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Frequent high telomerase reverse transcriptase expression in primary oral squamous cell carcinoma

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BACKGROUND: Gene copy number gain of chromosomal arm 5p is frequently found in oral squamous cell carcinoma (OSCC) suggesting the activation of proto-oncogenes. *TERT* is a candidate gene encoding for human telomerase reverse transcriptase (hTERT). The aim of the present study was to elucidate the relevance of *TERT* copy number gain and high hTERT expression in OSCC.
METHODS: Fluorescence *in situ* hybridization (FISH) for *TERT* and immunohistochemistry (IHC) for hTERT were performed to analyze *TERT* copy numbers and hTERT expression, respectively, on tissue microarray (TMA) sections including $n = 247$ OSCC and $n = 105$ pharyngeal and laryngeal squamous cell carcinomas (PSCC/LSCC).
RESULTS: Increased hTERT protein expression was more frequently found in OSCC (71.1%, 155/218) than in PSCC/LSCC (36.0%, 35/89) ($P < 0.001$). By contrast, specific *TERT* amplifications were less common in OSCC (2.1%, 4/191) compared with PSCC/LSCC (9.9%, 8/81) ($P = 0.047$).
CONCLUSIONS: High hTERT expression is a frequent finding in OSCC. It might be a promising target for the development of specific anti-neoplastic therapy approaches.
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Keywords: fluorescence *in situ* hybridization; human telomerase reverse transcriptase; immunohistochemistry; oral squamous cell carcinoma; *TERT*; tissue microarray

Introduction

Oral squamous cell carcinoma (OSCC) is a common malignancy affecting more than 500 000 individuals per year worldwide (1). A high rate of early relapse tumors due to minimal residual disease, and reduced 5-year overall and disease-free survival rates not exceeding 50% are typical clinical features of this neoplasm. During the last decade, genome screening approaches such as chromosomal comparative genomic hybridization (cCGH) were applied to disclose the molecular basis of oral carcinogenesis. Among others, gene copy number gain of the distal part of chromosome 5p was frequently found suggesting that proto-oncogenes localized in this region might be activated by gene copy number gain and contribute to OSCC initiation and progression (2, 3). Although the resolution of cCGH is low (~10 MB) (4) not allowing to pinpoint individual genes within this region, a number of promising candidate genes were suggested, which might be target genes of the 5p amplification in OSCC. One of these candidate proto-oncogenes is *TERT*, which is localized on chromosomal band 5p15.3 and encodes the human telomerase reverse transcriptase (hTERT). It is a well-known phenomenon of a variety of different cancer subtypes that high telomerase activity stabilizes telomere lengths, which possibly contributes to unlimited cell division and carcinogenesis (5–7). hTERT has been identified as a catalytic subunit of telomerase, the expression of which is crucial for telomerase activity. In OSCC, high telomerase activity as well as high TERT expression was found previously (8, 9), but the reason for high TERT expression remained unclear. As diverse specific oncogene amplifications were detected in OSCC (10–13), which led to increased protein amounts by a gene dosage effect, high hTERT protein expression might be the result of specific *TERT* amplification. To elucidate, whether *TERT* gene copy number gain results in increased hTERT protein expression, a clinically well-defined representative OSCC collection consisting of $n = 247$ tumor samples and a cohort of $n = 105$ pharyngeal and laryngeal squamous cell carcinomas (PSCC/LSCC) were screened for *TERT* gene copy number gain by fluorescence *in situ* hybridization (FISH) and for hTERT expression by immunohisto-

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chemistry (IHC) on tissue microarray (TMA) sections. In this respect, we aimed to enlighten the potential role of *TERT* and hTERT in the clinical course of oral carcinogenesis.

Material and methods

Tumor material and patients' characteristics

A total of 352 primary, paraffin-embedded head and neck squamous cell carcinomas (HNSCC) were obtained from the archives of the Institute of Pathology of the University Hospital Heidelberg after approval by the local institutional review board. A total of 247 tumors were primary OSCC and 105 tumors PSCC/LSCC. For all tumor samples, clinical and follow-up data of the patients were available from the Department of Oral and Cranio-maxillo-facial Surgery and the ENT Department of the University Hospital Heidelberg. All patients had a Caucasian phenotype. Mean age of the patients at the time of diagnosis was 60 years (range 16–92) for OSCC and 58 years (range 28–89) for PSCC/LSC respectively. Of OSCC patients, $n = 187$ were male and $n = 75$ were female. Of PSCC/LSCC patients, $n = 94$ were male and $n = 11$ female. TNM stadium and UICC stage of the different tumor subgroups analyzed are available from Tables 1 and 2. Five paraffin-embedded oral mucosa specimens from five healthy donors were used as control tissue.

Table 1 Summary of *TERT*-FISH results obtained from TMA analysis in different clinical subgroups of OSCC and PSCC/LSCC

FISH result	*BA no.*	*GCNG (AMPL) no.*
Total OSCC ($n = 191$)	81	110 (4)
T1/2 ($n = 95$)	40	55 (0)
T3/4 ($n = 96$)	41	55 (4)
N0 ($n = 80$)	35	45 (1)
N1–3 ($n = 111$)	46	65 (3)
SI–III ($n = 71$)	29	42 (0)
SIV ($n = 120$)	52	68 (4)
Total PSCC/LSCC ($n = 81$)	25	56 (8)
T1/2 ($n = 26$)	12	14 (3)
T3/4 ($n = 55$)	13	42 (5)
N0 ($n = 21$)	7	14 (2)
N1–3 ($n = 60$)	18	42 (6)
SI–III ($n = 18$)	8	10 (1)
SIV ($n = 63$)	17	46 (7)

BA, balanced; GCNG, gene copy number gain; AMPL, specific gene amplification.

Table 2 Summary of *TERT*-FISH results obtained from TMA analysis in different clinically subgroups of OSCC and PSCC/LSCC

Independent variables	*Coefficient*	*Standard error*	*Wald statistic*	*P-value*
Stage I–III vs. IV	1.488	1.065	1.951	0.162
OSCC vs. PSCC/LSCC	1.280	0.644	3.949	0.047
Age	−0.0420	0.0307	1.874	0.171
Gender	−0.854	1.075	0.630	0.427

TMA construction

The OSCC-TMA and the PSCC/LSCC-TMA were generated as previously described (10). Briefly, HE-stained sections were cut from each donor block to define representative tumor regions. Small tissue cylinders with a diameter of 0.6 mm were taken from selected areas of each donor block using a tissue chip microarrayer (Beecher Instruments, Silver Spring, MD, USA) and transferred to a recipient paraffin block. The recipient paraffin block was cut in 5-μm paraffin sections using standard techniques.

FISH analysis of TERT

For FISH experiments on TMA sections, BAC clone CTD-3080P12 (*TERT*) (Deutsches Ressourcenzentrum für Genomforschung, Berlin, Germany) was prepared from bacterial cultures using Qiagen-Plasmid-Kit (Qiagen GmbH, Düsseldorf, Germany) and labeled by nick translation in the presence of cyanine-3-dUTP (Perkin-Elmer Life Science, Boston, MA, USA). As internal control, a customer probe of chromosome 5 was differentially labeled with fluorescein-12-dUTP and co-hybridized. TMA slides were processed for FISH analysis as described recently (12). *TERT* gene copy number gain was defined if 30% of all cells analyzed obtained three to six signals per cell. If additionally at least 10% of all cells showed six or more signals or tight signal clusters, specific *TERT* gene amplification was scored. The signals of the centromeric probe were used as internal control for adequate hybridization efficiency.

IHC analysis of hTERT

Polyclonal mouse anti-hTERT antibody (Abcam, Cambridge, UK) was used in a dilution of 1:100 for IHC experiments. Standard avidin–biotin protocol was applied to TMA sections as described previously (11). The normal oral mucosa tissue specimens, which were available on both TMAs as controls, showed a slight nuclear and cytoplasmatic staining in the basal and suprabasal cell layer of the mucosa epithelium. As recent studies suggested that cytoplasmatic hTERT staining might represent the degraded form of the protein that retains hTERT antigenicity but not enzymatic function (14), only nuclear staining was considered for semi-quantitative evaluation of individual tissue cores (15). This was performed by calculating the percentage of stained tumor cell nuclei in the tumor specimen as follows: $<5\%$ positive cells: 'absent expression' (−); 5–25% positive cells: 'low expression' (+); 26–40% positive cells: 'moderate expression' (++); $>40\%$ positive cells: 'strong expression'. The average staining of the basal and parabasal layer of the five normal oral mucosa specimens was considered as 'low expression' (+). For statistical analysis, 'absent' and 'low' hTERT expression was tested against 'moderate' and 'high' hTERT expression.

Statistical analysis

Fisher's exact test was used to compare the prevalence of *TERT* gene copy number gain and differential hTERT expression in different tumor subgroups accord-

ing to the TNM system and the UICC stage. T1/T2 tumors were tested against T3/T4 tumors, N0 tumors against N1–3 tumors and stage I/II tumors against stage III/IV tumors. The prevalence of *TERT* gene copy number gain and hTERT high protein expression in primary OSCC was tested against the group of PSCC/LSCC by Fisher's exact test. To further calculate the influence of different clinical parameters on *TERT* copy numbers and hTERT expression, multivariate logistic regression analyses were performed including UICC stage, anatomic site, age and gender as independent variables. Estimations of the survival time distribution of patients with primary OSCC and PSCC/LSCC, respectively, were done by Kaplan–Meier analysis. Survival curves were compared by log-rank tests. Values of $P \leq 0.05$ were considered statistically significant.

Results

TERT copy number by FISH on TMA sections

For FISH analyses, $n = 191$ OSCC specimens were available on OSCC-TMA sections. The overall frequencies of CTD-3080P12 (*TERT*) copy number gain assessed by FISH analysis was 57.6% (110/191). Among those, specific gene amplification was detected in 2.1% (4/191). No significant difference concerning the frequency of gene copy number gain in OSCC subgroups T1/2 against T3/4, N0 against N1–3 and stage I–III against stage IV was found. An analysis of the overall and disease-free survival revealed no difference for primary OSCC with or without *TERT* gene copy number gain (all $P > 0.05$, Table 1). For primary PSCC/LSCC, for which $n = 81$ tumor specimens were available, there was a higher prevalence for *TERT* gene copy number gain (69.1%, 56/81) compared with primary OSCC. In addition, the number of specific *TERT* amplifications was higher in PSCC/LSCC (9.9%, 8/81) than in OSCC. In a multivariate logistic regression model, however, including UICC stage, anatomic site, age and gender as independent and *TERT* copy number gain and *TERT*-specific amplification as dependent variables, respectively, different anatomic sites influenced significantly the distribution of specific *TERT* amplification ($P = 0.047$) but not of sole *TERT* copy number gain ($P > 0.05$) in the tumor collection analyzed (Tables 2 and 3).

hTERT expression by IHC on TMA sections

To test whether OSCC shows an increase of hTERT expression, IHC analysis was performed (Fig. 1). For this TMA analysis, $n = 218$ OSCC specimens were available. The overall frequency of increased hTERT expression in OSCC was 71.1% (155/218). No significant difference concerning the frequency of increased hTERT expression in OSCC subgroups T1/2 against T3/4, N0 against N1–3 and stage I–III against stage IV were found (Table 4). For primary PSCC/LSCC, $n = 89$ tumor specimens were available for IHC analyses. In this collection of PSCC/LSCC, increased hTERT expression was significantly less frequently found (36.0%, 35/89, $P < 0.001$) compared with primary OSCC. This significant difference was also found in multivariate logistic regression analysis including UICC stage, anatomic site, age and gender as independent and hTERT expression as dependent variables ($P < 0.001$, Table 5). Kaplan–Meier analyses revealed no difference for overall and disease-free survival for OSCC and PSCC/LSCC with increased hTERT expression, respectively, compared with those tumors with low or no hTERT expression ($P > 0.05$).

To test whether *TERT* copy number gain might result in high hTERT expression in OSCC and PSCC/LSCC, a subset of $n = 227$ tumors, for which FISH as well as IHC results was available, was analyzed separately. No significant difference was found for the frequency of hTERT expression in tumors with *TERT* copy number gain (59.9%, 82/137) compared with tumors without *TERT* copy number gain (64.4%, 58/90). In addition, in a logistic regression model with hTERT expression as dependent variable and *TERT* copy number gain together with UICC stage, anatomic site, age and gender as independent variable, only anatomic site but not *TERT* copy number gain influenced significantly the distribution of high hTERT expression in this tumor subgroup analyzed (Table 6).

Table 3 Multivariate logistic regression model calculating the impact of UICC stage, anatomic site, age and gender (independent variables) on the distribution of *TERT* copy number gain in the tumor collection analyzed ($n = 272$)

Independent variables	*Coefficient*	*Standard error*	*Wald statistic*	*P-value*
Stage I–III vs. IV	0.0903	0.269	0.113	0.737
OSCC vs. PSCC/LSCC	0.491	0.291	2.839	0.092
Age	0.00296	0.0119	0.0616	0.804
Gender	−0.0249	0.297	0.00706	0.933

Discussion

High telomerase activity, which is absent in most normal somatic tissues, is a common feature of many tumor systems. By stabilization of telomere length, high telomerase activity prevents tumor cells from senescence and contributes to uncontrolled long-term proliferation. The catalytic subunit hTERT is an integrated part of the telomerase ribonucleoprotein complex and therefore essential for telomerase activity (7). An increase of hTERT expression was found associated with high telomerase activity in several tumor entities (16). For OSCC, high telomerase activity (17) as well as high hTERT mRNA expression (18) was detected, but a possible clinical significance remained unclear due to the limited number of tumor specimens investigated. In the present study, a representative well-defined OSCC collection was analyzed by IHC on TMA sections using a specific hTERT antibody. We were able to define the overall prevalence of high hTERT protein expression in OSCC with >70% of tumor specimens exhibiting high hTERT protein levels. Furthermore, as no difference in the frequency of high hTERT expression between tumors of early and late clinical stages was found, our data indicate that hTERT might be involved in early

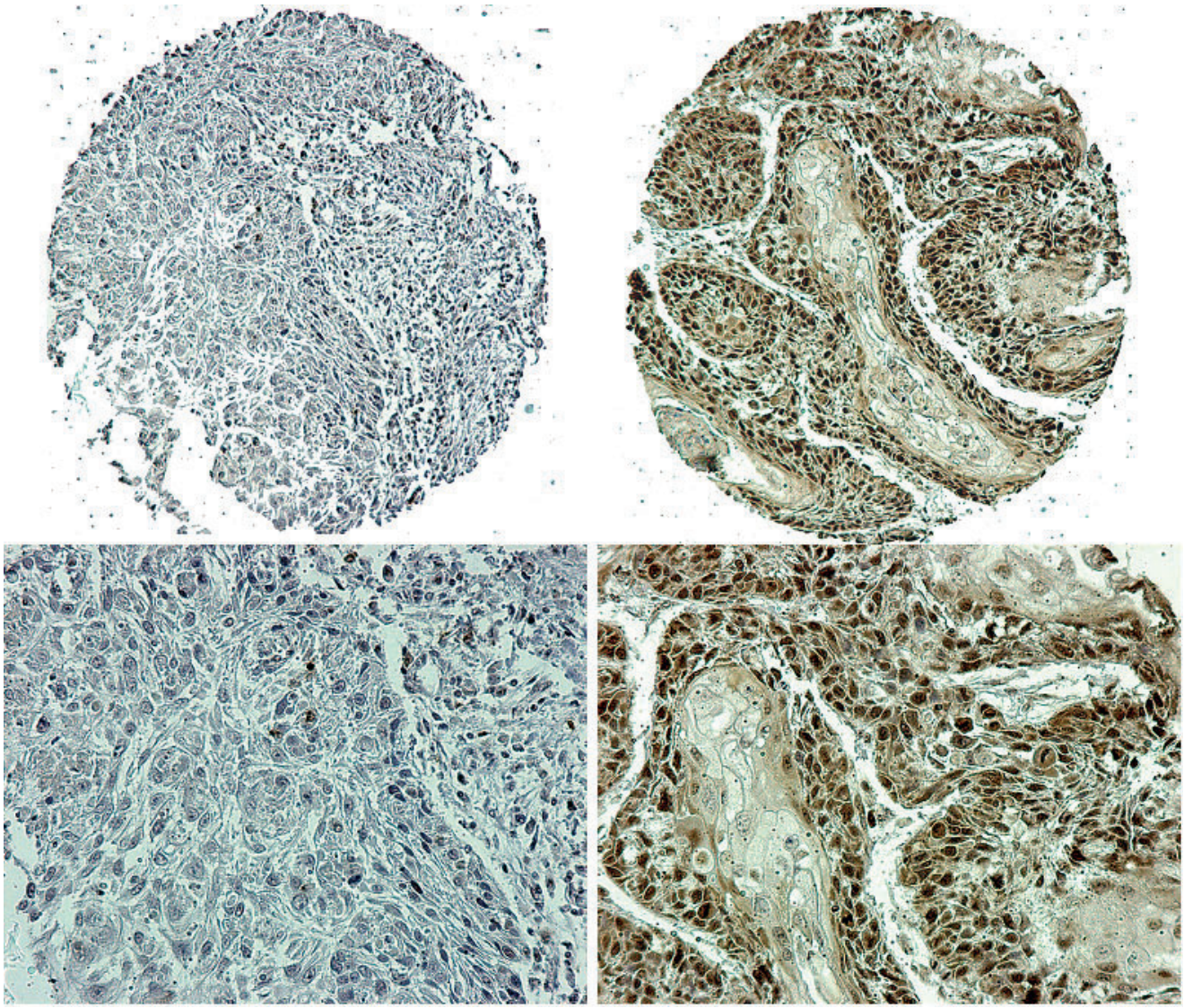

Figure 1 Detection of differential hTERT protein expression on TMA sections as detected by IHC. One tumor specimen with absent (−; left-top: 10×; left-bottom: 20×) and one with high (+++; right-top: 10×; right-bottom: 20×) hTERT expression are shown.

Table 4 Summary of TERT-IHC results obtained from TMA analysis in different clinically defined subgroups of OSCC and PSCC/LSCC

IHC staining	*Absent (−) no.*	*Low (+) no.*	*Moderate (++) no.*	*Strong (+++) no.*
Total OSCC ($n = 218$)	41	22	43	112
T1/2 ($n = 112$)	24	10	23	55
T3/4 ($n = 106$)	17	12	20	57
N0 ($n = 91$)	15	10	18	48
N1–3 ($n = 127$)	26	12	25	64
SI–III ($n = 86$)	17	10	17	42
SIV ($n = 132$)	24	12	26	70
Total PSCC/LSCC ($n = 89$)	39	18	16	16
T1/2 ($n = 26$)	14	2	4	6
T3/4 ($n = 63$)	25	16	12	10
N0 ($n = 23$)	8	3	7	5
N1–3 ($n = 66$)	31	15	9	11
SI–III ($n = 17$)	7	2	3	5
SIV ($n = 72$)	32	16	13	11

Table 5 Multivariate logistic regression model calculating the impact of UICC stage, anatomic site, age and gender (independent variables) on the distribution of high hTERT expression in the tumor collection analyzed ($n = 307$)

Independent variables	*Coefficient*	*Standard error*	*Wald statistic*	*P-value*
Stage I–III vs. IV	0.0196	0.271	0.00524	0.942
OSCC vs. PSCC/LSCC	−1.424	0.276	26.635	< 0.001
Age	0.0172	0.0118	2.131	0.144
Gender	0.381	0.314	1.476	0.224

oral carcinogenesis. Alternatively, as no correlation of high hTERT expression with such clinical parameters like advanced tumor stage or unfavorable outcome was determined, one might argue that these results might be biased due to the limited number of available tumor tissue on TMA sections. The assumption of hTERT playing a decisive role in the initiation of OSCC,

Table 6 Multivariate logistic regression model calculating the impact of *TERT* copy numbers in addition to UICC stage, anatomic site, age and gender (independent variables) on the distribution of high hTERT expression in the tumor collection analyzed ($n = 227$)

Independent variables	*Coefficient*	*Standard error*	*Wald statistic*	*P-value*
TERT BA vs. GCNG	0.0684	0.309	0.0489	0.825
Stage I–III vs. IV	−0.295	0.326	0.820	0.365
OSCC vs. PSCC/LSCC	−1.637	0.329	24.823	< 0.001
Age	0.00899	0.0149	0.365	0.546
Gender	0.534	0.363	2.162	0.141

BA, balanced; GCNG, gene copy number gain.

however, is corroborated by previous studies on oral precursor lesions. In moderate and severe oral dysplasia, high hTERT expression was found at a similar rate to invasive carcinoma specimens, whereas in normal oral epithelium hTERT expression was not detected (8). In addition, cells derived from oral dysplastic lesions exhibited immortal phenotypes in cell culture due to the activation of hTERT (19). Taken together, our data might provide further evidence for a significant participation of hTERT at an early stage of OSCC development.

Another aim of the present study was to elucidate, whether high hTERT expression was the result of gene copy number gain of the *TERT* locus. High-level amplification of *TERT* has been observed in several tumor entities, e.g. in cervical carcinoma (20), hepatocellular carcinoma (21) and neuroblastoma (22). For HNSCC, previous cCGH studies showed that especially the distal part of chromosomal arm 5p is subject of frequent gene copy number gain, which might eventually result in the activation of proto-oncogenes in this region (23). In the present study, gene copy number gain of the *TERT* locus was detected in the majority of OSCC specimens analyzed on TMA sections. Due to similar frequencies of *TERT* gene copy number gain and high hTERT protein expression in the collection analyzed, one might argue that increased *TERT* copy number led to high protein expression in OSCC. Distinct *TERT* high level amplification, however, was only found in four cases of OSCC. Furthermore, the prevalence of tumors exhibiting high hTERT protein expression was not significantly different in the group of tumors with *TERT* gene copy number gain compared with the group of tumors without *TERT* gene copy number gain. Eventually, although *TERT* gene copy number gain and distinct high-level amplification were more frequently present in the collection of PSCC/LSCC than in OSCC, the number of tumors exhibiting high hTERT protein expression was significantly lower in PSCC/LSCC than in OSCC. Therefore, we alternatively suggest that other proto-oncogenes residing in the vicinity of *TERT* on the distal part of chromosomal arm 5p might be target genes of the common 5p gains in OSCC and PSCC/LSCC. A promising candidate oncogene from the 5p consensus region is *TRIO*, encoding a potentially PTPRF-interacting triple functional protein, which was recently found amplified in a collection of OSCC (24). For *TERT*, other cellular mechanisms resulting in high hTERT expression might be active like transcriptional activation of the *TERT* promoter by viral integration as it was recently suggested for human papilloma virus type 16 in cervical carcinoma (25). As HPV infection might be involved in OSCC initiation and progression in at least a subgroup of tumors, it would be interesting to define the frequency of HPV 16 infection in the present collection with respect to the prevalence of high hTERT protein expression.

Although *TERT* might not be the target gene of 5p gains in HNSCC, the different frequencies of *TERT* aberrations and its differential protein expression in OSCC compared with PSCC/LSCC underline the recent findings of different molecular pathways, which are probably involved in initiation and progression of HNSCC of different anatomic localization (10). Our data suggests that 5p gains are more frequent in PSCC/LSCC than in OSCC. This finding is in concordance with a previous cCGH study, in which 5p copy number gains were most frequently found in PSCC and were defined as progression parameter (26). As our data preclude *TERT* as a relevant proto-oncogene from the 5p consensus region, which might be activated by copy number gain, it should be promising to analyze further 5p candidate genes in TMA experiments to define the relevant 5p proto-oncogene(s).

Due to the high frequency of high hTERT expression, which was especially found in OSCC in the present study, it might be a promising target for the development of specific anti-neoplastic therapy approaches. In cell culture experiments, it could be shown that the expression of a mutant catalytic subunit of human telomerase results in complete inhibition of telomerase activity limiting the cellular lifespan of human cancer cells (27). Recently developed specific hTERT inhibitors, such as the small molecule GRN163L, allowed to block telomerase activity *in vitro*, suggesting its translational potential as a novel anticancer therapeutic agent in breast cancer (28). For OSCC, promising results were obtained by using a combination of telomerase-specific antisense oligonucleotides and all-trans retinoid acid for specific hTERT inhibition (20). As our data indicate that high hTERT expression is frequent in OSCC of all clinical stages, the potential of these novel therapeutic agents should be evaluated in clinical phase I/II studies for early tumor stages preventing progression as well as for therapy refractory tumors.

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