].

Control of lymphocyte migration through chemokine receptor (CCR) expression enables the immune system to respond to peripheral sites of inflammation by ingress of cells responsible for surveillance and antigen uptake, while subsequent maturation programs lead to altered expression of CCRs in these cells, enabling their exit from the periphery to secondary lymphoid organs,

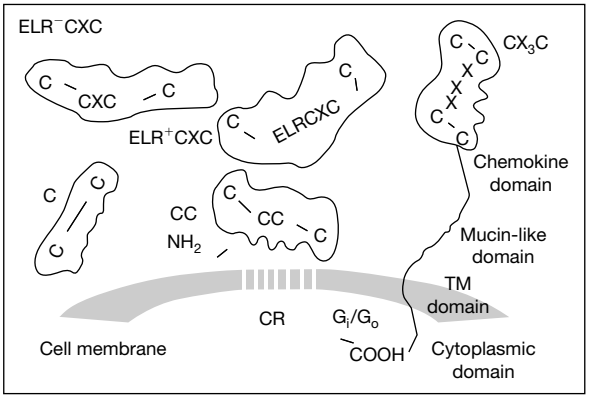

Fig. 1. Chemokine receptors are a G-protein-linked family of molecules.

where antigen-specific immunity may be initiated. Thus, normally chemokines and their receptors link innate and adaptive immune responses (fig. 2).

6Ckine/Exodus-2/SLC is constitutively expressed at high levels in lymphoid tissues such as lymph nodes, spleen and appendix. In the mouse, high levels of 6Ckine mRNA are also detected in the lung. The gene for human 6Ckine has been localized at human chromosome 9p13 rather than chromosome 17, where the genes of many human CC chemokines are clustered. The 6Ckine gene location is within a region of about 100 kb as the gene for MIP-3β/ELC, another recently identified CC chemokine. MIP-3β is constitutively expressed at high levels in the thymus, lymph nodes, appendix and tonsil. In addition, MIP-3β mRNA is also expressed by activated monocytes but not by dendritic cells (DCs) or peripheral blood mononuclear cells. The expression of MIP-3β is downregulated by the anti-inflammatory cytokine interleukin 10. Recombinant MIP-3β has been shown in vitro to be chemotactic for cultured human lymphocytes expanded with or without interleukin 2. Similar to 6Ckine, MIP-3β is not chemotactic for monocytes. MIP-3β has been shown to be a unique functional ligand for CCR7.

CCR6 is a β-chemokine-specific receptor for the chemokine ligand (CCL) 20 (MIP-3α/LARC/Exodus). In addition to having only 1 highly specific ligand, other features make CCR6 an interesting receptor. Human CCR6 is expressed in immature DCs derived in vitro from CD34+ precursors, and is downregulated as DCs mature; it is also expressed in memory T cells, cells positive for cutaneous lymphocyte-associated antigen and B cells. Similar CCR6 expression patterns are reported in the mouse, in which CCR6 is expressed in the myeloid but not in the lymphoid DC subpopulation, B cells and CD4+ T cells. Like its receptor, MIP-3/CCL20 has a restricted pattern of expression in vivo. MIP-3/CCL20 is constitutively expressed by keratinocytes in the basal and suprabasal layers of the

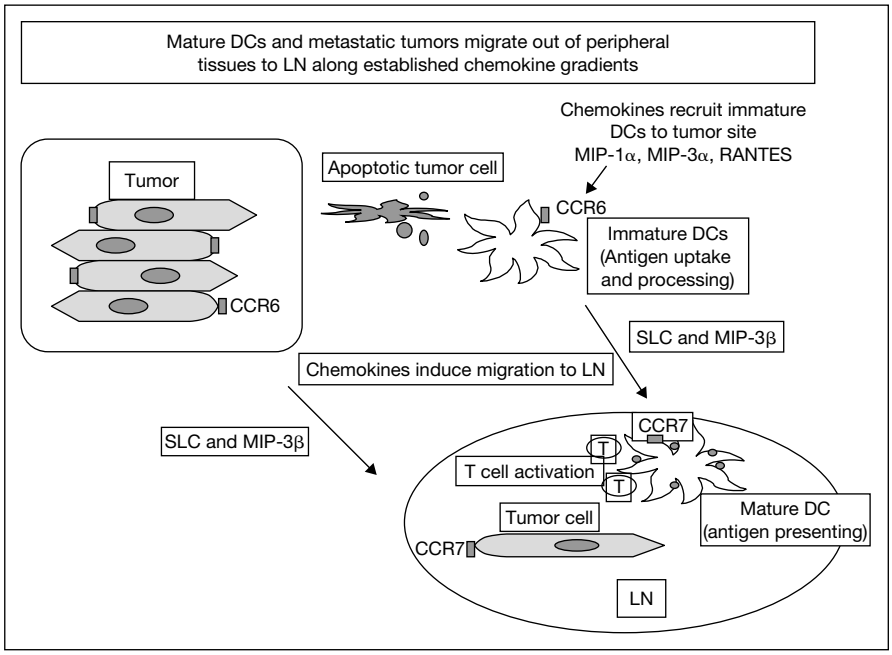

Fig. 2. Migration and invasion of HNSCC may occur in response to chemotactic gradients established for immune cell trafficking to secondary lymphoid organs.

epidermis and venular endothelial cells in skin. Likely involved in transendothelial migration and constitutive trafficking of Langerhans' cell precursors into the epidermis, MIP-3/CCL20 gene expression in peripheral blood mononuclear cells is strongly induced by inflammatory stimuli such as tumor necrosis factor α, lipopolysaccharide and phorbol 12-myristate 13-acetate, and it is a selective chemotactic factor for lymphocytes. Poorly expressed in the absence of inflammatory stimuli, MIP-3/CCL20 mRNA was found to be abundant in inflamed epithelial crypts of palatine tonsils and intestinal epithelial cells, especially those lying immediately over Peyer's patches and in other mucosal lymphoid structures, including the follicle-associated epithelium.

Mechanisms of Cancer Metastasis

Role of Chemokines in Tumor Metastasis

Several malignant cell types have been shown to express CCRs, which are expressed in these invasive cells in a nonrandom pattern [6–11]. Although these

receptors transduce signals, a thorough understanding of their true function in the tumor setting is still emerging. Chemokines and their receptors are likely to be involved in morphogenetic movements during organogenesis. It is possible that malignant transformation, and de-differentiation, may result in the reappearance of some receptors that had been involved in this process earlier in ontogeny. In the tumor microenvironment, it is likely that an extracellular growth factor-chemokine network represents a general mechanism connecting tumorigenesis and inflammation.

Recent studies have shown the involvement of CCRs in cancer metastasis, including HNSCC [12]. Metastatic tumor cells from breast [6], liver, prostate [11], ovarian [13] and gastric cancer [9] develop particular CCR expression, and this correlates significantly with CCLs produced within distant organs and lymphatic sites to which these cells metastasize. We have recently identified a unique, coordinate pattern of CCR expression on metastatic HNSCC [12]. CCR6 was nearly universally downregulated and CCR7 significantly upregulated (mean 50-fold by quantitative reverse-transcription polymerase chain reaction, qRT-PCR, and confirmed by flow cytometry and immunohistochemistry), in autologous metastases derived from primary HNSCC tumors. Thus, as selective CCR inhibitors are developed, this area of research may be translated into clinical benefit by effectively preventing this distal recruitment of metastatic tumor cells.

Chemokine Receptor Signaling Mechanisms

Due to the potential importance of chemokines in tumor growth and metastasis, recent work has focused on the expression of CCRs on tumor cells as they attempt to spread through lymphatic channels. Other analyses have also discovered that CCLs are secreted by tumor cells, paradoxically inviting lymphocytes to infiltrate peritumoral tissues. Interesting questions regarding local immunosuppressive effects of tumor cells on infiltrating lymphocytes are raised by such findings. Knowledge gained from these studies has enabled the development of vaccines that employ the strategy of CCL insertion into tumor cells and thus enhance tumor cell rejection by the recruited immune cells. These data also suggest that interference with downstream messenger molecules or direct receptor inhibition may prevent growth and/or metastasis of primary tumor cells.

Although CCR signaling has not been studied extensively in human cancers, analogy to lymphocyte CCR signaling may be drawn. Also, other G-protein-coupled receptors expressed by HNSCC cells appear to act in association with pathways dependent on epidermal growth factor receptor (EGFR),

Table 1. Clinical and demographic data of cell lines obtained from HNSCC patients in this study

Cell lines	Age	Sex	Primary site	Stage	Grade
PCI-4	51	male	larynx	$T_3N_1M_0$	moderately well
PCI-15	69	male	pyriform sinus	$T_2N_1M_0$	poor
PCI-37	62	male	larynx	$T_3N_2M_0$	moderate

Grade refers to degree of differentiation.

often through transactivation. For this reason, we have investigated whether phosphatidylinositol-3-kinase plays an important role in CCR7-dependent metastatic functions, including migration and invasion. Furthermore, we wished to clarify whether these functions, and downstream CCR7-dependent signals, are mediated through an EGFR-dependent mechanism, which has been shown previously to function in HNSCC. In preliminary studies, we have found that CCR7-mediated signals appear to act through a separate, EGFR-independent pathway, which may represent a novel target for therapeutic intervention against HNSCC metastasis [Wang J. and Ferris R.L., unpubl. data].

Unique Chemokine Receptor Expression Pattern of CCR6 and CCR7 Is Correlated with Head and Neck Cancer Metastasis

Differential Chemokine Receptor Expression in Primary and Metastatic Tumor Cells

Comparing the semiquantitative RT-PCR results of CCR expression in each pair of cells (table 1), a consistent pattern of CCR6 downregulation compared to metastases and robust CCR7 expression was found in metastatic tumor cell lines [12]. Importantly, the same pattern of CCR6 downregulation and CCR7 upregulation in metastatic as compared to primary tumor tissues was confirmed by standard RT-PCR analysis in 14 sets of paired primary and metastatic biopsy specimens, obtained from the same patients. CCR7 mRNA was detected in both primary and metastatic tumor lines and fresh specimens by RT-PCR analysis [12], raising the question as to whether levels of expression were similar between primary and metastatic tumor cells. Therefore, using real-time qRT-PCR, we determined the relative CCR7 expression between primary tumors and metastases, in paired cell lines and fresh tumor tissues. We carefully dissected tumor from the surrounding nodal architecture to remove contaminating immune cells. Since CCR6 expression was not detected in

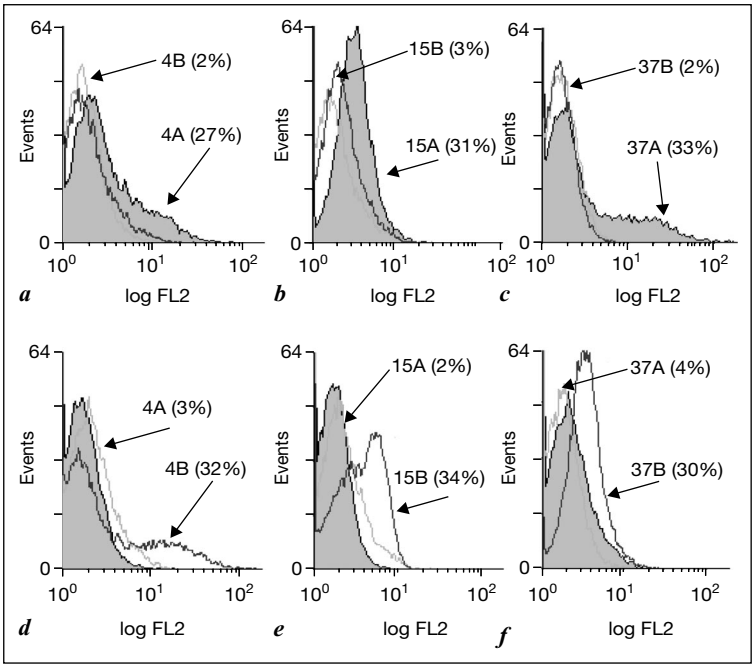

Fig. 3. Surface staining of CCR6 and CCR7 by flow cytometry in metastatic HNSCC cell lines. A = Tumor cell lines; B = metastatic cell lines. ***a–c*** Primary tumor. ***d–f*** Lymph node metastasis. Original magnification was ×200. ***a*** and ***d*** Anti-CCR6. ***b*** and ***e*** Anti-CCR7. ***c*** and ***f*** Isotype control mAb.

metastases, it was not analyzed using qRT-PCR. These experiments showed up to 50-fold higher expression levels ($p < 0.001$) of CCR7 in metastases than in primary tumors [12]. We have also confirmed this expression pattern by flow cytometry (fig. 3).

Immunohistochemical Staining of CCR6 and CCR7 in Fresh Tumor Biopsies Shows that a Novel Metastatic Expression Pattern Is Present in vivo

To study CCR expression in vivo, paraffin tumor blocks were stained for CCR6 and CCR7 [12]. Using immunohistochemistry, differential CCR6 and CCR7 expression in primary versus metastatic head and neck tumors (n = 4 patients) was confirmed from patients' tumors studied using qRT-PCR. Flow cytometry on autologous primary and metastatic cell lines (fig. 3) confirmed the presence of mature protein expression. Functional effects were initially indicated by receptor desensitization (downregulation only in response to the appropriate CCL treatment; data not shown).

Immunostained tissue sections indicated that, in vivo, primary tumors expressed high levels of CCR6, but metastatic tumors expressed high levels of CCR7. These results rule out the possibility that high CCR7 expression in metastatic tumors was due to lymphoid cells in tumor-metastatic lymph node specimens and is consistent with the retention of different migratory responses of primary and metastatic tumor cell lines, even after culture in vitro for months.

Tumor Cell Chemokine Receptors Mediate Chemotaxis in Response to Selective Chemokines

We next analyzed the capability of paired primary/metastatic cells to migrate in vitro in response to the respective ligands. Transwell migration assays followed by blocking of the receptors with receptor-specific monoclonal antibodies (mAbs) showed that both CCR6 (on primary cells) and CCR7 (on metastatic cells) surface receptors are functionally active. Media-pulsed and isotype-matched mAb-pulsed wells (data not shown) were used to control for CCR specificity of the blocking effect. An increase in expression of CCR6 in primary tumor cells was also observed in all 3 cell lines examined in response to MIP-3α. CCR7 upregulation in metastatic cells was observed in 3 of 3 cell lines tested suggesting their enhanced responsiveness to both CCR7 ligands, MIP-3β and SLC (fig. 4). This CCR-dependent migration was blocked using an mAb specific for the appropriate receptor, indicating the clinical potential of such inhibition pharmacologically (fig. 5).

Matrigel invasion assays using 686LN (poorly metastatic) cells and 686LN-M3a2 (highly metastatic derivative) as described previously [14] indicated a correlation between increased CCR7 expression detected by qRT-PCR in 686LN-M3a2 cells, with invasive potential.

Implications of these Findings

The overall 5-year survival rate for HNSCC has remained poor [15], largely because of the tendency of HNSCC tumors to disseminate to the regional lymph nodes, although they rarely metastasize to distant organs. In these patients, survival is decreased by one half as compared to patients without nodal metastasis [16]. A more aggressive or novel treatment of such cancers at the greatest risk of dissemination is likely to improve long-term survival. Hence, there is an urgent need to understand the mechanism(s) of metastatic spread of tumor cells to lymph nodes and eventually to other sites. Our aim in this study was to assess the role of CCRs in metastasis of HNSCC to regional lymph nodes.

Molecules regulating metastatic tumor dissemination to specific anatomical sites have to be constitutively expressed on these cells, and to have the ability

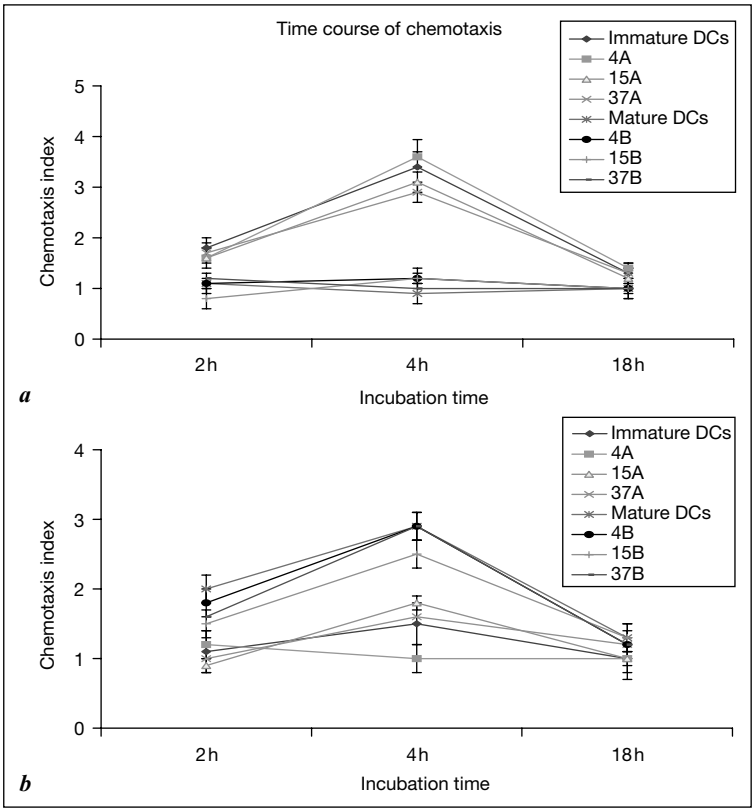

Fig. 4. Migration assay of HNSCC cells in response to chemokines in vitro. A = Primary cells; B = metastatic cells. Chemotaxis of 3 sets of paired HNSCC cell lines was measured in 96-well chemotaxis chambers at the concentrations previously determined to give optimal migration (500 ng/ml), after 2, 4 or 18 h of incubation with CCL. Immature and mature DCs were used as controls. CCR6 ligand (MIP-3α; ***a***) and CCR7 ligand (MIP-3β and 6Ckine/SLC; ***b***) were used as chemoattractants in this assay. Mean ± SD, n = 6.

to mediate tumor cell invasion into tissues that are needed to provide supportive microenvironments for tumor growth. Such a process requires the expression of a distinct receptor repertoire by the target cells. Chemokines perfectly fulfill these criteria [17, 18]. Indeed metastatic sites have been correlated with CCR expression in the metastatic tumor cells [8, 9], although our report is novel in indicating a role for loss of expression of the inflammatory CCR6 during the metastatic process. Whether metastasis takes place via hematogenous (similar to memory T lymphocytes) or lymphatic channels (such as DCs) is not presently clear from our findings (fig. 2), although certainly both mechanisms may be

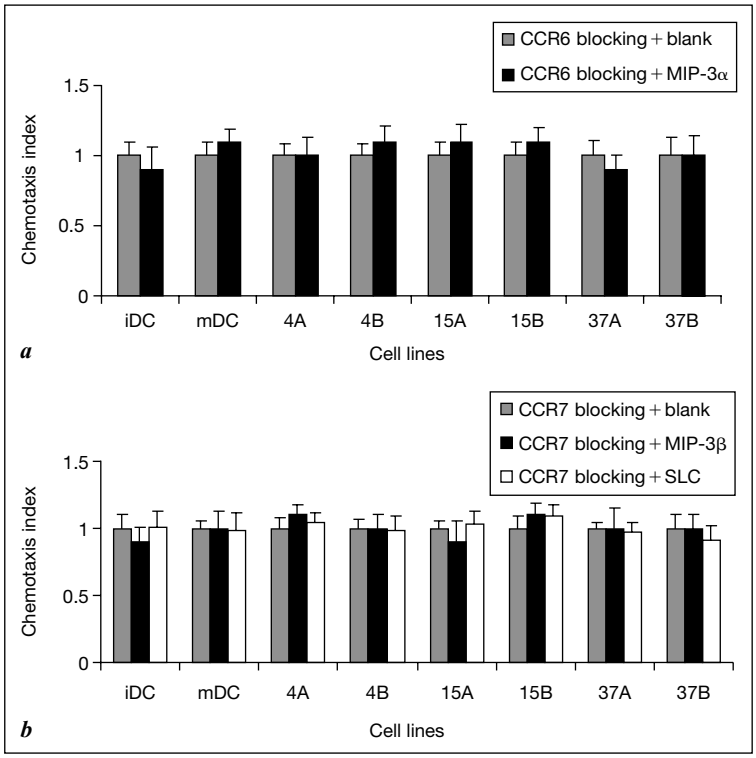

Fig. 5. Abrogation of CCR-induced migration in vitro by preincubation of metastatic HNSCC cell lines with blocking anti-CCR6 (***a***) and anti-CCR7 (***b***) mAbs. iDC = Immature DC; mDC = mature DC; A = primary cell line; B = metastatic cell line.

imported depending on angiolymphatic structures invaded in proximity of CCR7-expressing tumor cells.

By screening for CCR expression in HNSCC primary tumors and metastatic cells, we found a consistent pattern of the CCR6 downregulation and upregulation of CCR7 in selected metastatic cell lines and tissues. No other pattern was seen reliably in metastatic tumor specimens. On the other hand, our data indicate a remarkably consistent pattern of CCR6 and CCR7 expression in primary versus metastatic tumor cells, with rare exceptions in our analyses. While others have suggested that CCR7 upregulation in tumor cells might facilitate their emigration from tissues, the consistent loss of expression of CCR6 in our system is novel and reminiscent of the response of immune cells to inflammatory stimuli (fig. 4). For example, after antigen uptake or other stimuli that activate their progression to mature DCs, CCR6 expression is lost, while CCR7 is

significantly upregulated. Emigration from peripheral sites and trafficking to regional lymph nodes usually leads to interaction with other immune cells. While CCR7 is also used by circulating naïve and memory T cells, we would expect that metastatic HNSCC cells are likely to access cervical lymph nodes using afferent lymphatics, similar to DCs. However, it is important to note that our data do not currently distinguish between vascular pathways used for this phenomenon, as our studies only used DCs as controls in qRT-PCR and migration studies [12] (fig. 3 and 4). Lymphangiogenesis is not well understood currently, including the ligand-mediated signals related to embolism and implantation into regional lymph nodes. Growth of lymphatic channels and blood vessels may predispose tumors with metastatic potential, through CCR7 upregulation, to access lymph nodes by one of these routes. This mechanism points to the ability of more aggressive tumor cells to cooperate functional characteristics of normal leukocytes (fig. 2), and our CCR blocking studies suggest novel antimetastatic therapies for patients with early tumors (fig. 5), perhaps enabling less aggressive or more successful surgical therapy.

The abundant expression of the homeostatic chemokine SLC (a ligand of CCR7) in lymph nodes makes it a likely candidate to attract CCR7+ tumor cells [19], as suggested previously for dissemination of melanoma [10] and adult T cell leukemias [20]. On the other hand, CCR6 downregulation in metastatic tumor cells indicates that the coordinate expression of CCR6 and CCR7 may be important in orchestrating HNSCC metastasis. Remarkably, our observations in fresh tumor specimens were maintained in cell lines cultured in vitro for months at a time. This also helps rule out that, in fresh metastatic tumor biopsies, CCR7 upregulation was simply the result of immune cell contamination of our tumor RNA. In addition, the pattern of this coordinate CCR expression in metastases indicates that the upregulation of CCR7 in these tumor cells is not due to the lymph node microenvironment in fresh tissues, since even in metastatic tumor cell lines, this effect is maintained over long periods in culture. The finding that increasingly metastatic tumor derivatives in a metastatic mouse model showed quantitatively increased CCR7 expression demonstrates the in vivo importance of this phenomenon, in a completely separate experimental system.

We also show that CCR7 is functional and appears to lead to expected signals after ligand binding [12]. The normal response to ligand occupancy of cytokine and chemokine receptors is ‘desensitization’ or downregulation [21]. Using paired autologous HNSCC primary/metastatic cell line pairs, we studied CCR6 and CCR7 responses after treatment with their ligands, MIP-3α and MIP-3β, respectively, at a concentration of 500 ng/ml for 4 h by flow cytometry. Figure 5 shows that primary tumor cells downregulate (internalize) CCR6 in response to MIP-3α treatment, while metastatic cells manifest this effect after

treatment with the CCR7 ligand (MIP-3β). Treatment of each cell line (primary tumor cells with MIP-3β and metastatic cells with MIP-3α) did not result in receptor downregulation.

Thus, a specific and differential expression pattern of CCR6, CCR7 and their ligands appears to play an important role in determining the metastatic potential of HNSCC cells. This program is manifested by tumor cells, as their migration appears to be regulated by the loss of CCR6 expression, acting as the brake, and gain of expression of CCR7, acting as the accelerator, leading to migration of tumor cells to proximal lymphoid tissues. Although the organ predilection of metastatic cells might ultimately depend on multiple CCR-CCL interactions as we encountered here, rather than a single interaction, our data suggest that inhibition of CCR7 signaling could be a clinically useful target for therapy in the prevention of HNSCC metastasis. Further work is under way to determine metastatic pathways responsible for tumor cell CCR-associated metastasis, in proinflammatory, nuclear-factor-κB-mediated signaling pathways [22, 23]. Preventing such a poor prognostic feature in this disease would likely enable more successful locoregional tumor control and improve survival, even with the current surgical and/or chemoradiotherapeutic modalities.

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p53-Based Immunotherapy of Cancer

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Abstract

Genetic alterations in *p53* are common to a wide range of human tumors, including squamous cell carcinomas of the head and neck. Given the need for novel adjuvant therapies for this disease and the renewed interest in immunotherapy as an adjuvant therapy, p53 has become an attractive candidate for vaccines to treat patients. Although *p53* is frequently mutated, the remainder of the molecule keeps its wild-type sequence (wt). As a consequence, several nonmutated peptides can be processed from the altered p53 molecules and presented by tumor cells for T cell recognition. Thus, the targeting of wt p53 peptides represents an approach to developing broadly applicable cancer vaccines. Like most things, however, targeting p53 seems more difficult than originally thought. Whether these difficulties can be circumvented remains to be determined. The development of p53-based vaccines over the past decade is reviewed together with the promising initial findings of their clinical introduction.

The potential of augmenting the immune system of patients with cancer to eradicate their tumors, which has been facilitated by advances in molecular immunology, is attracting greater attention now than at any previous time. As the survival of patients with squamous cell carcinoma of the head and neck (HNSCC) has not improved significantly in over 30 years, novel adjuvant therapies are being sought for the treatment of this disease [1]. Preclinical and clinical evidence strongly indicates that host defense against progressive tumor growth is dependent on T-cell-mediated antitumor immune responses [2]. Whereas CD8+ cytotoxic T lymphocytes (CTLs) are considered the critical effectors for eradication, CD4+ T lymphocytes or T helper (Th) cells have been found to be critical for the maintenance of antitumor immunity [3–5].

Both of these T cell subsets recognize short peptides or 'epitopes' derived from proteins present in cells that presented on the cell surface in association with class I or II major histocompatibility complex allelic molecules [6, 7]. In humans, these molecules are termed human lymphocyte antigens or HLA. The development and clinical introduction of melanoma vaccines for inducing and/or enhancing T-cell-mediated antitumor immune responses have created an opportunity for developing comparable vaccines for carcinomas, including HNSCC.

Nearly all the human tumor antigens being used in developing cancer vaccines are tumor-associated determinants. They represent nonmutated peptides derived from 3 distinct groups of proteins [8]. These groups can be distinguished by their patterns of expression in tumors and normal adult cells. One determinant group is derived from tissue-specific or differentiation antigens that are overexpressed in tumors relative to normal cells. They are 'self' epitopes. The second group is derived from proteins expressed in the testes, but not normal cells. Epigenetic and/or genetic events result in activation of genes encoding these 'cancer-testes' or 'cancer-germline' proteins. Their lack of expression on normal cells and inappropriate expression in a wide range of tumors makes them immunologically 'nonself' in nature and enhances their potential for use in cancer vaccines. The third determinant group is derived from a variety of gene products involved in cell cycle regulation. Loss of their functional activities is a critical event in transformation. Many of these proteins are products of oncogenes or tumor suppressor genes; p53 is a prime example of the latter group of gene products.

Immune Targeting of p53

p53 was initially identified as a transformation-related antigen using antibodies present in the sera of mice hyperimmunized against chemically induced sarcomas [9]. In these studies, p53 expression was detected at elevated levels in cells transformed by a variety of agents. A subsequent study by Crawford et al. [10] identified anti-p53 IgG antibodies in the sera of some patients with cancer and, thereby, established the immunogenicity of p53 in humans and the concomitant presence of anti-p53 T cell as well as B cell responses in these individuals. In nearly all cases, the tumors of p53-seropositive patients have been found to express elevated levels of p53 [11–14].

A key function of p53 is to prevent DNA replication following DNA damage due to a variety of causes, such as radiation [15]. It does so by blocking replication until DNA repair has occurred. Following the identification of p53 as a tumor antigen, alterations in *p53* have been shown to be the most

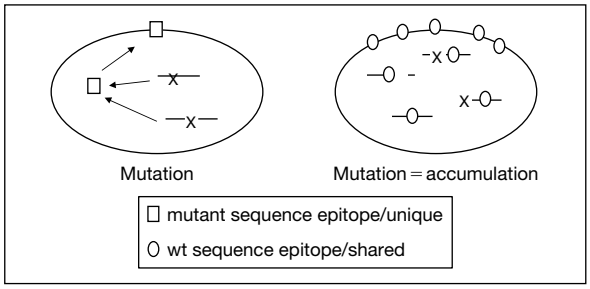

Fig. 1. Classes of CTL-defined p53 tumor antigens.

frequently occurring genetic event associated with human cancer [16–18]. At least 50% of all human tumors analyzed contain genetic alterations in *p53.* For the most part, the frequency of *p53* genetic alterations is based on sequence analysis of *p53* exons 5–8, which encode the DNA binding region of the molecule. When all the *p53* exons (exons 2–11) as well as intron/exon junctions are analyzed, as has recently been done for a group of HNSCC tumors [19, 20], the incidence of genetic defects in *p53* approached 80%.

In normal cells, wild-type (wt) p53 molecules are sequestered in the nucleus and have a relatively short half-life. Missense mutation of *p53*, however, is frequently associated with stabilization (increased half-life) of mutated p53 molecules in the cytosol of tumors, termed 'accumulation or overexpression'. As the accumulation of mutated p53 in tumors resembles the overexpression phenotype associated with many shared tumor-associated antigens being targeted for use in cancer vaccines, one can presume that enhanced processing of the accumulating mutant p53 molecules in the tumor cytosol could also occur. In contrast to most other tumor-associated antigens, however, 2 classes of p53 peptides can be presented: epitopes containing the missense mutation(s) and nonmutated, wt sequence epitopes (fig. 1). The former would be 'nonself' epitopes, whereas the latter are 'self'. In contrast to the missense mutation, which could generate a single mutant epitope, an array of wt p53 peptides can be derived from the remainder of the mutated protein. Since the constraints of antigen processing and presentation would limit the presentation of mutant p53 peptides to tumors of only a few individuals, vaccines targeting these determinants would essentially need to be 'custom made' and of limited applicability. Quite the opposite applies to wt p53 epitopes, which would have greater probabilities of being presented by many tumors for T cell recognition. They represent 'shared' tumor-associated antigens, and vaccines targeting them would be broadly applicable [21, 22]. The development and clinical introduction of p53-based vaccines, however, need to surmount many of the difficulties

that face cancer immunotherapy [2, 23–25], as well as several issues and concerns that are specific to targeting p53 and evolve from the genetic events leading to loss of its function, which is a key event in oncogenesis.

Critical Issues in the Development of p53-Based Cancer Vaccines

When the target of an immunization is a 'self' tumor antigen, rather than an infectious agent, concerns and issues involving immunological tolerance and its impact of host responsiveness to 'self' tumor antigens need to be considered [26, 27]. This is in addition to the variety of mechanisms that can contribute to 'tumor escape', including immunoselection or immunoediting [28, 29]. All of these concerns and issues impact on any effort to develop effective cancer vaccines. In addition, the options that are available for preparing cancer vaccines need to be evaluated. The relative merits of employing intact recombinant proteins, protein fragments or peptides need to be evaluated as well as various chemical, viral and cellular vehicles for their delivery. In many respects, the development of p53-based immunotherapy has greatly benefited from the knowledge and insights gained from preclinical studies using p53-based vaccines in murine tumor model systems [22, 26, 27, 30, 31].

CTL-Defined wt p53 Peptides

Unlike most of the CTL-defined human tumor peptides identified, no HLA-class-I-restricted CTL-defined wt p53 peptides have been identified using antitumor effectors derived from peripheral blood mononuclear cells (PBMCs) obtained from patients with cancer. Instead, they have been identified by 'reverse immunology'. Essentially all the CTL-defined wt p53 peptides have been identified using a combination of sequence motifs of peptides binding to specific class I HLA allelic molecules and algorithm predictions of binding to these molecules [7, 32–41]. Due to its high frequency of expression in the patient populations being studied, the focus of this effort has been to identify HLA-A*0201 (HLA-A2)-restricted epitopes. Using HLA-A2 transgenic mice as well as PBMCs obtained from normal donors, a series of CTL-defined wt p53 peptides have been identified. In vitro studies are then required to define which peptides can be classified as naturally processed and presented HLA-A2-restricted CTL-defined wt p53 peptides. In this manner, wt $p53_{65–73,\ 149–157,\ 189–196,\ 217–225,\ 264–173}$ and $p53_{322–330}$ are being studied for their potential use in p53-based cancer vaccines for patients expressing the HLA-A2.1 allele.

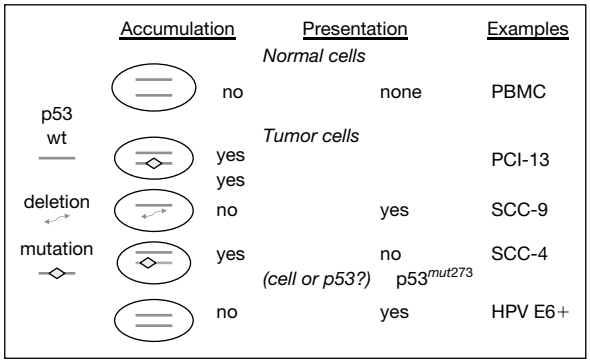

Fig. 2. Phenotypes of p53 expressed in tumors and their abilities to present wt p53 peptides for CTL recognition.

Nature and Sites of Genetic Alterations in p53 Can Influence Presentation of wt p53 Epitopes

A surprising outcome of the initial studies identifying CTL-defined wt p53 peptides were the repeated observations that (1) accumulation of mutant p53 in a tumor cell did not automatically confer sensitivity to cytolysis by anti-wt p53 CTLs and (2) a variety of mechanisms can influence the processing and presentation of wt p53 epitopes for immune recognition (fig. 2). It has become increasingly apparent that the nature and sites of p53 missense mutation influence antigen processing of p53 molecules [34, 36, 42, 43]. Furthermore, oncogenic events involving human papillomavirus (HPV) infection or *mdm2* amplification can also impact on processing of p53 epitopes for T cell recognition [43–45]. The HPV E6 contributes to transformation by binding to p53 and enhancing its ubiquitation and proteasomal degradation. This can lead to loss of p53 function and contribute to the transformation process at the same time enhancing the presentation of wt p53 epitopes. Consequently, an HPV+ HNSCC could present wt p53 epitopes in the absence of accumulation of wt p53. Genetic alterations in *mdm2*, an oncogene that controls ubiquitation and proteasomal degradation of p53, are frequently encountered in tumors. Many of these tumor cells can accumulate wt p53 and present wt p53 epitopes. Aside from the defects in the antigen processing machinery components in tumors [46], which can hinder the successful outcome of any T-cell-based immunotherapy, a particularly critical challenge for a successful outcome for wt p53-based immunization is knowledge of p53 expressed in the tumor and predicting tumor presentation of the targeted wt p53 peptides.

Immunological Tolerance Plays a Role in Defining Patients' Responsiveness to 'Self' Tumor Peptides

By the very nature of the 'self' versus 'nonself' concept that governs the immune system, effective immunity against 'self' tumor antigens, such as wt p53 peptides, must breach the barriers to protect the body from inappropriate and potentially deleterious autoimmune responses. While a controlled 'antitumor autoimmune' response may be the desired effect of vaccination targeting 'self' tumor antigens, it must break the immunological tolerance to these epitopes. This subtlety of targeting 'self' tumor peptides is particularly important in the case of wt p53 epitopes. Most of the other 'self' tumor antigens being targeted with vaccines, such as those derived from tissue-specific or differentiation antigens, have limited tissue distribution. As a result, these antigens are not readily available in the thymus, and immune responsiveness to them is governed by peripheral tolerance. In contrast, p53 is expressed by all nucleated cells and readily available in the thymus for induction of tolerance to p53. Based on in vitro immunological studies involving PBMCs, it is apparent that cells from only about a third of PBMCs obtained from normal donors and patients with HNSCC were responsive to in vitro stimulation (IVS) with autologous dendritic cells (DCs) pulsed with wt p53 peptides. The DCs were chosen for this assay as they are considered the only antigen-presenting cells capable of inducing antigen-specific responses from naïve T cells. The anti-wt p53 T cells induced from these individuals displayed a low-to-intermediate affinity for their ligands and a limited repertoire of T cell receptor usage [36, 47–49]. To what extent the weak immunogenicity of wt p53 peptides reflects deletion or anergy of anti-wt p53 T cells is an open question [26, 27], but preclinical murine studies have clearly demonstrated that the anti-wt p53 CTLs induced in p53 null mice are more reactive than the anti-wt p53 CTLs induced in normal p53 (+/+) mice [30, 31]. Most importantly, the adoptive transfer into normal mice of these high-affinity anti-wt p53 CTLs as well as anti-wt p53 Th cells obtained from p53 null mice did not result in deleterious autoimmune side effects [30, 31].

Circumventing the Nonresponsiveness of PBMCs to CTL-Defined wt p53 Peptides

One well-established method for enhancing the immunogenicity of a T-cell-defined epitope is to identify amino acid exchanges in the peptide sequence that enhance its binding to HLA molecules and/or interaction with the T cell receptor and permit expansion of T cells capable of recognizing the parental peptide [50–53]. For the wt $p53_{264–272}$ and $p53_{149–157}$ peptides, this approach was

Table 1. IVS responses of PBMCs obtained from HLA-A2+ normal donors to wt and the T50L variant p53 peptides

Donor No.	$p53_{25–35}$	$p53_{65–73}$	$p53_{149–157}$	$p53_{217–225}$	$p53_{264–272}$	$p53_{322–330}$	p53 T150L
1	−	+	+	−	−	−	n.d.
2	−	+	+	−	−	−	n.d.
3	−	−	−	−	−	+	−
4	−	+	+	+	+	+	−
5	−	−	−	−	+	+	+
6	−	−	−	−	+	−	−
7	−	+	+	−	−	−	n.d.
8	−	+	−	−	−	−	n.d.
9	−	−	−	−	−	−	+
10	−	−	−	−	+	−	−
Total	0/10	5/10	4/10	1/10	4/10	3/10	2/6

Responder cell reactivity was tested in ELISPOT and/or cytotoxicity assays. n.d. = not determined.

successful in optimizing the immunogenicities of these two epitopes. In the case of the wt $p53_{264–272}$ peptide (LLGRNSFEV), which contains a favorable amino acid (leucine) at the anchor positions 2 and 9, the exchange of tryptophan for phenylalanine at position 7 of the peptide, L270W, increased its immunogenicity by enhancing the stability of the HLA/peptide/T cell receptor complex, as evidenced by an increased affinity for the parental peptide of anti-wt $p53_{264–272}$ CTL induced using the optimized peptide. The amino acid exchange of the favorable amino acid (leucine) for an unfavorable amino acid (threonine) in anchor position 2 of the wt $p53_{149–157}$ peptide (STPPPGTRV) increased the binding affinity of the T150L peptide for HLA-A2.1 molecules and enhanced its immunogenicity [54]. These findings demonstrate the utility of using amino acid exchanges to optimize the efficacy of cancer vaccines targeting 'self' tumor peptides.

Patterns of IVS Responsiveness of Normal Donor PBMCs to CTL-Defined wt p53 Peptides

As multiple CTL-defined wt p53 peptides have been identified and IVS responsiveness to two of them have shown to be sporadic [36, 47–49], the question of defining their relative immunogenicities becomes an issue that needs to be resolved to proceed with the development of multiepitope p53-based vaccines. In this regard, we examined the IVS responsiveness of PBMCs obtained

from 10 normal donors to a panel of 5 wt p53 peptides and the optimized $p53^{T150L}$ peptide [Chikamatsu and De Leo, unpubl. results]. The reactivities of the outgrowing lymphocytes for wt p53 peptides were determined using ELISPOT and cytotoxic assays with peptide-pulsed T2 target cells as the target cells [36]. As indicated in table 1, the most frequently detected IVS responses were to the wt $p53_{65–73}$, $p53_{149–157}$ and $p53_{264–272}$ peptides. The response rate to wt $p53_{149–157}$ was further enhanced by the use of the optimized T150L peptide. The results of this ongoing analysis require IVS analysis of additional PBMC samples to be significant. Nonetheless, they indicate that all PBMCs from all the 10 donors tested responded to either wt $p53_{65–73}$, $p53_{149–157}$ or $p53_{264–272}$ peptides, most of the donors responding to 2 of the peptides. We are concluding, therefore, that these 3 wt p53 peptides would be the most suitable consideration in the construction of a multi-CTL peptide p53 vaccine.

Th-Cell-Defined wt p53 Peptides

The identification of anti-p53 IgG antibodies in the sera of some patients with cancer is indicative of anti-p53 CD4+ Th cell responses induced in these individuals. Unfortunately, it is also associated with a poor prognosis, which might be attributable to a predominating Th2 antitumor immune response in these patients rather than the Th1-biased response that is generally associated with tumor eradication. Preclinical studies have demonstrated that vaccines that employ Th- as well as CTL-defined epitopes derived from the same tumor antigen show enhanced efficacy due to the established role of the antigen-specific CD4+ T cells in the induction and maintenance of effective antitumor immunity [55, 56]. Consequently, the identification of Th-cell-defined p53 peptides would be useful for not only enhancing the efficacy of p53-based immunization but also to possibly 'reverse' the Th2-biased responses of p53-seropositive patients. Several in vitro studies have focused on proliferative T-cell-mediated responses to intact p53 protein or p53 peptides [13, 14, 57]. The study of Fujita et al. [58] identified several immunogenic HLA-class-II-restricted wt p53 peptides. The abilities of these peptides to be naturally presented, however, were not established in their study. In our recent study, which utilized recombinant wt p53 protein-pulsed DCs as the antigen presenting cells and algorithm-predicted HLA-DRB1*040-binding 15-mer peptides [59], we identified wt $p53_{110–124}$ peptide as a naturally presented HLA-DRB1*0401-restricted epitope [60]. In in vitro experiments using the autologous PCI-13 HNSCC system available in our laboratory, the addition of anti-wt $p53_{110–124}$ CD4+ T cells to PBMCs was shown to increase the total number of CD8+ T cells in the IVS cultures and, more relevantly, to enhance the induction of anti-PCI-13 effectors (fig. 3). This effect was dependent

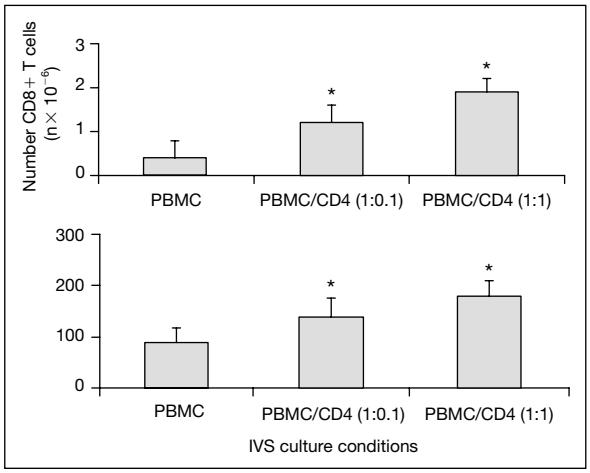

Fig. 3. IVS of PBMCs with autologous anti-wt $p53_{110–124}$ CD4+ Th cells enhances the expansion of CD8+ T cells and the generation of anti-PCI-13 effectors. *$p < 0.05$.

on the ratio PBMC/CD4 cells in the cultures. These results are consistent with the concept of developing a multi-epitope p53 vaccine that would employ Th-defined as well as CTL-defined p53 peptides to maximize its efficacy.

Immunoselection or Immunoediting Relative to p53: Sites of Mutation in p53 in Tumors of HLA-A2+ Patients with HNSCC

Although missense mutations in *p53* in an incredible number of human tumors have been identified, the HLA haplotype of these tumors is unknown. Several years ago, Wiedenfeld et al. [61] broached the question of whether the sites of *p53* missense mutation occurring in lung tumors outgrowing in HLA-A2+ individuals showed a potential for a disproportionate incidence of mutations occurring within putative HLA-A2-restricted p53 epitopes. Hoffmann et al. [47, 49] have shown that an interesting inverse correlation appears to exist between the IVS responsiveness to the wt $p53_{264–272}$ peptide and/or frequencies of anti-wt $p53_{264–272}$ CTLs present in PBMCs obtained from HLA-A2+ patients with HNSCC and the mutational site/level of p53 expressed in their tumors. The results obtained from 30 patients tended to distinguish these individuals into 2 groups: nonresponsive and responsive. The tumor specimens obtained from these 30 patients were then analyzed for p53 accumulation by immunohistochemistry and genotyped for alterations in *p53* exons 5–8. The tumors of 'nonresponsive' patients had a p53 phenotype consistent with presentation of the wt $p53_{264–272}$

peptide (accumulation of mutant p53), while the 'responders' had tumors expressing normal levels of wt p53 and, presumably, a low potential to present the epitope. While several explanations are possible, these results are consistent with immunoselection of 'epitope loss' tumors. Another involves the possibility that the SCCHN tumors expressing wt p53 might also be HPV+. Presumably, in the presence of HPV E6, tumors need not accumulate mutant p53 to present the $p53_{264–272}$ peptide for CTL recognition [43].

Of the 30 tumor specimens analyzed, 15 had genetic alterations within *p53* exons 5–8. Six of the tumors had missense mutations within 3 CTL-defined epitopes, $p53_{217–225}$, $p53_{149–157}$ and $p53_{264–273}$, and 1 had a mutation at codon 273, which is known to block processing of the $p53_{264–272}$ peptide [34, 42]. The mutation in the $p53_{217–225}$ epitope, Y220C, was detected in 2 of the tumors. Codons 273 and 220 are considered p53 mutational 'hot spots'. Mutation at *p53* codon 273 is the most frequently detected *p53* mutation in human cancers (~12%), while mutation at codon 220 ranks 6th with a frequency of approximately 1% [18]. These values are independent of tumor type and do not take into account the HLA haplotype of the tumor. Consequently, it would appear that a disproportionate number of missense mutations are occurring within the regions encoding the wt $p53_{149–157}$ and $p53_{217–220}$ epitopes in HNSCC of HLA-A2+ patients. The question of whether the altered p53 sequences yield T-cell-defined mutant p53 epitopes needs to be studied. Of interest, however, is the fact that 2 of the 3 missense mutations detected that occurred within the $p53_{149–157}$ epitope were nonconserved amino acid exchanges at the anchor positions of the peptide. These mutant peptides, even if processed, would most likely be weakly immunogenic due to unfavorable amino acid substitutions at anchor residue positions and unable to induce a robust antimutant p53 immune response and tumor eradication. Overall, the skewed pattern of *p53* missense mutation in the tumors of HLA-A2+ patients with HNSCC strongly suggests that wt p53 peptides, although 'self' antigens, are surprisingly immunogenic. Given that mutation of *p53* is considered an early event in the development of HNSCC, the immunological pressure exerted by anti-wt p53 CTL cells could readily promote the outgrowth of 'p53 epitope loss' tumors. These observations imply that robust multiepitope anti-p53 immune responses are needed in order to minimize outgrowth of 'epitope loss' variants, which tumors can readily achieve this due to their inherent genetic instability and heterogeneity.

Active and Passive p53-Based Immunizations

As in any vaccine development program, the optimization of the immunogen and vaccine vehicle is critical. A wide range of p53-based vaccines and

immunization protocols has been evaluated. Murine studies have shown that effective anti-wt p53 T-cell-mediated antitumor responses could be induced by (1) wt p53 peptides or recombinant p53 protein admixed with chemical adjuvants or pulsed onto bone-marrow-derived DCs [22, 26], as well as (2) DCs transfected with nonviral plasmids or viruses encoding intact p53 or fragments [62–64]. In addition, p53 nonviral plasmid DNA vaccines biolistically (gene gun) delivered as well as recombinant viral vectors expressing p53 have also been shown to be effective in inducing antitumor immunity [65–67]. In vitro studies using PBMCs obtained from normal donors and patients have shown the utility of peptide or protein-pulsed DCs [36, 47, 60] or DCs transfected with recombinant adenoviral constructs expressing p53 for induction/expansion of anti-wt p53 CTL and Th cells [68]. Complementing these active p53-based immunizations has been the evaluation of adoptive transfer of anti-wt p53 CTLs and Th cells to induce tumor eradication. The use of high-affinity anti-wt p53 T cells derived from p53 null mice and HLA-A2.1-transgenic p53 null mice has been shown to be very effective in inducing tumor eradication [30, 31, 69]. Although of high affinity, these cells do not react with normal cells and did not induce any detectable evidence of autoimmunity. The concept of genetically engineering high-affinity anti-p53 human T cell effectors by transfecting PBMCs with cDNA encoding the T cell receptor derived from antihuman p53 murine CTLs is being actively pursued [70].

Clinical p53 Vaccine Trials

Several phase I/II studies on p53-based immunotherapy of cancer patients utilizing peptide-based or recombinant viral vectors expressing wt p53 are in progress. They are based on the promising results of preclinical murine tumor model studies using transplantable tumors that have repeatedly demonstrated the efficacy of peptide as well as recombinant viral vector-based p53 vaccines targeting mutant as well as wt p53 epitopes in tumor eradication. Of particular translational relevancy were the murine studies employing DCs pulsed with protein or peptide or transfected with adeno/p53 as vaccines.

The concept of replacing mutant p53 in a patient's tumor with functional wt p53 delivered using recombinant adenoviral/p53 constructs preceded the use of this construct in p53-based immunotherapy. An underreported aspect of replacement adeno/p53 gene therapy trials is whether any 'bystander' anti-p53 immune responses were induced in these patients receiving this gene therapy [71]. The great advantage of using recombinant protein or viral vectors encoding p53 as immunogen is that it permits multiple CTL and Th epitopes to be presented independently of knowing the identity of these epitopes and patients'

HLA haplotypes. The initial findings of phase I/II immunization trials of patients using either a recombinant adenovirus or canarypox virus encoding wt p53 have been reported on. Kuball et al. [72] detected antiviral immune responses but no anti-wt $p53_{264–272}$ peptide CTL responses in HLA-A2+ patients treated with a recombinant adeno/p53 vaccine. In contrast, a clinical vaccine trial involving patients with metastatic colon cancer using canarypox virus (ALVAC) encoding wt p53 administered intravenously induced or augmented humoral anti-p53 IgG responses in 3/16 patients and anti-p53 cellular responses in 4/16 [73, 74]. A clear distinction between the two trials is that the anti-p53 T cell responses in the latter trial were detected in assays using mixtures of overlapping wt p53 peptides to stimulate the T cells. The responses detected in these assays were independent of the patients' HLA haplotypes and identity of the epitopes. A third virus-based vaccine trial, which is modeled on murine studies using adenoviral/p53-transfected DC vaccine, is listed by the NCI for patients with lung cancer as being in progress.

One wt p53 peptide-based vaccine trial has been initiated using either DC or chemical adjuvants. This vaccine trial is for HLA-A2+ patients with low burden ovarian cancer, and the patients are receiving wt $p53_{264–272}$ peptide-based vaccines [75]. One group of 5 patients received the peptide pulsed onto autologous DCs and administered intravenously, while the second group of 6 patients received the peptide subcutaneously admixed with ISA-51 and granulocyte-macrophage colony-stimulating factor. Both groups of patients also received low-dose interleukin 2 for 10 days beginning with cycle 3 of the vaccination protocol. Immunological monitoring showed the induction in individuals of both groups of anti-wt p53 CTL responses in ELISPOT and tetramer assays. This was accompanied by increased progression-free survival times.

Conclusion

High stakes and expectations are associated with any experimental cancer therapy. The numerous concerns and issues associated with the development of immunotherapy of cancer are well documented. Advances in molecular immunology and improved and more detailed immunological monitoring of immunized patients are enhancing the development of cancer vaccines. Compared to many of the other tumor antigens being targeted, p53 is unique in many respects. It is truly a 'self' antigen and is expressed in all nucleated cells. Despite this, no evidence of deleterious autoimmune reactions has been detected in the patients participating in the initial phase I/II p53-based immunotherapy trials. Whether the 'autoimmune response' that signals the efficacy of some melanoma trials will also be evident in patients receiving

p53-based immunotherapy is a critical unknown [76]. In addition, p53 seems to have the ability to readily 'dodge the immunological bullet'. The very nature of the genetic instability that is initiated by genetic alterations in *p53* coupled with the pressure of immunoselection/immunoediting represents a combination of influences that promote tumor escape and are, at the very least, challenging. Nonetheless, the stakes are high and nothing is impossible. It is just more difficult than initially thought.

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p53 as an Immunotherapeutic Target in Head and Neck Cancer

Approaches to Reversing Unresponsiveness of T Lymphocytes and Preventing Tumor Escape

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Abstract

Squamous cell carcinomas of the head and neck (HNSCCs) are characterized by a high frequency of mutations in the *p53* gene often leading to p53 protein accumulation. Since accumulation of p53 is associated with enhanced presentation of wild-type sequence (wt) p53 peptides to immune cells, the development of 'pan' vaccines against HNSCC has focused on wt p53 epitopes. We used the HLA-A2.1-restricted wt $p53_{264–272}$ epitope pulsed on autologous dendritic cells to generate cytotoxic T lymphocytes (CTLs) ex vivo from circulating precursor T cells of HLA-A2.1+ patients with HNSCC. CTLs specific for the wt $p53_{264–272}$ peptide were generated from leukocytes obtained from a cohort of patients with HNSCC (group A). Paradoxically, none of those patients had tumors which adequately presented the epitope, i.e. accumulated p53. In contrast, patients who did not generate CTLs (group B) had tumors which accumulated altered p53 and potentially could present the $p53_{264–272}$ epitope. When $p53_{264–272}$-specific T cells were directly enumerated in the peripheral circulation of patients with HNSCC using tetrameric $p53_{264–272}$/HLA-A2.1 complexes by multicolor flow cytometry, group A had high and group B low percentages of tetramer+ CD3+ CD8+ T cells. These findings suggested that in vivo p53-specific CTLs in group A might play a role in the elimination of tumor cells expressing the $p53_{264–272}$ epitope ('immunoselection'), leading to the outgrowth of 'epitope loss' tumor cells. On the other hand, precursor CTLs specific for the wt $p53_{264–272}$ peptide in group B are unresponsive to the p53 antigen. Unresponsiveness of CTLs specific for the wt $p53_{264–272}$ peptide detected in group B could be reversed by using more immunogenic variant peptides of the $p53_{264–272}$ epitope. In vivo, immunoselection of tumors which become resistant to anti-p53 immune

responses has important implications for future p53-based vaccination strategies. It calls for modified approaches, in which altered peptide variants of the wt sequence $p53_{264–272}$ epitope are used in a vaccine in order to overcome unresponsiveness of T lymphocytes to the native epitope.

The current treatment of squamous cell carcinoma of the head and neck (HNSCC) consists of surgery or combinations of surgery with radiotherapy and/or chemotherapy [1]. However, the prognosis of patients with HNSCC treated with these therapies has not changed for the last 30 years [2]. New therapies are needed to improve patient survival, and tumor vaccine development is considered a promising therapeutic strategy. Since missense mutations of *p53* occur in the majority of HNSCC [3–5], this tumor suppressor gene product could be an attractive candidate for antitumor vaccination strategies [6, 7]. Initially, the effort to develop p53-based vaccines focused on missense mutations, which are tumor specific in nature. However, they have limited clinical usefulness, because of the requirement that they need to occur within epitopes which could be presented to immune cells by human leukocyte antigen (HLA) molecules expressed by the individual patient. On the other hand, the majority of *p53* mutations involve the alteration of a single amino acid. Therefore, in tumors accumulating p53, the majority of p53 epitopes processed and presented to immune cells can be expected to be wild type in sequence and could be candidates for the use in the development of broadly applicable cancer vaccines [8–17].

To evaluate the quality of immune responses specific for the HLA-A2.1-restricted, human wild-type sequence (wt) epitope $p53_{264–272}$ in patients with HNSCC, T cells specific for this epitope were generated ex vivo and evaluated relative to the presence of *p53* gene mutations and protein expression in the patients' tumors. In addition, $p53_{264–272}$-specific T cells were directly enumerated in the peripheral circulation of patients with HNSCC, using novel tetrameric $p53_{264–272}$ peptide/HLA-A2.1 complexes in multicolor flow cytometry. The data revealed an unexpected dichotomy in T cell responses to wt $p53_{264–272}$ epitope among patients with HNSCC that was related to p53 expression in the tumor. In this paper, we consider the significance of this dichotomy for the success of future p53-peptide-based vaccines in these patients.

Methods

Generation of Anti-p53 Cytotoxic T Cells using Peptide Pulsed Autologous Dendritic Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from HLA-A2.1+ HNSCC patients and healthy individuals. Dendritic cells were generated from monocytes in the

presence of granulocyte-macrophage colony-stimulating factor + interleukin (IL) 4, pulsed with the $p53_{264–272}$ peptide (LLGRNSFEV) and cocultured with autologous PBMCs in the presence of low doses of IL-7 and IL-2. After weekly in vitro stimulation (IVS), the reactivity of generated T cells was tested against various targets (peptide pulsed T2 cells and different HNSCC lines, which naturally present the $p53_{264–272}$ epitope) in γ-interferon ELISPOT as well as cytotoxicity assays. The specificity was confirmed in antibody-blocking experiments and confirmed by tetramer staining.

Tetrameric Peptide/HLA-A2.1 Complexes ('Tetramers')

The streptavidin-phycoerythrin-labeled tetramers were applied in 4-color flow cytometry assays as previously described by us in detail [18].

p53 Analysis in HNSCC

Exons 5–8 of the *p53* gene were analyzed using a PCR-based technique [19]. For p53 protein, immunohistochemistry with D0-7 (Dako) was performed.

Variant Peptides

The basis for the selection of variant peptides (Protein Identification Resource, National Biomedical Research Foundation, Washington, D.C., USA) for synthesis was a bias towards retention of a high degree of similarity to $p53_{264–272}$ in the central region of the peptide.

Results

In vitro Generation of Anti-p53 CTLs

Generated T cells were HLA class I restricted and reacted against T2 cells pulsed with $p53_{264–272}$ peptide and, to a lesser extent, against HLA-A2.1 matched HNSCC cell lines which naturally present the epitope [20]. In contrast, HNSCC cell lines, which do not express the epitope, were only minimally lysed, and their killing was not blocked by anti-HLA-A2 antibody. Tetrameric $p53_{264–272}$/HLA-A2.1 complexes were used to confirm the anti-$p53_{264–272}$ specificity of CTLs present in bulk IVS cultures. In 1 HNSCC patient, up to 35% out of all CD8+ lymphocytes were found to be CD8+/tetramer+ after IVS.

Table 1 summarizes the results obtained with PBMC of HNSCC patients after IVS with the $p53_{264–272}$ peptide. T cells of 3/6 patients with HNSCC were found to be reactive against peptide-loaded T2 cells as well as against tumor cells naturally presenting the wt $p53_{264–272}$ epitope. This reactivity was HLA class I restricted, as it was blocked by anti-HLA-class-I or anti-HLA-A2 antibody.

p53 Analysis in Patients' Tumors and Association with Anti-p53 Response

Immunohistochemistry for p53 as well as sequencing of the *p53* gene in the patients' tumors were performed in order to investigate a possible association between the presence of a CTL response specific for the $p53_{264–272}$ epitope

Table 1. Summary of ex vivo anti-p53 responses in patients with HNSCC [20]

Patients	p53 status		Anti-p53 response after IVS			
	tumor *p53* genotype	tumor p53 protein	parental wt $p53_{264–272}$	variant $p53_{6T}$	variant $p53_{7P}$	variant $p53_{7W}$
1	wt in exons 5–8	–	+	–	–	+
2	wt in exons 5–8	–	+	–	–	–
3	mutation in exon 8	accumulation[1], no presentation	+	–	–	+
4	mutation in exon 7	accumulation	–	–	–	–
5	mutation in exon 5	accumulation	–	–	–	–
6	mutation in exon 8	accumulation	–	–	–	+

PBMCs were obtained from HNSCC patients and stimulated with the peptide-pulsed autologous dendritic cells or PBMCs in 2–4 IVS cycles. Effector cell reactivity was tested in ELISPOT or cytotoxicity assays, and T cell specificity for the $p53_{264–272}$ epitope was confirmed using tetramer technology. – indicates that there was no specific effector cell reactivity against the $p53_{264–272}$ peptide; + indicates that specific reactivity was observed against the $p53_{264–272}$ peptide. For T cells which were stimulated with a variant peptide, + indicates that specific reactivity against the variant peptide as well as cross-reactivity against the parental wt $p53_{264–272}$ epitope were observed.

[1]The R273H mutation has been shown to prevent processing of the $p53_{264–272}$ epitope [21].

and the p53 status of the tumor. Although it is generally considered that HLA-A2+ tumor target cells sensitive to lysis by CTLs recognizing the wt $p53_{264–272}$ epitope do accumulate mutant p53, this phenotype is not an absolute prerequisite for their recognition by the CTL. In particular, mutation at codon 273 is known to prevent the processing and presentation of $p53_{264–272}$ epitope, due to interference with the proteasome pathway [21] (table 1). Of the 3 patients who did not show CTL responses to the wt $p53_{264–272}$ epitope (No. 4, 5 and 6), mutations in *p53* exons 5–8 were detected in all 3 tumors. The tumors of patients 4, 5 and 6 accumulated p53 and presumably could present the epitope. In contrast, among the 3 patients who showed CTL responses (No. 1, 2 and 3), patients 1 and 2 had tumors with the wt in exons 5–8 of the *p53* gene and no p53 accumulation. The tumor of patient 3 accumulated p53 expressing a

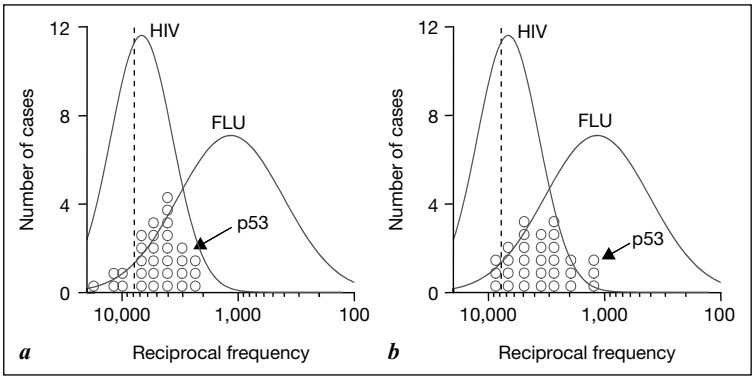

Fig. 1. Reciprocal frequencies of $p53_{264–272}$ tetramer+ CD8+ CD3+ T cells in the peripheral circulation of normal donors (***a***) versus patients with HNSCC (***b***) [22]. Patients had higher frequencies of such tetramer+ T cells than normal donors. Open circles show individual data points. Superimposed normal distribution curves were calculated from sample means and standard deviations of HIV and FLU tetramer data from the normal control group. The dashed line represents the cutoff for the lower detection limit of tetramer frequency.

missense mutation at codon 273, known to prevent the processing and presentation of the $p53_{264–272}$ epitope [21]. All 3 tumors were, therefore, unlikely to present the wt $p53_{264–272}$ epitope. Therefore, it would appear that the CTL response to the wt $p53_{264–272}$ epitope was demonstrable in patients bearing a tumor unable to present the epitope.

In vivo Frequency of $p53_{264–272}$-Specific T Cells in HNSCC Patients

We next extended the study to 30 HNSCC patients and 31 healthy individuals (all HLA-A2.1+) and determined the frequency of $p53_{264–272}$-specific T cells by multicolor flow cytometry, using tetramer technology. We determined that the mean of $p53_{264–272}$-specific T cells was higher than that of T cells specific for HIV (ILKEPVHGV, $pol_{476–484}$) but lower than that of those specific for influenza (GILGFVFTL, $FLU_{58–66}$) [22] (fig. 1).

Patients who responded to IVS with the $p53_{264–272}$ peptide had higher frequencies of $p53_{264–272}$-specific precursor T cells than ‘nonresponders’ (not shown). Furthermore, patients had significantly higher proportions of $p53_{264–272}$-specific CD8+ T cells in the circulation relative to normal donors (fig. 2). Patients with particularly high frequencies of wt $p53_{264–272}$-specific T cells had p53– tumors. In contrast, tumors of patients with very low frequencies of wt $p53_{264–272}$-specific T cells showed p53 overexpression in most cases (fig. 2).

Finally, we sought to increase the response rate obtained with the parental $p53_{264–272}$ peptide during IVS (fig. 3). In order to reverse the unresponsiveness

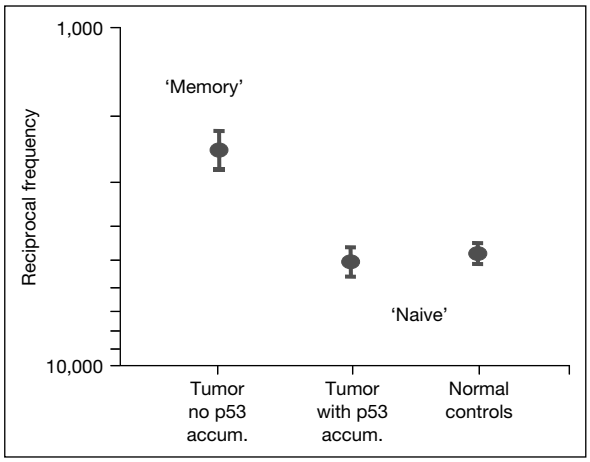

Fig. 2. Reciprocal frequency of $p53_{264–272}$-specific CD8+ T cells in patients with HNSCC and normal controls [22]. The mean frequencies for normal controls and for patients with HNSCC found to accumulate p53 or not to accumulate p53 were determined. PBMCs of 30 HLA-A2.1+ patients with HNSCC and 31 normal controls were evaluated. Tumors of these patients showed either (1) normal p53 protein expression, (2) accumulated p53 protein or (3) a mutation within or next to the $p53_{264–272}$ epitope, most likely preventing presentation of the epitope [21]. In this instance, the tumor was considered to have normal p53 expression since the $p53_{264–272}$ epitope could not be presented; bars indicate ±SD.

of T lymphocytes to the $p53_{264–272}$ epitope in patients whose tumors accumulate p53, we tried to increase the immunogenicity of the parental wt $p53_{264–272}$ epitope by introducing single amino acid exchanges at nonanchor positions. A total of 19 variants of the wt $p53_{264–272}$ peptide were synthesized and tested. Two of these variant peptides [substitutions at positions 6 (6T) and 7 (7W)] were found to be capable of inducing specific T cells in a proportion of normal donors and cancer patients, including an individual (patient No. 6, table 1) who did not respond to the parental peptide and whose tumor was able to present the p53 epitope. As determined by tetramer staining (fig. 4), ELISPOT assays for γ-interferon and cytotoxicity, these T cells were cross-reactive against the parental wt $p53_{264–272}$ epitope pulsed on T2 target cells as well as naturally presented by tumors [23].

Discussion

Novel therapeutic strategies for patients with HNSCC include the development of vaccines. An immunogenic antigen is a requirement for a vaccine. Using

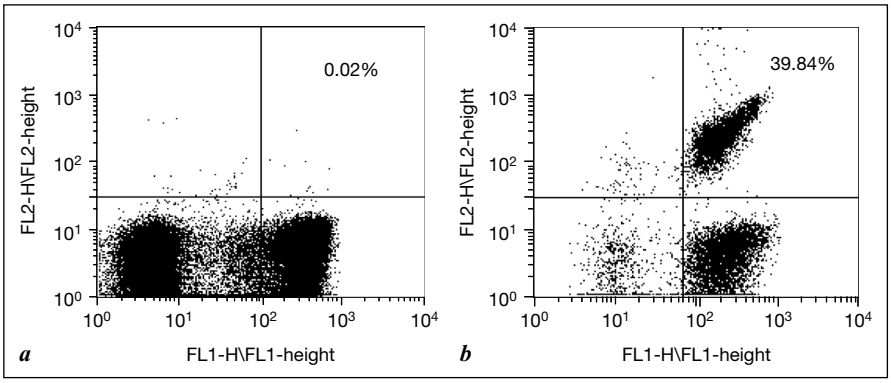

Fig. 3. Staining of T cells with tetrameric peptide/MHC-class I complexes before (***a***) and after IVS (***b***; 4 × IVS with 270W) with 7W variant peptide [23]. Cells were progressively gated by forward and side scatter for lymphocytes and CD3+/CD8+ T cells. Gated populations are plotted as CD8 staining (horizontal axis) versus tetramer staining (vertical axis). Tetramer staining was performed with p53 variant peptide 7W/HLA-A2.1 complexes. The upper right quadrant shows the percentage of tetramer+ T cells.

apoptotic tumor cells [24] or a known tumor-associated antigen, wt $p53_{264–272}$ [20], we were able to induce antitumor responses in vitro from PBMCs of normal donors. However, it was not always possible to induce an antitumor response with PBMCs obtained from HNSCC patients. In the case of p53, we expected that PBMCs of HNSCC patients whose tumors accumulate p53 would readily generate anti-p53 responses, while PBMCs of patients whose tumor cannot present the $p53_{264–272}$ epitope would not. Surprisingly, anti-p53 CTLs were only generated from PBMCs obtained from patients with tumors unlikely to present the wt $p53_{264–272}$ epitope (wt *p53* expression or *p53* mutation which prevents presentation of the epitope) and not from PBMCs obtained from patients whose tumors accumulated mutant p53 and most likely could present the epitope. These findings suggest that in vivo, preexisting CTLs specific for the wt $p53_{264–272}$ peptide play a role in the elimination of tumor cells expressing this epitope. This type of immune selection by the preexisting CTLs could lead to the outgrowth of 'epitope loss' tumor cells, as illustrated in figure 4.

The observed reciprocal relationship between the frequency of $p53_{264–272}$-specific T cells in the patients' peripheral circulation and the p53 tumor status lends further credence to the immunoselection hypothesis. In the presence of wt $p53_{264–272}$-specific T cells, epitope loss tumor variants are selected in vivo during tumor progression [22]. Immunoselection contributes to tumor escape from the immune system, and thus has important implications for future p53-based immunization strategies. Patients whose tumors have lost expression of the targeted

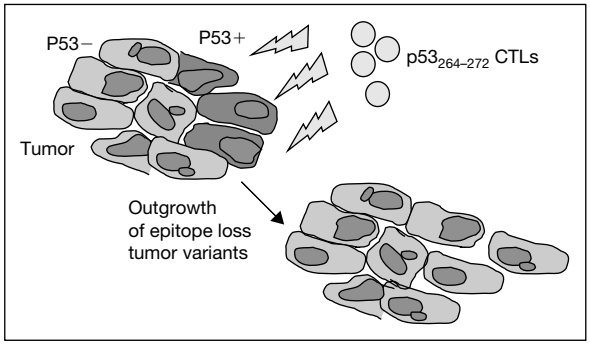

Fig. 4. A model for immunoselection of epitope loss tumor variants. The initial tumor is heterogenous in p53 expression, consists of a p53 heterogeneity with some cells accumulating p53 (p53+) whereas others do not accumulate p53 (p53–). The p53-accumulating cells are subsequently subject to physiologically significant selection pressure by certain expandable $p53_{264–272}$-specific CTLs in situations where such CTLs arise in vivo. This subsequently could lead to the outgrowth of p53– tumors.

epitope represent unlikely responders to p53-based vaccines. On the other hand, PBMCs of patients whose tumors had a high potential to express the target antigen also had low numbers of p53-specific precursor cells, which were not expandable by IVS. These cells can be considered to have been tolerized in vivo [25–27]. It appears that it might be necessary to break tolerance of immune cells to the self-antigen, $p53_{264–272}$ epitope, to achieve responses to a p53-based immunotherapy in many patients with HNSCC. A potential strategy for this is to seek and use a potent immunogen. We, therefore, sought to increase the immunogenicity of the wt $p53_{264–272}$ peptide by introducing single amino acid exchanges and indeed were able to increase response frequencies in vitro [23]. Most importantly, these variant peptides or altered peptide ligands were found to be capable of inducing specific T cells in a proportion of PBMCs obtained from HNSCC patients who did not respond to the parental peptide. Significantly, this was the case in a patient whose autologous tumor was able to present the p53 epitope.

The possibility that immunoselection of epitope loss tumor cells might occur during p53-based immunotherapy has to be considered in designing future clinical trials. In this context, the use of vaccines capable of targeting multiple tumor-associated antigens might be necessary to prevent tumor escape from the immune system [28].

Finally, the use of variant peptides of the wt $p53_{264–272}$ epitope represents a promising approach to overcoming the unresponsiveness of certain cancer patients to this self-epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients.

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Imbalance in Absolute Counts of T Lymphocyte Subsets in Patients with Head and Neck Cancer and Its Relation to Disease[1]

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Abstract

Apoptosis of circulating CD8+ T cells seen in patients with squamous cell carcinoma of the head and neck (HNSCC) suggests a possibility of lymphocyte imbalance. Therefore, absolute numbers and percentages of T lymphocyte subsets were examined in the peripheral blood of patients with HNSCC and age-matched controls. Venous blood was obtained from 148 patients with HNSCC and 54 normal volunteers. Absolute numbers of CD3+, CD4+ and CD8+ T lymphocytes were determined using fluorobeads in a flow-cytometry-based technique. Percentages of T lymphocyte subsets were also evaluated by flow cytometry. The patients were grouped, at the time of blood draw (active vs. no evident disease, NED), type of therapy administered and the length of follow-up. Patients with HNSCC were found to have significantly lower absolute numbers of CD3+, CD4+ and CD8+ T cells than normal controls (NC). However, no differences in the percentages of T cell subsets between patients and NC were observed. Patients with active disease had significantly lower CD3+ and CD4+ T cell counts than those with NED. Patients with NED after surgery and radiotherapy had lower T cell counts than those treated by surgery alone. Patients who remained without evident disease for more than 2 years did not recover their T cell counts, and the T cell imbalance was evident many years after curative surgery. Patients with recurrent disease at the time of blood draw tended to have the lowest CD4+ T cell counts. The TNM stage or site of the disease were not related to the absolute T cell count. Our data indicate that patients with HNSCC have altered lymphocyte homeostasis, which persists for months or years after curative therapies.

[1]The data presented and text originate in part from the paper by Kuss et al. entitled 'Decreased absolute counts of T lymphocyte subsets and their relation to disease in squamous cell carcinoma of the head and neck', Clin Cancer Res 2004;10:3755–3762.

Despite the constant input of new lymphocytes from the bone marrow and the thymus and the exponential generation of specific cells in response to antigens, the size of the peripheral lymphocyte pool remains relatively stable. To maintain homeostasis, the peripheral blood T lymphocyte pool is regulated in complex ways that have not yet been fully defined [1]. In general, clonal expansions of specific cells are balanced by programmed cell death in other subpopulations and the maintenance of a relatively constant total peripheral blood T lymphocyte count. We have reported previously that patients with squamous cell carcinoma of the head and neck (HNSCC) as well as melanoma and breast cancer have increased proportions of circulating T cells that bind annexin V and are, therefore, destined to apoptose [2–5]. Due to this extensive apoptosis in the peripheral circulation and concomitant repopulation of the blood compartment with T cells from the immature cell pool, rapid turnover of effector cells takes place that is reminiscent of that described for patients with HIV [6, 7]. These observations suggest that the evaluation of the proportions and absolute numbers of lymphocytes and their subsets in the peripheral circulation of cancer patients is important and might provide insights into the redistribution of those lymphocyte subsets that mediate antitumor defense.

In order to evaluate a possible impact of spontaneous apoptosis of circulating T lymphocytes [2–5] on the peripheral T cell pool, we investigated *both* the percentages and absolute numbers of CD3+, CD4+ and CD8+ T lymphocyte subsets in a large cohort of patients with HNSCC, using a single-platform flow-cytometry-based method. The study objectives were: (a) to determine whether absolute T cell numbers and percentages are significantly decreased in patients relative to normal controls (NC); (b) to correlate the imbalance in absolute T cell counts to disease activity and oncologic therapy, and (c) to obtain insights into a possible predictive role of T cell counts in patients with HNSCCHN. We found that the presence of tumor as well as its recurrence had a significant impact on the absolute number of T cell subsets. Both the type of therapy and length of the posttherapy period were considered, and the results suggested that regardless of these factors, lymphopenia was a persistent feature of the disease.

Material and Methods

Patients and Controls

148 patients with HNSCC, who were consecutively seen between July 2001 and June 2003 at the Outpatient Otolaryngology Clinic at the University of Pittsburgh Oral Cancer Center were entered into the study. The Institutional Review Board has approved the protocol for collection of patient blood samples. Normal healthy donors (NC) were recruited among the laboratory personnel, family members of patients and other volunteers, with an intent to match controls and patients for age. Subjects who served as NC were interrogated

Table 1. Clinical characteristics of HNSCC patients at the time of blood draw (n = 148)

Active disease (47)
Surgery for recurrence (7)
Surgery of primary disease (34)
Recurrent disease (6)
NED (101)
After surgery alone (73)
<2 years (40)
>2 years (33)
After surgery + RT (28)
<2 years (14)
>2 years (14)

The numbers of patients are indicated in parentheses.

for the general state of health, use of medications, smoking and alcohol consumption. A written informed consent was obtained from each individual participating in this study.

The clinical characteristics of the patients included in this study are shown in table 1. The cohort of 148 patients included 111 men and 37 women with a median age of 62 years (range 24–86), and the group of 54 volunteers comprised 25 males and 29 females with a median age of 54 years (range 22–88).

Collection of Blood Samples

Venous blood (10 ml) was obtained from the patients either in the morning before surgery or during the outpatient visits and prior to any therapies. Blood samples were collected into heparinized tubes. Samples were also collected from age-matched NC. Blood samples were hand-carried to the laboratory and immediately used for experiments.

Absolute Lymphocyte Count Determination

A standard single platform technique, the tetraONE™ System (Beckman Coulter, Miami, Fla., USA), based on 4-color flow cytometry in the presence of counting beads was used. The identification of lymphocytes by expression of bright CD45 and low side scatter signals was followed by the identification of T cell subsets based on the expression of CD3, CD4 and CD8, as previously described [8]. The one-platform method was validated in our laboratory by comparisons with the previously established two-platform method.

Statistical Analysis

Differences in percentages and counts of lymphocyte subsets between patients and normal controls were age adjusted by linear regression models after suitable data transformations. If age adjustment was unnecessary, differences were tested with the t test or the Wilcoxon test. The associations among lymphocyte subsets and clinical (disease status, site of disease), pathologic (stage) and behavioral (smoking) characteristics were tested with the t test or Wilcoxon test for 2-group differences or the Kruskal-Wallis test for 3 or more groups. Tests of trend with ordinally scaled endpoints such as T and N stages were conducted with the Jonckheere-Terpstra test.

Table 2. Absolute numbers and percentages of T cells and T cell subsets in patients with HNSCC and NC

	CD3+	CD4+	CD+
Absolute number			
Patients (n = 148)	1,081 ± 601	670 ± 412	392 ± 269
NC (n = 58)	1,512 ± 494	1,005 ± 360	476 ± 208
p value	<0.0001	<0.0001	0.0012
Percentages			
Patients (n = 148)	71 ± 9	44 ± 9	26 ± 11
NC (n = 58)	70 ± 9	47 ± 9	22 ± 7
p value	0.6374	0.1141	0.0917

The data are means ±SD. The p values are for the differences between patients and NC.

Results

Analysis of T Cell Subsets in Patients and NC

Using the single-platform method, we initially compared both the percentages and absolute numbers of CD3+, CD4+ and CD8+ cells in all patients to those in NC. Although percentages of T lymphocyte subsets were not found to be age associated, the absolute counts of all subsets decreased with age. For this reason, all comparisons of absolute lymphocyte counts in patients versus NC were adjusted for age. In comparing patients with NC, no significant differences were observed in the percentages of T cell subsets (table 2). However, all absolute T cell numbers were significantly different in patients versus NC. The patients had 382 fewer CD3+ cells, 297 fewer CD4+ and 85 fewer CD8+ cells per cubic millimeter than NC. These significant differences ($p < 0.0001$ for CD3+ and CD4+, $p < 0.0012$ for CD8+ cells) were detectable at any age. Overall, the patients had significantly lower absolute T cell counts but not percentages than NC, and the absolute numbers of CD8+ T cells were the least depressed in patients with HNSCC relative to NC.

Comparisons of CD4 and CD8 T Cell Counts in Patients and NC

Although the mean CD4/CD8 ratio was not different between patients and NC, we undertook a more detailed analysis of the relationship between the numbers of CD4+ and CD8+ T lymphocytes in individual patients, as shown in figure 1. Relative to NC with the mean CD4/CD8 ratio of 2.4 (fig. 1a), the

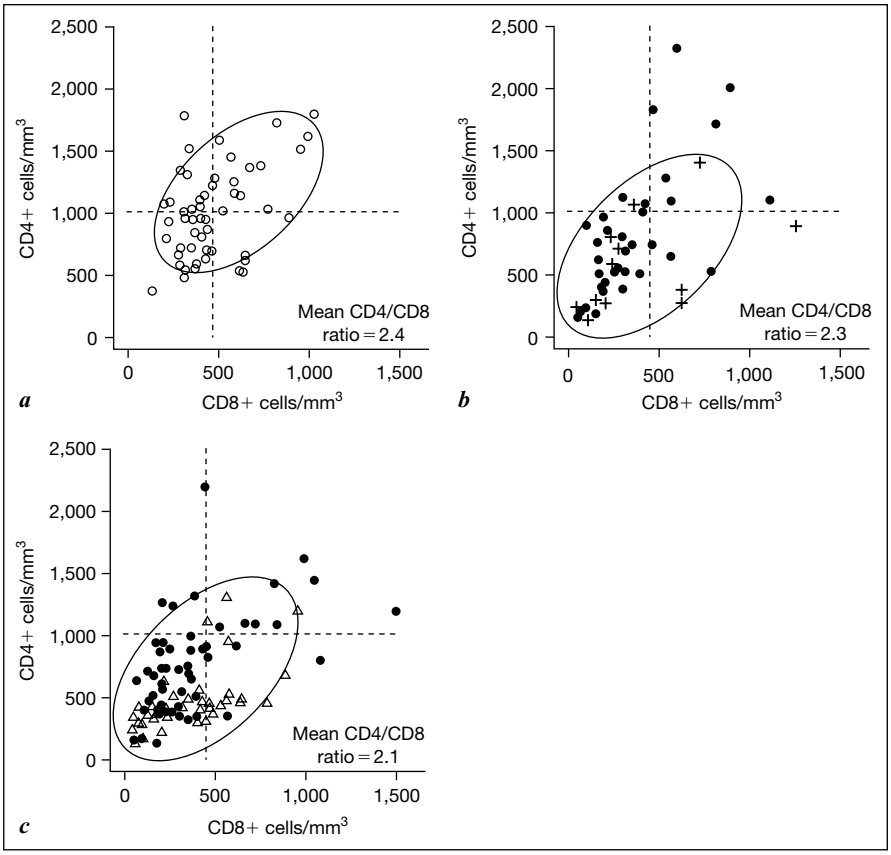

Fig. 1. Distribution of the individual CD4+ and D8+ T cell numbers among NC (***a***), HNSCC patients with active disease (***b***) and HNSCC patients with NED (***c***). The horizontal and vertical reference lines indicate mean values for CD4+ and CD8+ T cells of NC tested in the same assays as the patients. The circles indicate the prevalent distribution of cell counts. Note very low numbers of both CD4+ and CD8+ T cells in ***b*** and ***c***. In ***b***, patients treated surgically for disease recurrence or second primary tumors are indicated by a cross. In ***c***, patients treated with postoperative radiotherapy are denoted by a triangle.

patients with active disease, whose mean CD4/CD8 ratio was 2.3, had decreased numbers of CD4+ as well as CD8+ T cells. As a result, there was a dramatic shift of the individual CD4 and CD8 counts to the left lower quadrant in figure 1b. Not surprisingly, the patients with recurrent disease had the lowest absolute counts of CD4 and CD8+ T cells (fig. 1b). Among the patients with active disease, a small subgroup (8/47) with high numbers of CD4+ T

cells and relatively normal counts of CD8+ T cells (right upper quadrant in fig. 1b) is evident. A somewhat different relationship emerges when CD4+ and CD8+ T cells are examined in the patients who at the time of blood draws had no evident disease (NED) either after curative surgery or surgery plus radiotherapy (fig. 1c). Here, it can be seen that patients previously treated with radiation therapy (RT) in addition to surgery had the lowest CD4+ counts. Other NED patients had somewhat depressed CD4+ counts but normal CD8+ counts when compared to NC (fig. 1a).

Effects of Postoperative Radiation on Absolute Lymphocyte Counts

Among 101 patients with NED at the time of blood draws, 28 had received postoperative RT. The postoperative RT consisted of 66–70 Gy divided into 1.8- to 2.0-Gy fractions over 35 individual daily sessions. This RT was applied within 6 weeks of surgery. The time duration between the last RT and a blood draw for this study was variable, ranging from 1 month to more than 10 years. The patients who had RT also had significantly lower absolute numbers of CD4+ T cells ($p < 0.0001$) as well as CD3+ T cells ($p = 0.0001$) as compared to 73 NED patients without prior RT (fig. 2). In contrast, the count of CD8+ T lymphocytes remained unchanged after RT, an indication that CD8+ T cells were not sensitive to RT. Nevertheless, it should be noted that the NED patients treated with surgery alone also had comparably low CD4+ and CD8+ T cell numbers, and, thus, RT was only one of the factors contributing to T cell cytopenia in the patients.

Effects of Surgery on Absolute Lymphocyte Counts

The cohort of 73 NED patients who underwent curative surgery alone were divided into those treated more than 2 years before the blood draw and those studied within 2 years of surgery. As seen in figure 3, the absolute numbers of the T cell subsets were significantly decreased in both groups relative to NC (fig. 1a; $p < 0.0001$). The data suggest that effects of the tumor on the homeostasis of lymphocytes are observed long after the tumor has been removed and in the absence of any other lymphoablative therapy.

Lymphocyte Counts and Disease

Among 47 patients with active disease at the time of blood draw prior to surgery, 34 were diagnosed as having primary tumors and 13 as recurrent or second primary tumors (table 1). CD4+ and CD8+ T cell counts were significantly depressed in both these groups relative to normal controls (fig. 1b). However,

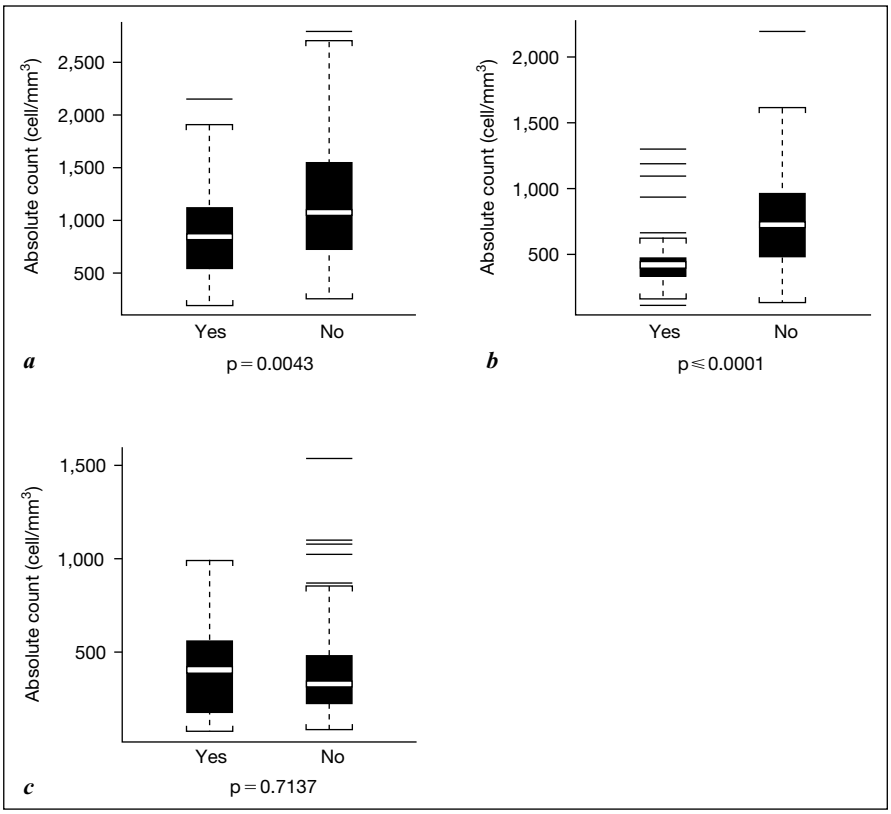

Fig. 2. Box plots showing effects of postoperative RT on the absolute numbers of CD3+, CD4+ and CD8+ T cells in patients with HNSCC. The absolute counts of patients who received RT are compared to counts in the patients treated with surgery without RT. The bars are median values, the box indicates the interquartile range (25–75%), and the 'whiskers' extend to 1.5 times the interquartile range.

patients with documented disease recurrence or second primary tumors at the time of blood draw had the lowest CD4+ T cell counts of all the groups studied ($p = 0.0001$).

Neither the disease stage, site nor nodal involvement defined for these patients at the time of curative surgery had any discernible effects on absolute lymphocyte counts determined at the time of blood draws (data not shown). It has to be stressed, however, that blood draws for lymphocyte counts were obtained at different time points relative to surgery and in many cases, 4 or more years after curative therapy.

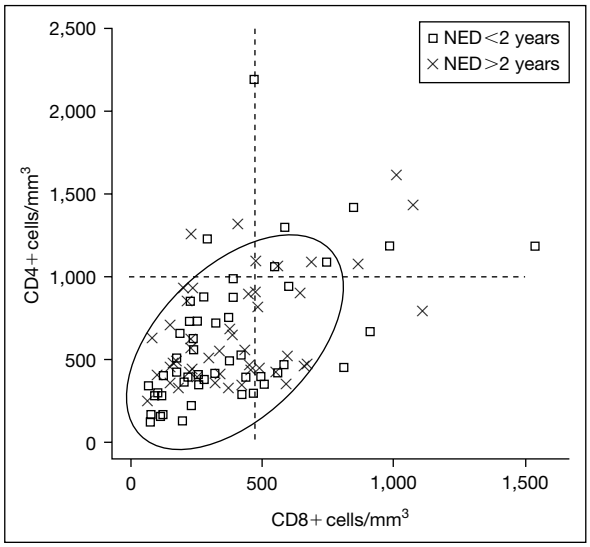

Fig. 3. Absolute counts of CD4+ and CD8+ T cells in individual patients with NED after curative surgery. The patients studied <2 years after surgery are represented by open squares, while those studied >2 years after surgery are represented by x. Note low absolute counts of both CD4+ and CD8+ T cells in the patients with NED regardless of the length of follow-up. No difference in the CD4+/CD8+ ratio was observed between these two groups of NED patients ($p = 0.56$). The stippled lines represent the mean values for CD4+ and CD8+ T cell numbers in normal donors.

Discussion

Patients with cancer who do not receive conventional therapies are not generally considered to be lymphopenic. Nevertheless, abnormalities in T cell counts have been observed in patients with breast cancer, ovarian cancer, myeloma, head and neck cancer or liver cancer [9–11]. More importantly, some reports show associations of T cell subset abnormalities with poor clinical outcomes [12–14]. However, there is no consensus on the extent of changes in different T lymphocyte subsets in the course of cancer progression or its relationship with response to tumor-specific therapy or patient survival.

The rationale for the study of absolute T-cell counts in patients with HNSCC was based on our earlier reports of significant levels of spontaneous apoptosis consistently observed among circulating T lymphocytes in these patients [2, 3] and suggesting that their absolute lymphocyte counts might be depressed. Through the use of single-platform flow cytometry it was possible to independently analyze the percentages and absolute numbers of the

T cell subsets in the patients' peripheral circulation. Interestingly, absolute numbers of CD3+, CD4+ and CD8+ T cells were found to be significantly decreased in the patient group, emphasizing the existence of a relative lymphopenia in the patients versus age-adjusted NC, despite the apparently normal percentages of circulating T lymphocytes. This observation emphasizes the necessity of determining absolute counts, not percentages, of lymphocyte subsets in patients with cancer. The usually reported cell percentages are misleading, because they do not consider the total white blood cell count, which might be, and frequently is, altered in these patients particularly after anticancer therapies.

The most interesting finding of this study, was the observation that the absolute count of CD8+ T cells, although significantly decreased in the patients versus NC, and especially so in patients with active disease, appeared to recover and normalize in most patients with NED, while the CD4+ T cell count did not. We had expected to find significantly decreased numbers of circulating CD8+ T lymphocytes rather than CD4+ T lymphocytes in these patients based on previous data of selective apoptosis of CD8+ T cells [2, 3]. A possible explanation for this unexpected finding could be that the homeostatic mechanisms compensate for the selective apoptosis by a rapid expansion of CD8+ T cells in the periphery of patients with HNSCC. Our data reported here and elsewhere [15] indicate that the homeostasis of CD8+ T cells in the peripheral circulation of patients with HNSCC appears to be maintained by their rapid turnover, resulting in a relatively stable mean peripheral CD8+ cell count for the patient population as a whole. A situation associated with profound depression of the peripheral CD8+ T cell count occurred in patients with stage IV disease, who failed to normalize the CD8+ T cell count after surgery. This observation suggests that in advanced disease, patients might fail to effectively compensate for the loss of effector cells. Patients with documented recurrence of disease or second primary tumor at the time of blood draw had the lowest T cell counts of all studied. The observed trend was for CD8+ T cell counts to increase and for CD4+ T cell counts to decrease after tumor removal by surgery. But one of the most interesting findings of this study was that HNSCC patients who remained without evident disease for 2 years or >2 years after curative surgery alone (no radio- or chemotherapy) still showed an imbalance in the T cell subsets. This imbalance was highly significant for CD4+ T cells, while CD8+ T cells tended to normalize. This observation suggests that the disease process has a profound and long-lasting impact on T lymphocyte homeostasis in patients with HNSCC. Others have suggested previously that lymphocyte counts have prognostic value in HNSCC [16].

Another factor that had a significant impact on T cell counts was the history of previous RT. Only CD4+ but not CD8+ T cells appeared to be

affected by previous RT (fig. 4). While this has been previously reported [17], the finding of decreased counts of CD4+ T cells long after the administration of RT was somewhat surprising. Apparently, CD4+ T cells are not only more sensitive to RT than CD8+ lymphocytes, but the restoration of the peripheral CD4+ T cell pool after RT is very slow. As indicated above, many patients with NED and no previous RT also had reduced CD4+ T cell counts long after curative surgery. Thus, RT was not the only factor modulating the number of circulating CD4+ cells in a cohort of patients with HNSCC previously treated for their disease.

While the current study was not designed to address survival as endpoint, we considered the possibility that disturbed lymphocyte homeostasis might be especially prominent in patients with poor prognosis, i.e. stage IV disease and nodal metastases. However, we could not establish a statistically significant relationship between disease stage or its severity and lymphocyte counts based on retrospective data analysis. It should be noted that no predictive value for absolute lymphocyte counts or CD4/CD8 ratios for larynx preservation, response to therapy or survival of patients with HNSCC was seen in another study [18].

Our results for patients with HNSCC are complementary to the findings of others who also report lymphocyte imbalance in cancer [11–14]. Combined with the presence of functional abnormalities in T cells of patients with HNSCC, as reported by us previously [3, 5, 19–21], the overall impression is that of decreased immune competence in these patients. Lowered T cell numbers in the circulation could predispose the patients to infections, disease recurrence or a second malignancy. It is, therefore, advisable to pay attention to T cell counts during posttreatment visits, even when the patients have NED long after curative therapy. Our data emphasize that decreased T cell counts reflect effects of the disease process on T cell homeostasis and not just therapy-mediated alterations and that the imbalance is long-lasting. In view of the possibility that such persistent changes in homeostasis of T cell subsets might adversely influence antitumor responses and promote recurrence. Therapies designed to increase lymphocyte counts could be considered even in patients with NED.

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Antitumor Immunization of Head and Neck Squamous Cell Carcinoma Patients with a Virus-Modified Autologous Tumor Cell Vaccine

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Abstract

Background: Head and neck squamous cell carcinomas (HNSCCs) are aggressive tumors with poor 5-year survival rates, thus demanding new treatment concepts. **Methods:** In a nonrandomized study, 20 HNSCC patients were preconditioned with interleukin (IL) 2 and subsequently vaccinated with virus-modified autologous tumor cells prepared from short-term tumor cultures. Antitumor reactivity was determined by delayed-type hypersensitivity (DTH) skin reaction. **Results:** Preconditioning of tumor patients with IL-2 prior to vaccination was associated with an increased number of T cells especially after a radiation-induced marked decrease, and levels of mitogen stimulation capacity were almost as high as before surgery. MHC class I molecules expressing autologous tumor cell cultures were successfully infected. Vaccination with virus-modified tumor cells was able to increase systemic antitumor reactivity as revealed by augmentation of DTH reactivity to unmodified tumor cells. **Conclusion:** We provide evidence that a combination of preconditioning of HNSCC patients with IL-2 to improve their immune competence with subsequent vaccination with virus-modified autologous tumor cells leads to augmented antitumor DTH reactivity.

The incidence of malignant tumors of the head and neck (HNSCC) has increased all over the world during the last decades [1, 2]. Despite progress in surgical techniques and an improved application of radio- and chemotherapy,

the mortality due to these tumors is still high, therefore demanding the development of new treatment modalities.

Immunotherapy of HNSCC is very appealing because it offers the potential for tumor specificity with low side effects [3, 4]. Certain proteins are selectively overexpressed in HNSCC [5], making these tumor antigens possible targets for immune recognition and reaction. In an animal model, it was shown that postoperative vaccination with irradiated, modified tumor cells could cure about one third of the animals, even at the time when the tumor had already metastasized to the first draining lymph nodes [6].

For optimal efficacy, tumor cell vaccines require the addition of danger signals by adjuvants to increase their immunogenicity [7]. For this purpose, we used Newcastle disease virus (NDV) [8], an avian paramyxovirus that does not integrate into the host DNA and is well known for its low pathogenicity. It selectively replicates in tumor cells and exhibits pleiotropic immune-modulatory properties [9]. In the infected cells, it induces danger signals such as double-stranded RNA, interferons and chemokines [10]. Our strategy of tumor vaccine design is based on the use of patient-derived autologous live tumor cells from cell culture (ATV), which may express common as well as unique tumor-associated antigens. For vaccine production, 10 million cells are infected with NDV and then inactivated by irradiation. When applied intradermally, such an ATV-NDV vaccine is well tolerated and can induce delayed-type hypersensitivity (DTH) skin reactions. Table 1 contains a summary about postoperative long-term survival of patients treated with such a vaccine. Over the last 10 years, 9 phase II clinical studies were performed on different tumor types, and improvements of survival were observed in most of them.

With regard to HNSCC, it is well documented that these tumors release substances that cause immunosuppression, such as transforming growth factor β and interleukin (IL) 10 [18]. In addition, surgery and postoperative radiation therapy are associated with a marked decrease in T lymphocytes [19, 20], which are of major importance for an efficient immunological antitumor response. Therefore our aim was first to rescue lymphocytes from radiation- and surgery-induced apoptosis by applying low-dose IL-2. When the T cell numbers improved, active specific immunization with the ATV-NDV vaccine was performed. Here we describe the effects of conditioning the patients with IL-2 and show some characteristics of the HNSCC-NDV vaccine.

Materials and Methods

Patients

Twenty patients with pathologically confirmed HNSCC were recruited from January 1996 to December 1997 to receive antitumor vaccination. Eligibility criteria included a

Table 1. Clinical studies with NDV-infected autologous tumor cells

Type of cancer	Type of study	Patients, n	Benefit	Reference
Colorectal, metastatic	phase II trial	23	improved DFS	Schlag et al. [11], 1992
Colorectal, locally advanced	phase II trial	57	improved OS	Ockert et al. [12], 1996
Breast, early	phase II trial	63	improved OS	Ahlert et al. [13], 1997
Ovary, metastatic	phase II trial	82	improved DFS	Ahlert et al. [13], 1997; Möbus et al. [14], 1993
Renal, metastatic	phase II trial	40	improved OS	Pomer et al. [15], 1995
Pancreas, stage G3	phase II trial	9	improved OS	Schirrmacher et al. [16], 1998
Stomach	phase II trial	7	improved OS	Schirrmacher et al. [16], 1998
Melanoma, recurrent metastatic	phase II trial	41	improved OS	Schirrmacher et al. [16], 1998
Glioblastoma multiforme	phase II trial	23	improved DFS and OS	Steiner et al. [17], 2004

DFS = Disease-free survival; OS = overall survival.

Karnofsky performance score of 60 or greater and normal baseline hematological parameters 2 weeks before the first vaccination (hemoglobin, total granulocyte count, platelet count, creatinine, transaminases and thromboplastin time), a patient age over 18 years and a written informed consent. Exclusion criteria were pregnancy, severe pulmonary, cardiac or other systemic disease associated with an unacceptable operative risk, presence of an acute infection, autoimmune disorders or other malignancies. Each patient received maximal surgical resection (20/20) followed by radiotherapy in 15/20 cases (total dose 60 Gy). Vaccination therapy was started 3 months after surgery, when radiotherapy was completed and the subsequent preimmunization period was finished. Patients were required to use a medically accepted form of birth control during the study. The study was approved by the institutional review board, in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Total Counts and Mitogen Stimulation Capacity of T Cells

At different time points, total T lymphocyte counts were determined in the peripheral blood by FACS analysis as described [20].

For mitogen stimulation tests, mononuclear cells were exposed for 3 days to different concentrations of concanavalin A and phytohemagglutinin (PHA) or for allogeneic stimulation incubated for 6 days in a mixed lymphocyte culture as described [19]. Then cells were pulsed with ^{3}H-thymidine, and incorporation was determined. Relative responses were calculated as ratio of counts per minute from tumor patients to counts per minute from healthy donors.

Autologous Tumor Cell Culture and Characterization

Tumor samples were mechanically dissected within 2 h after resection. The cell suspension was cultured in Dulbecco's minimal essential medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and antibiotics. We only used pretested serum batches from countries in which bovine spongiform encephalopathy has never been diagnosed. Mycoplasma contamination was excluded by 4,6-diamidino-2-phenylindole staining (Roche Diagnostics, Mannheim, Germany), and cells were routinely tested for lack of fungal and yeast contaminations. All short-term cultures were characterized for their epithelial origin by the immunohistochemical detection of tissue-specific markers using antibodies recognizing a broad spectrum of cytokeratins (clone MNF 116, Dako, Hamburg, Germany) and for expression of MHC class I molecules.

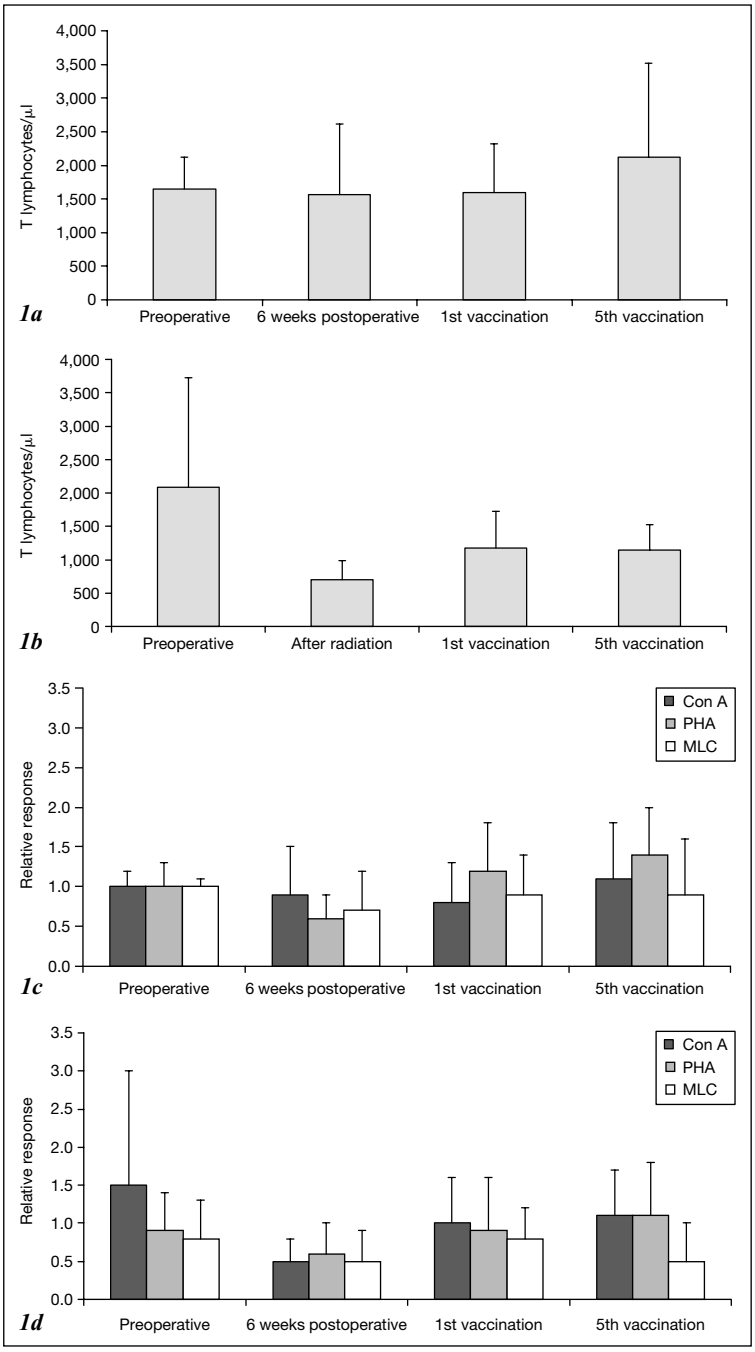
Preparation of NDV-Modified Autologous Tumor Cell Vaccine

Per vaccine, 1×10^{7} tumor cells were incubated for 1 h with 64 hemagglutinating units of the avirulent strain Ulster of NDV. Successful infection was proven by immunohistochemical staining with anti-NDV-HN monoclonal antibody recognizing the viral protein hemagglutinin neuraminidase, kindly provided by Dr. Iorio, Worcester, USA. For a DTH test, non-virus-modified autologous tumor cells as well as NDV-modified tumor cells were used. Finally, cells were irradiated with 200 Gy. In case of the ATV-NDV vaccine, a dose of 400,000 IU of recombinant IL-2 (Chiron, Ratingen, Germany) was added to the vaccine prior to application.

Vaccination Procedure and DTH Test

Vaccination started 12 weeks after radiotherapy, consisting of up to 5 applications with 1×10^{7} ATV-NDV cells intradermally on the upper thigh (alternately left and right). In addition 1×10^{6} IU IL-2 were subcutaneously applied 4 times over a time period of 2 days at the vaccination site. Vaccinations 1–4 were given at 3-week intervals followed by a 6-month interval for vaccination 5. To determine antitumor reactivity against unmodified tumor cells,

Fig. 1. Levels and mitogen stimulation capacity of T cells in nonirradiated (***a***, ***c***; n = 5) and irradiated patients (***b***, ***d***; n = 15) at different time points. Relative responses determined at the 1st vaccination were obtained after completion of the IL-2 preconditioning period. In irradiated patients (***b***, ***d***), T cell numbers and mitogen stimulation responses were markedly decreased after radiotherapy. They recovered to normal values after IL-2 treatment. In nonirradiated patients (***a***, ***c***), especially postoperative values for stimulation with different mitogens decreased slightly and were as high as before surgery after IL-2 preconditioning. Con A = Concanavalin A; MLC = mixed lymphocyte culture.


T lymphocytes/μl
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Preoperative
6 weeks postoperative
1st vaccination
5th vaccination
1a
T lymphocytes/μl
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Preoperative
After radiation
1st vaccination
5th vaccination
1b
Relative response
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Con A
PHA
MLC
Preoperative
6 weeks postoperative
1st vaccination
5th vaccination
1c
Relative response
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Con A
PHA
MLC
Preoperative
6 weeks postoperative
1st vaccination
5th vaccination
1d

challenge tests were performed with 1×10^6 non-virus-modified cells on the upper thigh before the first and before the last vaccination. DTH reactions at the vaccination and at the challenge sites were recorded 24 h after injection by measuring the area of induration.

Immunohistochemistry

Immunohistochemical staining was performed on HNSCC cells and on cryostat sections of the frozen specimens. Fixation and staining were carried out as described [21].

Results

T Cell Levels and Mitogen Stimulation after IL-2 Application

In order to avoid an unwanted decrease in T cell levels at the time of vaccination, during the preconditioning period the patients received 10^6 IU IL-2 four times a week, which was applied in the abdominal wall. T lymphocyte counts were determined at different time points (fig. 1a, b). Mean T cell levels of nonirradiated patients were not reduced after surgery and even slightly increased at the end of the vaccination treatment (fig. 1a). In contrast, T lymphocyte counts of patients who underwent surgery and subsequent radiotherapy showed a marked decrease in mean levels (694 T cells/μl) as determined after the completion of radiotherapy (fig. 1b). This is far below the normal range of 1,017–1,862 T cells/μl. Application of IL-2 was associated with a normalization of T lymphocyte count with a mean value of 1,176/μl at the time of the first vaccination. Interestingly, mean T cell levels remained constant during the vaccination period although the IL-2 dose, which the patients received together with the vaccine, was much lower than during the preconditioning period.

To analyze the functional activity of the T cells, we performed a mitogen stimulation test with concanavalin A and PHA and performed allogeneic stimulation in a mixed lymphocyte culture. Six weeks after surgery we observed a reduced stimulation capacity. This was most pronounced in irradiated tumor patients (fig. 1d). However, at the end of the preconditioning period, mean stimulation values were almost as high as before surgery and thus in the normal range of ≤ 0.5. During the vaccination period, mean values for at least concanavalin A and PHA remained high, while mean mixed lymphocyte culture stimulation in irradiated patients decreased again.

Establishment and Characterization of HNSCC Short-Term Cultures

To obtain vaccine in sufficient quantity and quality, ATV-NDV was prepared for each patient from autologous HNSCC cell cultures. All HNSCC short-term cultures showed homogenous expression of epithelium-specific markers as determined by cytokeratin staining. As shown in figure 2a, expression of MHC

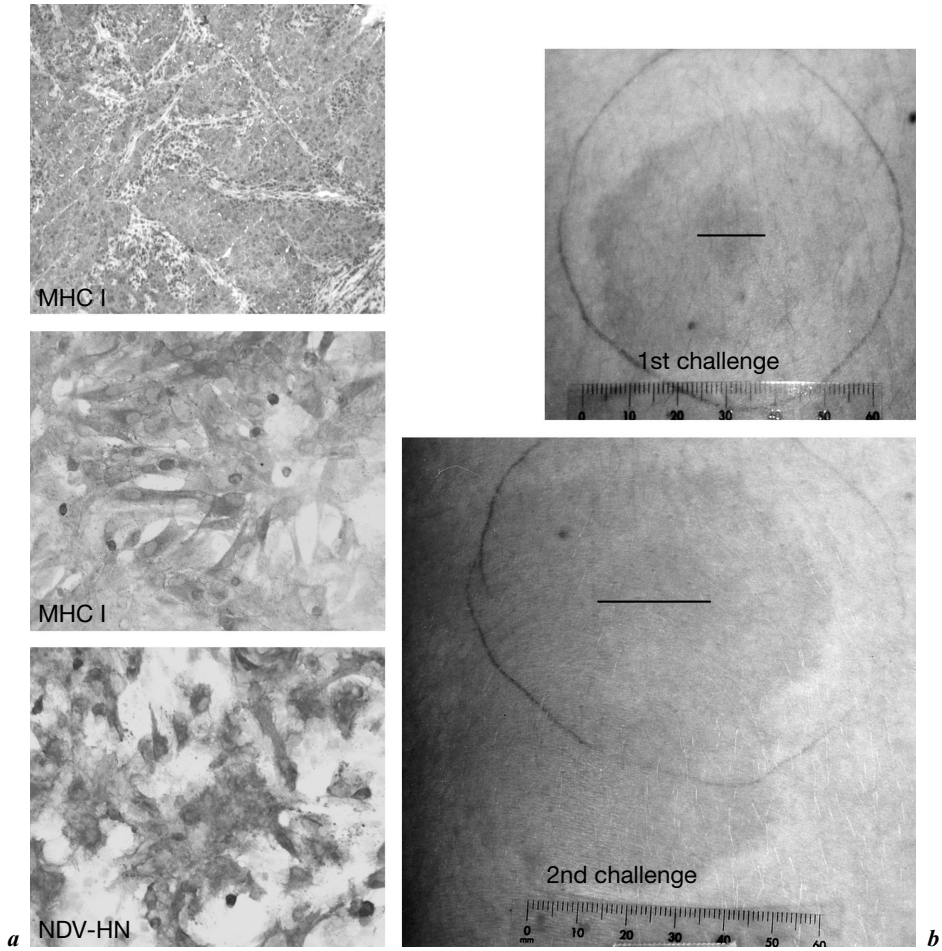


Fig. 2. Vaccine characteristics and skin reactivity before and after vaccine application. ***a*** Immunohistochemical analysis demonstrates expression of MHC class I molecules in tumor cells of the native tumor tissue (upper part), their maintained expression in short-term tumor cell cultures (middle part) and the successful infection of cultured tumor cells with NDV (lower part). ***b*** DTH reactivities to unmodified tumor cells (black bars) before first vaccination (1st challenge) and before the last vaccination (2nd challenge). The increased area of induration in the 2nd challenge test is of particular relevance since it indicates systemic reactivity to autologous tumor cells as they may have remained in the patient.

class I molecules, which are important for the presentation of tumor-associated antigens, was not only observed in the original tumor tissue (upper part) but was maintained in all HNSCC short-term cultures (middle part). Successful infection of HNSCC tumor cells with NDV could be demonstrated in all cases

by staining with an antibody recognizing the viral molecule hemagglutinin neuraminidase (NDV-HN, lower part).

Effects of Vaccination Demonstrated by Immune Monitoring in the Skin

DTH skin reactions – defined as the area of induration at the application site – were determined 24 h after vaccination with 10^7 virus-modified tumor cells as well as after challenge with 10^6 unmodified irradiated tumor cells. Figure 2b shows a representative example of antitumor reactivity against unmodified tumor cells before the first and before the last vaccination (1st and 2nd challenges). Antitumor vaccination was associated with a markedly increased area of induration (see bars in fig. 2b). The increased reactivity to unmodified tumor cells is of particular relevance, since it indicates systemic reactivity to autologous tumor cells, which may have remained in the patient.

Discussion

In the present antitumor vaccination study, we demonstrate in 20 HNSCC patients that postoperative treatment with low-dose IL-2 increases the number of T cells and restores their functional activity. This is of major importance since HNSCC patients have been noted to develop marked surgery- and radiation-induced cellular immune defects [12, 13] that may be unfavorable especially for immunotherapeutic approaches. Effects observed after IL-2 conditioning were maintained during the subsequent antitumor immunization period, in which patients repeatedly received an autologous virus-modified tumor cell vaccine and thus were able to develop an increased systemic cell-mediated immune responsiveness as evidenced by antitumor DTH reactivity.

Although the IL-2 dose we have chosen for preconditioning has marked effects on T cell numbers and functional capacity, it has been reported that even higher IL-2 doses did not improve the prognosis of HNSCC patients [22]. However, in accordance with our observations on the functional capacity of T cells, IL-2 was shown to rescue antigen-specific T cells from radiation-induced apoptosis [23]. Preconditioning with IL-2 may thus be a good way to enhance the immune competence of HNSCC patients, especially after radiation-induced immunosuppression.

With regard to the vaccine preparation, we decided to use individual intact tumor cells obtained from short-term tumor cultures as the source of tumor antigens. This allowed us to produce a vaccine with a standardized high number of tumor cells. The high number of 10 million autologous viable tumor cells

per vaccine might include individually unique tumor antigens derived from mutations or other genetic alterations and might also be representative of the heterogeneity of tumor antigens in an individual tumor of a patient. The use of whole tumor cells eliminates the need to first identify the respective tumor antigens, which would require sophisticated techniques. Since even multiple applications of the vaccine did not induce autoimmune disease, this approach can be considered to be safe [Karcher et al., unpubl. data].

As adjuvant in the tumor vaccine, we employed NDV strain Ulster, based on good experiences in various animal tumor models [16]. Recently, interest in the use of tumor-selective replication-competent viruses such as NDV, which has already been safely applied to many cancer patients in Europe and the USA, has been reviving [8]. NDV possesses antineoplastic, oncolytic as well as immunestimulatory properties [8, 9]. In tumor cells, it induces T-cell-costimulatory activity [24], upregulates MHC and adhesion molecules and induces IFN-α and -β as well as the chemokines RANTES and IP-10 [10]. These factors lead to proinflammatory effects at the vaccination site and thus contribute to the augmentation of cytotoxic antitumor effects [25].

Regarding the clinical outcome of the vaccinated patients, we have indications for an improved overall survival in patients suffering from stage IV tumors that is associated with an increased antitumor memory [Karcher et al., unpubl. data].

Altogether, the present study provides evidence that the preconditioning of HNSCC patients with IL-2 might improve the responsiveness to a tumor vaccine combining multiple tumor antigens with NDV-induced danger signals [7]. This combined approach seems to be an interesting new concept for the treatment of HNSCC patients.

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Prognostic Factors

Confusion Caused by Bad Quality of Design, Analysis and Reporting of Many Studies

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Abstract

In contrast to therapeutic research guidance to design, conduct, analyse and report studies on prognostic factors is less developed and often several deficiencies are stressed. For the assessment of the importance of a factor of interest a systematic review of the corresponding studies would be required, however, this is hardly possible because of many weaknesses in the individual studies. In this article I will discuss several deficiencies of the analysis of prognostic factor studies and shortly discuss problems of reporting and of a summary assessment. By using 3 studies in cancer and a hypotheitcal study as examples I will discuss categorization respectively the determination of a functional form for a continuous factor, sample size, multivariable analysis and data quality. The message of this paper is that serious improvements of prognostic factor studies are required. This can be achieved by a closer collaboration between several disciplines and a closer collaboration at the international level. Specifically, experienced statisticians have to play a central role in the planning, analysis, interpretation and reporting of these studies.

Cancer is a heterogeneous disease with tumours being highly variable in their growth rates, patterns of metastases and other biological characteristics. Several molecular markers that might complement the TNM system have been investigated in the last two decades in order to improve the knowledge of disease evolution, but the results of published studies are often inconsistent or even contradictory, thus limiting their value and potential application [1].

About 10 years ago, Altman [2] has heavily criticized the quality of medical research. In one sentence he nicely summarized several deficiencies. He stated that in numerous studies of the medical literature, in both general and specialist journals, researchers ‘use the wrong techniques (either wilfully or in

ignorance), use the right techniques wrongly, misinterpret their results, report their results selectively, cite the literature selectively and draw unjustified conclusions'. He discussed several reasons for this situation.

Concerning therapeutic research with the aim to find a treatment strategy which is in some sense 'better' than the current 'standard' treatment, the situation has improved, at least for some diseases such as breast cancer or gynaecological cancer. This assessment is based on personal experiences for about two decades in these diseases.

Concerning prognostic factors, the quality of research is still poor. Despite a huge research effort and thousands of papers, the prognostic value of most traditional factors under discussion is uncertain and the usefulness of prognostic indices or classification schemes in oncology is often unproven [3]. Because of the relevance for the treatment of patients, clinicians are now more interested in a predictive factor, which is in methodological terminology an interaction of treatment with a factor of interest. Unfortunately, in the medical community the value of specific predictive factors is also controversial. Many statements concerning the predictive value of baseline variables are based on subgroup findings, but often without appropriate statistical tests for interaction [4].

For both prognostic and predictive markers, summary assessments are required in order to identify the most valuable markers for a disease of interest. Obviously, this requires a number of important steps, starting with systematic reports of all studies. Relevant studies have to be identified, and the required information has to be extracted from each study, or even better, original data should be available for re-analyses. As the latter may be time-consuming, costly and very difficult, summary assessments are often based on reports in the literature. Obviously this requires standards of reporting. For randomized trials, the CONSORT (Consolidated Standards of Reporting Trials) statement was developed and improved reporting [5–7]. In contrast, reporting of prognostic factor studies is hardly addressed in the literature, a meta-analytic approach to assess the prognostic value of a marker is still an exception. To investigate the practicality of such an approach, an empirical investigation of a systematic review of tumour markers for neuroblastoma was performed by Riley et al. [8]. Based on 260 studies identified they concluded that the reporting was often inadequate, in terms of both statistical analysis and presentation, and that there was considerable heterogeneity for many important clinical/statistical factors.

Although guidelines for the evaluation of prognostic factors have been published more than a decade ago [9], the quality of research in this area is still bad. This has also been recognized by clinicians. In a review of prognostic and predictive factors in breast cancer, Gasparini [10] mentions 'the methodology of studies on prognostic and predictive indicators that are, at present, the most disappointing, controversial, and debatable aspect of this field of research. In fact,

most of our knowledge on the usefulness of novel biomarkers for prognostic and predictive purposes is from retrospective studies that often report repetitive and inconclusive results due to methodological biases related to: inadequate specificity and sensitivity of the assay, lack of quality control for inter/intra-laboratory variations, inadequate sample size or length of follow-up, suboptimal statistical analyses, co-determination of confounding variables or heterogeneous therapy.'

Some researchers may argue that these are problems from the past which may be less relevant in the 'new era' of molecular markers, where data from thousands of genes are collected in order to develop new and better prognostic classification schemes and to identify groups of genes predicting the usefulness of therapeutic strategies. However, many of the issues are also relevant, some of them will be even more critical. In the summary of a review article on the future of cancer prognostic studies, Hall and Going [11] state: 'Many studies have attempted to define useful prognostic and predictive factors in cancer but few have achieved acceptance in clinical practice because of methodological weaknesses. These include failure to test clearly formulated hypotheses, inadequate sample size, inappropriate multiple significance testing, arbitrary definition of patient groups, inadequately reproducible assays, and failure to verify prognostic factors with data independent of the data which suggested the original hypothesis. This unsatisfactory situation will persist until critical attention is routinely paid to study design and prospective validation of supposed prognostic and predictive factors, without which classical approaches will be suboptimally exploited and the flood of data from new molecular technologies will not be used effectively. We propose that prognostic factors should be evaluated in three phases: I, assay definition; II, retrospective testing; III, prospective testing, ideally as a designed part of clinical trials.'

Many critical issues are mentioned in these summaries, several challenges and barriers from the laboratory and clinical side were discussed by Pritzker [12], and for further methodological issues see Altman and Lyman [13].

The main aim of this paper is to demonstrate some deficiencies of the analysis of these studies. Furthermore I will discuss problems of a summary assessment for a prognostic factor of interest.

Issues in Data Analysis

Data Sets Used for Demonstration

Freiburg DNA Study

The database of the study consisted of all patients who had surgery for primary breast cancer between March 1982 and December 1987 at the

Department of Gynaecology of the University of Freiburg. Paraffin-embedded material was available for 372 patients. Some exclusion criteria (e.g. pretreated patients or history of malignancy) were defined retrospectively, which left 266 patients with a median follow-up time of 82 months for the analysis.

Eight important patient characteristics were investigated. Besides S phase fraction (SPF), we consider here only lymph node status and ploidy status. Except for SPF in aneuploid tumours, the data are nearly complete. According to the treatment policies of the clinic and the exclusion criteria, none of the node-negative patients had adjuvant chemotherapy or hormonal therapy. One hundred fifteen events (39 in node-negative and 76 in node-positive; 48 in diploid and 67 in aneuploid tumours) have been observed for recurrence-free survival, which was defined as the time from surgery to the first locoregional recurrence, distant metastasis, second malignancy or death. More details about the study can be found in Pfisterer et al. [14].

Prognostic Factors in Node-Positive Breast Cancer

From July 1984 to December 1989, the German Breast Cancer Study Group (GBSG 2) recruited 720 patients with primary node-positive breast cancer into a comprehensive cohort study [15]. Randomized and non-randomized patients were eligible, and about two thirds were entered into the randomized part. The effectiveness of 3 versus 6 cycles of chemotherapy and additional hormonal treatment with tamoxifen were investigated in a 2×2 design. After a median follow-up time of nearly 5 years, 312 patients had had at least 1 event (recurrence of the disease or death). The event-free survival time of the 686 patients (with 299 events) who had complete data for the factors age, tumour size, number of positive lymph nodes, progesterone and oestrogen receptor status, menopausal status and tumour grade is considered in this paper. The prognostic factors comprise 5 continuous, 1 binary and 1 ordinal variable (tumour grade). Categorizations were based on medical considerations and were used by Schumacher et al. [16] for the analysis of the randomized trial.

Glioma

A randomized trial to compare two chemotherapy regimes included 447 patients with malignant glioma. At the time of the analysis, 293 patients had died and the median survival time from the date of randomization was about 11 months. Besides therapy, 12 variables (age, 3 ordinal and 8 binary variables) which might influence the survival time were considered. The 3 variables measured on an ordinal scale (the Karnofsky index, the type of surgical resection and the grade of malignancy) were each represented by 2 dummy variables, resulting in a total of 15 predictors denoted by X_1, ..., X_{15}. For these

predictors, complete data were available for 413 patients (274 events) used here in a complete case analysis. A detailed description of the study and a comparison of several approaches to investigate the influence of prognostic factors and therapy are given by Ulm et al. [17], Sauerbrei and Schumacher [18] and Sauerbrei [19].

Continuous Factor – Categorization or Determination of Functional Form?

Often variables are measured in a continuous form; however, changing it to a categorical form is common practice in many analyses. An important reason is that categorization makes it easier for clinicians to use information about the relationship between an outcome and a predictor variable in making treatment decisions [20]. Another reason is that the functional form of the influence is unknown and that analysis based on categorization data is easier.

In particular, values of a variable are frequently divided into just two groups. Categorization enables researchers to avoid strong assumptions about the relation between the marker and risk, but at the expense of throwing away information. The information loss is greatest with only two groups, but this approach is common, often by splitting at the sample median [21]. It is well known, however, that the results of analyses can vary if different cutpoints are used. Often, several cutpoints are investigated, and the one that corresponds to the most significant relation with outcome is chosen. In other words, the cutpoint defining 'low' and 'high' risk is chosen that minimizes the p value relating the prognostic factor to outcome. The cutpoint so chosen is often termed 'optimal', but this description is inadvisable because of the well-known problem of multiple testing. Altman et al. [21] prefer to call it the 'minimum p value approach'.

This approach requires the systematic variation of the cutpoint when categorizing a continuous covariate like SPF in the Freiburg DNA study and computing a p value for each cutpoint. In figure 1a we show that the p value is unstable and that the cutpoint corresponding to the minimal p value is a result of chance.

Even more importantly, this approach clearly leads to a serious problem of multiple testing [22]. When a series of statistical tests, each with a prespecified nominal type 1 error, for example 5%, is performed on the same data, then this procedure leads to a global error rate for the whole procedure that might be much higher than 5%. Theoretical arguments [23, 24] and results from simulation studies [21, 25] demonstrate that the false-positive rate can be inflated to values exceeding 40% when a nominal level of 5% is used. A correction, valid for large sample sizes, of the minimal p value to allow for the multiple testing can be derived [21].

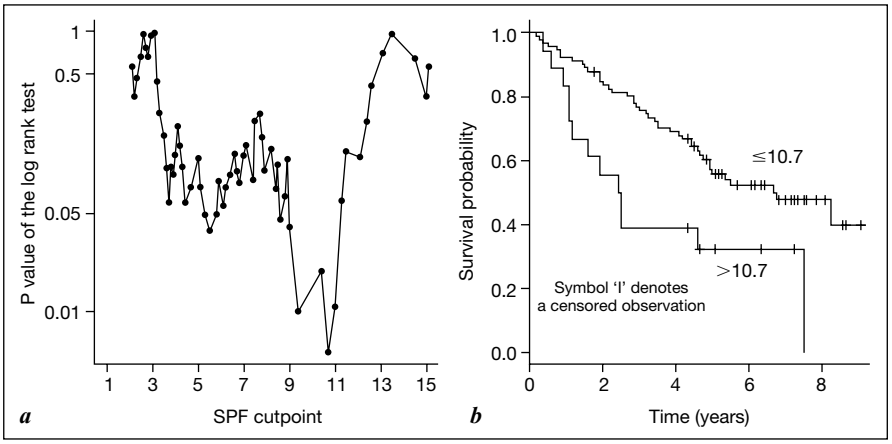

Fig. 1. DNA example, node-positive population. ***a*** Dependence of the p value of the log rank test on the SPF cutpoint chosen. ***b*** Kaplan-Meier estimates for 'low' and 'high' SPF values, respectively, categorization determined by the 'optimal' cutpoint approach.

A simple approximation for the corrected p value [21] is

$p_{cor} \approx -1.63\ p_{min}(1 + 2.35 \log_e p_{min})$ for $\varepsilon = 10\%$
$p_{cor} \approx -3.13\ p_{min}(1 + 1.65 \log_e p_{min})$ for $\varepsilon = 5\%$

where p_{min} denotes the minimum p value of the log rank statistic, taken over the selection interval characterized by the proportion ε of smallest and of largest values of the prognostic factor that are not considered as potential cutpoints.

For example, to reach a value $p_{cor} = 0.05$ requires $p_{min} = 0.002$ when $\varepsilon = 10\%$ and even $p_{min} = 0.001$ when $\varepsilon = 5\%$. Using the correction formula, the corrected p value for their optimal cutpoint in the full data set of the SPF example was 0.403, in contrast to the minimum p value of 0.037 which would indicate a significant influence of SPF.

Using this approach, most likely different cutpoints will be termed 'optimal' in different subpopulations. For the node-positive population, the optimal cutpoint for SPF is from 10.7 to 10.9 (fig. 1). In the node-negative population, the optimal cutpoint is from 9.0 to 9.1; analysing both populations together results in an optimal cutpoint of 5.4. In further populations, e.g. defined by ploidy status, other optimal cutpoints were derived [21]. Analysing the data in a multivariate analysis, e.g. adjusting for tumour size and age, will furthermore change the optimal cutpoint, originally intended as cutpoint to classify patients into two groups with 'low' and 'high' SPF values, respectively.

Ten years ago, SPF was seen by many researchers as an important prognostic factor in breast cancer. Altman et al. [21] found 19 cutpoints used to

classify SPF into high and low values; some of them were derived by an optimal cutpoint approach. Nowadays, it is well known that the prognostic value of SPF was heavily overestimated by many researchers. Overestimation of the effect is illustrated by the two Kaplan-Meier estimates for 'low' and 'high' SPF values, the categorization for SPF is based on the 'optimal' cutpoint approach (fig. 1b). Statistical methods have been proposed to correct for the overestimation [26]. More details on problems of the optimal cutpoint approach are discussed in Holländer and Schumacher [27].

To avoid information loss and to avoid the unrealistic way of describing a smooth relationship between a predictor and an outcome variable by a cutpoint model, it is preferable to investigate the functional influence for a continuous prognostic factor.

Usually, in regression models, the effect of continuous covariates is assumed to be linear. However, for some prognostic factors this assumption may be wrong, leading to wrong conclusions or even declaring a factor erroneously as uninfluential. A systematic investigation for sensible non-linear relationships is a better way to analyse the data. We propose to use the fractional polynomial (FP) approach developed by Royston and Altman [28]. Here, one or two terms of the form X^p are fitted, the exponents p being chosen from the small preselected set of integer and non-integer values $\{-2, -1, -0.5, 0, 0.5, 1, 2, 3\}$. Although only a small number of transformations is considered (7 for FPs of degree 1 and 36 for FPs of degree 2, where the values from the set are combined), FP functions provide a rich class of possible functional forms leading to a reasonable fit to the data in many situations. Royston and Altman [28] dealt mainly with the case of a single predictor, but they also suggested and illustrated an algorithm for fitting FPs in multivariable models. By combining backward elimination, which aims to exclude from a model all factors without influence on the outcome, with the search for the most suitable FP transformation for continuous predictors, Sauerbrei and Royston [29] propose modifications to this multivariable FP procedure. A further extension of the multivariate FP procedure aims to reflect basic medical knowledge of the types of relationship to be expected between certain predictors and risk. Sauerbrei et al. [30] use this approach to investigate the functional influence of 7 standard prognostic factors in a prospective study on node-positive breast cancer patients. They show that the FP approach can provide a clearer insight into the nature of the relationship between the values of the factors and the risk of recurrence or death than usual approaches based (i) on the assumption of a linear relationship for continuous variables or (ii) categorized data (fig. 2). With both approaches age would not have a significant effect on recurrence-free survival, p values are 0.9 for a linear relationship and 0.2 for the categorized variable. For the latter, the 2 cutpoints were prespecified. The figure clearly demonstrates a strong non-linear effect of age on recurrence-free survival time ($p = 0.001$). Sauerbrei et al. [30] discuss that

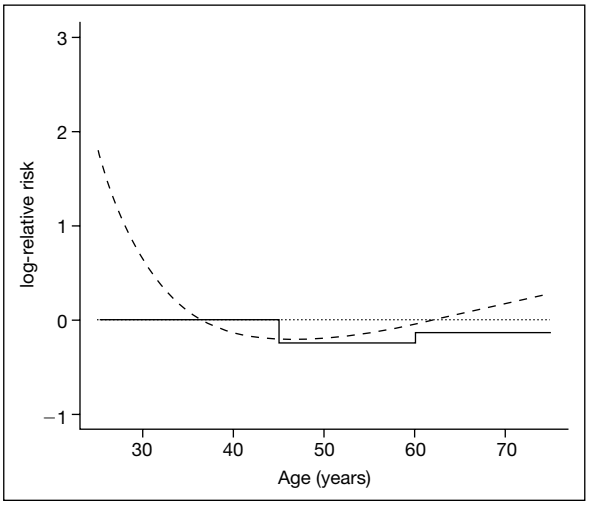

Fig. 2. Estimated log-relative risk function for age obtained by the FP– (- - -), categorization (———) and linear (·····) approach in the breast cancer study.

the proposed functional form explains the current controversial discussion about the prognostic value of age in the literature. They argue that the controversies are caused by differences concerning the use of other prognostic factors considered in a multivariable framework and by different cutpoints used to define 'young age' for a breast cancer patient.

Sample Size

If the role of a new prognostic factor is to be investigated, a careful planning of an appropriate study is required. This includes an assessment of the power of the study in terms of sample sizes. An adequate analysis of the independent prognostic effect of a new factor has to be adjusted for the existing standard factors [31]. With survival or event-free survival as the endpoint, this will often be done with the Cox proportional hazards model. Sample size and power formulae in survival analysis have been developed for randomized treatment comparisons. In the analysis of prognostic factors, however, the covariates included are expected to be correlated with the factor of primary interest. In this situation, the existing sample size and power formulae are not valid, a simple extension recently proposed by Schmoor et al. [32] can be used.

In reality, studies on prognostic factors are often not carefully planned, and consideration of sample size and power are an exception. See some of the cited

Table 1. Hypothetical study: influence of a binary prognostic factor (F) on observing an event

Factor	Event		Total
	yes	no	
F+	30 (10%)	270 (90%)	300 (66.6%)
F–	6 (4%)	144 (96%)	150 (33.3%)
Total	36 (8%)	414 (92%)	450

critique in the introduction [10, 11, 13]. Often it is ignored that the number of events – sometimes called 'effective sample size' – determines the power of a study and not the number of patients recruited. Therefore the prognosis of a population and the lengths of follow-up determine the required number of patients to enter in a study. With a small number of events it is only possible to detect very large effects which are rather unlikely in reality, at least if the assessment is done correctly by also considering the 'standard' factors in a multivariable context. For example, to detect an effect with a relative risk of 1.5 for a binary prognostic factor (prevalence 30%), 227 events are required for a power of 80%, type I error 5%. If in the intended follow-up time an event is expected only in about half of the patients, the required sample size will be 454. A large effect (relative risk, RR = 2.0) can be detected with a much smaller effective sample size of 78.

The required number of events increases if a variable of interest is correlated with other variables included in a multivariable model. For a population with a good prognosis, even large effects can only be detected with sample sizes of several hundred patients and with a long-term follow-up.

A large study is also required if the prevalence of a factor is low. For example, with a prevalence of 10%, the required number of events increases to 530 (RR = 1.5) and to 182 (RR = 2.0), respectively, other assumptions unchanged. For more details, see Schmoor et al. [32] or Schumacher et al. [33]. Based on these calculations, it is obvious that many prognostic factor studies have only a very low power to detect a prognostic effect of relevant size.

The seriousness of this issue is partly presented in a systematic review of tumour markers for neuroblastoma [8] (see also the summary assessment below). For 13 tumour markers of interest, they identified 318 reports which would have been suitable to estimate an effect of the marker. However, they had to exclude 122 (38.4%) reports because the sample size was 25 or lower! Probably the percentage of reports with a sufficient power, say 80%, was very low for an effect with a usual size.

With a simple hypothetical study, we will further illustrate the necessity of a sufficient sample size. In table 1 we give the result of a study with a binary prognostic factor (F) and a binary outcome.

In a study with 450 patients, we observe a prevalence of 66.6% for the factor F. So far, 8% of patients have an event. The frequency of an event is 10% if the factor is present, but only 4% if it is not present. The estimated RR is 2.5, and a χ^2 test of independence gives a p value of 0.027, indicating that F has an influence on the probability of observing an event. Even this large effect would not have been significant ($p = 0.118$), if the study had included 225 patients only, with all percentages in the table unchanged (halving absolute numbers in the table).

Doubling the numbers in the table gives a study with 900 patients and a highly significant p value of 0.002. Often, the p values for the observed RR of 2.5 would be interpreted as being 'non-significant ($n = 225$)', 'significant ($n = 450$)' and 'highly significant ($n = 900$)'. This illustrates that the p value is highly dependent on the sample size (respectively the effective sample size in studies with survival data) and that erroneous conclusions are possible if an assessment is based on p values only. They have to be complemented by estimating the size of the effect with the corresponding confidence interval. In our example the RR is always 2.5, the 95% confidence intervals are 0.75–8.37 for $n = 225$, 1.06–5.87 for $n = 450$ and 1.37–4.57 for $n = 900$.

Despite the large sample size, confidence intervals for the estimate of the RR are still large. One reason is the small number of events observed so far. Let us assume that we observe 4 times the number of events in the example, with the RR and the prevalence unchanged (120 events in 300 F+ patients and 24 events in 150 F– patients). In the study with 450 patients, this larger number of events substantially reduces the 95% confidence interval of our estimate of the RR to 1.69–3.70, the p value is now <0.001.

Multivariate Analysis

Data analysts are often faced with many predictor variables which may have an influence on outcome. Variable selection strategies are needed in order to identify the subset of 'important' predictors. In regression models, several strategies such as sequential procedures (e.g. stepwise selection or backward elimination) or all-subset selection with different optimization criteria (e.g. Akaike or Bayesian information criteria) are available, but they may give different answers. Expert knowledge is required for a sensible application. Although subject matter knowledge should guide selection, some variables will inevitably be chosen mainly by statistical principles – essentially, p values for

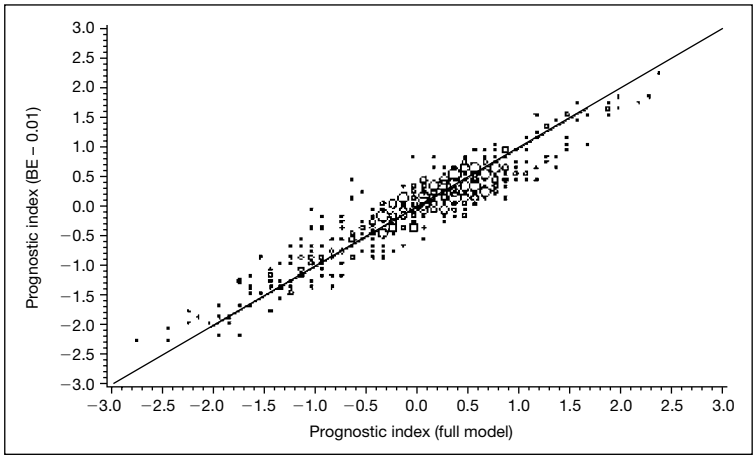

Fig. 3. Scatter plot of the prognostic indices from the full model (15 variables) and from a model selected by backward elimination (BE) with 4 variables for 413 patients in the glioma study: the radius of each circle represents the number of cases. From Sauerbrei [19].

including or excluding variables. The definition of a 'best' strategy to produce a model which has good predictive properties in new data is difficult. A model which fits the current data set may well be too much data driven to give adequate predictive accuracy in other settings. Several difficulties and issues how to handle them are discussed in the statistical literature [19, 34, 35].

Generalizability and practical usefulness are other issues which have to be kept in mind when developing a predictive model. Because it would require measuring all variables in an identical or at least in a similar way, it is obvious that a prognostic model based on many variables is 'not clinically useful' and is 'quickly forgotten' [3].

For patients with a glioma, Sauerbrei [19] developed several prognostic models by using backward elimination and discussed several issues of model complexity. The scatter plot of the prognostic indices from the full model with 15 variables and from the most simple model with 4 variables only shows the high correlation (Pearson's correlation coefficient 0.94) between these two indices.

Concerning the practical usefulness the index based on 4 variables is clearly preferable; concerning the predictive ability there is hardly any difference between them. Sauerbrei [19] also illustrates several problems of models which are too complex and argues for greater simplicity of final regression models.

With statistical packages it is easy to perform a multivariable analysis, but all approaches have several more or less obvious assumptions. Violations of the

assumptions can result in wrong models and wrong conclusions. Multivariable modelling is a task for the statistical expert.

Tree-based approaches [36] and artificial neural nets [37, 38] are often used as alternatives to regression models for the analysis of prognostic factor studies. In some data sets, Schumacher et al. [33] compare results between the approaches and assess the prognostic ability of classification schemes derived from them. The authors give several arguments that regression models are the standard tool for analysing the prognostic relevance of various factors. As the other approaches are substantially different in their assumptions and in their way of modelling, they can complement this analysis.

To improve the understanding of the basics of multivariable analysis for clinical readers, Katz [39] discusses issues such as what multivariable models are, why they are used, what types exist, what assumptions underline them, how they should be interpreted and how they can be evaluated. For other readable papers for clinicians on the use of the Cox model, see Baker et al. [40], and on multivariate data analysis of survival data, see Bradburn et al. [41].

Data Quality

It is obvious that data quality has a strong influence on the assessment of a prognostic factor. Many papers are published on the reproducibility of measurements, and statistical investigations have shown that errors in measurement will result in underestimating a true effect. This will be illustrated with an extension of the hypothetical example from the section on 'sample size' above. If in table 1 the status of factor F is misclassified in 10% of the patients (3 events and 27 non-events as F– instead of the correct F+; 15 non-events from F– to F+), the estimated RR is reduced to 1.74. Even for n = 450, the corresponding p value (p = 0.130) would now be much larger than the usual significance criteria of 5%.

Another important issue of data quality is the completeness of data. For studies with interest in survival data, completeness of follow-up is necessary in order to observe all events. The influence of the effective sample size (number of events) on the power of a study was discussed earlier. For patients without an event, observed survival time is calculated up to the last information, the term 'censored' survival time is used. Because methods for the analysis of survival data, e.g. Kaplan-Meier estimator or Cox model, usually assume that the mechanism of censoring does not depend on the event time, it can be very critical if incomplete follow-up of a patient depends on a prognostic factor of a patient. For example, a patient with a bad status of health may not come (may not be able to come) to an intended follow-up visit. The simplest (and usual!) way is

to use the information up to the last visit; however, this can (seriously) bias the analysis of survival time if the bad health status is the reason for the missing follow-up information.

A further issue is completeness of data for prognostic factors, which is a frequent complication. Most often it is assumed that the data are 'missing at random' and the standard approach in most statistical packages is a complete case analysis, that is, all subjects with at least one missing value in the covariates are excluded from the analysis [42]. In a multivariate analysis this may result in a substantial reduction of the sample size, even if for each variable only a small percentage of patients has missing values. For example, if 97% of the data are available for each of 7 variables of interest, up to 21% (3×7) of the patients will be excluded in a complete case analysis. Obviously, with such an approach a lot of information is wasted. Other issues arise in a multivariable analysis if information is missing for one variable in a substantial part of the patients, e.g. in a third. There are several ways to impute values for the missing values starting with simple approaches, e.g. the mean from patients with information, to more complicated multiple imputation techniques where the imputed value depends on the correlation with other variables in the study [42, 43]. Because the missing value issue is specific in each study, it is not possible to give general guidelines; experienced statisticians have to handle this issue.

Summary Assessment of a Prognostic Factor

For all diseases a large number of prognostic markers is discussed. Systematic reviews and meta-analytical approaches to identify the most valuable prognostic markers are needed because usually conflicting evidence relating to markers is published across a number of studies. Obviously, this requires a number of important steps, starting with systematic reports of all studies. For randomized trials the CONSORT statement [5] was developed and improved reporting. Weaknesses of the original version were addressed in a revised version [6, 7].

Although there is hardly any empirical evidence on the issue of publication bias in prognostic factor studies, it is most likely that it is more serious than in the context of randomized trials. For the latter situation, the problem has been known for a long time [44–46]. As prognostic factor studies are often based on historical data, it is much easier to conduct such a study; often, it is even done by a small group of researches and students within several months. If the result is 'positive', e.g. a large effect of the intended direction is found, a paper will be submitted and may be published. If the result is 'negative', researchers may not even submit a manuscript on the study, or it is more likely

Table 2. Published reports for 3 prognostic factors for neuroblastoma [8]

Markername	Papers	OS and DFS reports	Total successful estimates	Estimates U/A	Different cut-off groups	Different stage groups	Different age groups
MYC-N	151	194	94	77/17	9	9	4
CD44	8	8	3	3/0	1	1	2
MDR	16	30	16	13/3	8	3	3

For more explanations, see the text. OS = Overall Survival; DFS = Disease Free Survival; U/A = estimates from unadjusted/adjusted (multivariate) analysis.

that editors reject the manuscript. Within the current situation of research in prognostic factor studies, it is obvious that identification of all studies for a specific marker of interest is impossible.

A further critical issue is the reporting of prognostic factor studies. A large systematic investigation of this issue highlights that reporting is in general insufficient [8]. In a systematic review of tumour markers for neuroblastoma, 260 studies which considered 130 different markers were identified. The authors concluded that 'the reporting of these studies was often inadequate, in terms of both statistical analysis and presentation, and there was considerable heterogeneity for many important clinical/statistical factors. These problems restricted both the extraction of data and the meta-analysis of results from the primary studies, limiting feasibility of the evidence-based approach.'

As key problems they identified:

(1) no appropriate statistical analysis performed or reported;
(2) hazard ratio not calculated or not reported;
(3) inexact p values provided;
(4) group numbers and group events not given;
(5) marker studies too small.

Some issues are obvious by considering reports for the markers MYC-N, CD44 and the multi-drug resistance protein (MDR) in table 2. For MYC-N, 151 papers were identified for which 194 reported results for overall survival and disease-free survival, respectively. However, successful estimates of the effect could only be extracted for 94 (48.5%) of them. Only 17 of these estimates could be derived from a multivariable analysis, adjusting for other factors. The majority of these estimates (77) is only from a univariate analysis. In these papers, 9 different cut-off values were found to categorize MYC-N; 9 different stage groups and 4 different age groups were used.

For CD44 and MDR, the number of reports is much smaller. The limited information from multivariable analysis is obvious.

The paper by Riley et al. [8] clearly demonstrates the seriousness of problems of research on prognostic factors. I agree with their statement that their findings for tumour markers within the neuroblastoma literature can be generalized to other prognostic markers and other disease areas. The resulting problems for a sensible systematic assessment of the prognostic ability of markers are obvious [47]. Improvement of reporting is a prerequisite for an improvement of research for prognostic factors. Some proposals towards guidelines for improved reporting are discussed by Riley et al. [8], specific guidelines are currently under development by a joint working group of the National Cancer Institute of the USA and by the EORTC. In the future, the guidelines may play a similar role to the CONSORT statement for the reporting of randomized trials, hoping that they can help to avoid some important and obvious mistakes in the design and analysis of these studies and that they can lead to a substantial improvement of reporting of these studies.

Beside improvement of reporting, a summary assessment for a marker of interest seems to require availability of full individual patient data for a 'well-defined' group of studies which should be incorporated in the summary. In order to avoid serious problems caused by publication bias, this latter issue is important.

It may also be sensible that researchers work together towards planned pooled analyses, a concept considered important in epidemiological research. Together with further issues of systematic reviews of observational studies this is discussed in detail by Blettner et al. [48].

Conclusion

In this paper, I have considered several critical issues of prognostic factor studies. Specifically, I have tried to show several basic statistical issues which are still handled insufficiently in many studies. Obviously, this represents a subjective choice and view of the author.

Much more on this topic can be found in Schumacher et al. [33] and other papers and books (e.g. Simon and Altman [31], Altman and Lyman [13]). The obvious messages of these papers are: firstly, that serious improvement is required (and is possible) in this type of research; secondly, that improvement requires the close collaboration between several disciplines – experienced statisticians have to play a central role in the planning, analysis, interpretation and reporting of these studies; thirdly, that summary assessments of prognostic factors require a close collaboration at an international level. In the research on treatment strategies, this has been successfully done for many years in several diseases.

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Author Index

Subject Index