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Parotid carcinoma: expression of kit protein and epidermal growth factor receptor

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OBJECTIVES: Our aim is to investigate the expression of kit protein (KIT) and epidermal growth factor receptor (EGFR) in parotid carcinomas in order to correlate the expression to histology and prognosis. Further we want to perform mutation analysis of KIT-positive adenoid cystic carcinomas.

PATIENTS AND METHODS: Formalin-fixed paraffin-embedded sections from 73 patients with parotid gland carcinomas were used for the study. The sections were stained with both KIT and EGFR polyclonal antibodies. Twelve KIT-positive adenoid cystic carcinomas were examined for *c-kit* mutation in codon 816.

RESULTS: Of all carcinomas 25% were KIT-positive and 79% were EGFR-positive. Ninety-two percentage of the adenoid cystic carcinomas were KIT-positive. None of the adenoid cystic carcinomas had mutations in codon 816 of the *c-kit* gene.

CONCLUSION: Neither KIT- nor EGFR-expression seem to harbour significant prognostic information. Adenoid cystic carcinomas express KIT, but no mutations in codon 816 of the *c-kit* gene were identified.

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Keywords: adenoid cystic carcinoma; *c-kit*; EGFR; kit protein; polymerase chain reaction; salivary gland carcinomas

Introduction

Primary parotid cancer makes up 1–3% of the head and neck carcinomas (1) and the incidence is estimated to be 0.7/100 000 inhabitants per year (2). It is a very heterogenic group comprising more than 10 different morphological types (3, 4). Some tumours are less malignant and have a good prognosis while others are very aggressive with recurrences, metastases and a fatal course. The long-term survival for adenoid cystic carcinoma as a whole is particularly poor with an overall-survival after 5, 10 and 15 years of 72%, 44% and 34%, respectively (5). The treatment of these tumours is surgery, which can be combined with radiotherapy, while chemotherapy still has a palliative role only (5, 6).

Immunohistochemical procedures can be important in classifying parotid carcinomas, since the choice of treatment depends on the tumour type. Furthermore, expression of specific membrane attached antigens have proved to contain prognostic information (7). Finally, overexpression of some proteins might open the possibility of new treatment modalities.

C-kit is a proto-oncogene located to chromosome 4 (long arm). It encodes a transmembranous tyrosine kinase receptor (KIT or CD117). KIT enters – after binding to its ligand – in the regulation of cell growth. Many cells express KIT – mast cells, interstitial cells of Cajal (the pacemaker cells of the gastrointestinal tract), epithelium cells of the breast and among others it plays an important role in the development of germ cells and melanocytes. KIT-expression has been found in many neoplasms – malignant melanoma, breast cancer, gastrointestinal stromal tumour (GIST), both small cell and non-small cell lung cancer, gynaecological cancers, thyroid neoplasms, myeloid leukaemia and seminomas (8, 9). C-kit mutation in the juxtamembranous domain (codon 560, exon 11) is a known mechanism in GIST where additional mutations have been found in exon 9 or exon 13. These tumours are now treated with the potent c-kit inhibitor Imatinib (STI571, Gleevec) with success (10–12). In adult systemic mastocytosis, there is also a known mutation, but in this case it is located in the phosphotransferase domain (codon 816, exon 17), and very rare in exon 11 (12). Attempts have been made to treat patients with aggressive adult systemic mastocytosis with Imatinib – but without success (13). Therefore, it has been concluded that Imatinib does not have effect on diseases caused by a mutation in codon 816. Several centres have now introduced a routine genetic investigation of patients with aggressive adult systemic mastocytosis as exclusion of mutation in codon 816 can result in a consideration of Imatinib treatment (11). It should be emphasized that the usual

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mutation in codon 816 is D816V, in which asparagine is replaced by valine. More infrequent mutations in the codon 816 exist – D816Y, D816N and D816H. In patients with those mutations, Imatinib has a lesser effect (12). Today Imatinib is also approved for treatment of patients with chronic myeloid leukaemia (CML) (11).

Epidermal growth factor receptor (EGFR) is one of four receptors in the human EGF receptor (HER)-family, namely HER-1. It is encoded by a proto-oncogene located on the short arm of chromosome 7 and it is like KIT – a transmembranous glucoprotein with tyrosine kinase activity. It has an influence on the initiation of DNA-synthesis and cell replication and it is expressed in a variety of normal tissues including salivary gland tissue (7, 14, 15). Overexpression is seen in different carcinomas, for example head and neck squamous cell carcinoma and in cancer of the breast, brain, lungs and the colon (15). EGFR inhibitors are known and studies concerning these have had particular focus on head and neck cancers. One inhibitor is the human monoclonal antibody, Cetuximab (16).

The purposes of this study are to investigate the expression of KIT and EGFR in parotid carcinomas in order to correlate the expression to histology and prognosis and to perform mutation analyses of KIT-positive adenoid cystic carcinomas in order to identify patients who might benefit from biological targeted therapy.

Patients and methods

The study is based on a regional database covering 1/5 of the Danish population (Funen and the southern part of Jutland). From 1975 to 1994, 85 patients with parotid cancer were treated at the Center of Head and Neck Oncology, Odense University Hospital. The material has been used in an earlier study by Godballe et al. (17). All specimens were then histologically revised by the same pathologist. Classification was carried out according to the World Health Organization (WHO) guidelines (3). After revision, 10 of the 85 (12%) were excluded (benign tumours, metastases). The histological distribution appears from Table 1. The median age at the time of diagnosis was 63 years (22–89 years) and the female–male ratio was 1/1. Two paraffin blocks did not contain enough tumour tissue for the present study. Samples from the remaining 73 patients were included in the study.

Sections of 4 μm thickness were cut from neutrally buffered formaldehyde-fixed paraffin-embedded tissue blocks. Sections were mounted at ChemMate^{TM} Capillary Gap Slides (DakoCytomation, Glostrup, Denmark) dried at 60°C, deparaffinized and hydrated. Prior to antigen retrieval, blocking of endogenous peroxidase was carried out in 1.5% hydrogen peroxide in Tris Buffered Saline (TBS) buffer, pH 7.4 for 10 min. Antigen retrieval was performed using microwave heating in 10 mM Tris with 0.5 mM Titriplex VI (EGTA) at pH 9.0. Three Tissue-Tek containers (Miles Inc, Elkhart, IN, USA), each with 24 slides in 250 ml buffer, were placed on the edge of a turntable inside the microwave oven. Slides were heated for 9 min at full power (900 W), then for 15 min at 440 W. After heating, the slides remained in buffer for 15 min. Incubation with KIT polyclonal rabbit antibody (DakoCytomation, code no. A 4502, dilution 1/800) and EGFR polyclonal mouse antibody (NovoCastra, Newcastle upon Tyne, UK, code no. NCL-EGFR, dilution 1/50) was carried out for 60 min at room temperature. Immunostaining was automated using the EnVision+^{TM} HRP detection systems K4003 for KIT-expression and PowerVision+^{TM} HRP detection system DPVB+500HRP (ImmunoVision Technologies, Brisbane, CA, USA) for EGFR-expression on the TechMate^{TM} 500 instrument (DakoCytomation). As substrate-chromogen system DAB+ K3468 was used (DakoCytomation). Immunostaining was followed by brief nuclear counter staining in Mayer's haematoxylin. Finally, cover slips were mounted. For all sections also, negative controls were made with antibody diluent.

For both KIT and EGFR colour intensity (0, 1+, 2+, 3+) and the percentage of positive coloured tumour cells were noted. On the basis of earlier published results (8, 9, 14, 18–24) tumours with over 10% positive tumour cells with a colour intensity 2+ or 3+ were considered positive. Both colouring in the cytoplasm and in the cell membrane were considered. Figures 1 and 2 shows KIT and EGFR colouring, respectively.

Mutation analysis of codon 816 was performed on KIT-positive adenoid cystic carcinomas. For c-kit

Table 1 Expression of KIT and EGFR in relation to histology

Histology	*KIT+ [n (%)]*	*KIT– [n (%)]*	*EGFR+ [n (%)]*	*EGFR– [n (%)]*	*Total [n (%)]*
Acinic cell carcinoma	1 (5)	19 (95)	14 (70)	6 (30)	20 (27)
Adenoid cystic carcinoma	12 (92)	1 (8)	10 (77)	3 (23)	13 (18)
Malignant mixed tumour	0 (0)	11 (100)	9 (82)	2 (18)	11 (15)
Mucoepidermoid carcinoma	2 (22)	7 (78)	7 (78)	2 (22)	9 (12)
Adenocarcinoma NOS[a]	1 (13)	7 (87)	6 (75)	2 (25)	8 (11)
Squamous cell carcinoma	1 (17)	5 (63)	6 (100)	0 (0)	6 (8)
Salivary duct carcinoma	0 (0)	3 (100)	3 (100)	0 (0)	3 (4)
Undifferentiated carcinoma	0 (0)	1 (100)	1 (100)	0 (0)	1 (1)
Epithelial–myoepithelial carcinoma	1 (100)	0 (0)	1 (100)	0 (0)	1 (1)
Basal cell adenocarcinoma	0 (0)	1 (100)	1 (100)	0 (0)	1 (1)
Total material	18 (25)	55 (75)	58 (79)	15 (21)	73 (98)

[a]Not other specified.

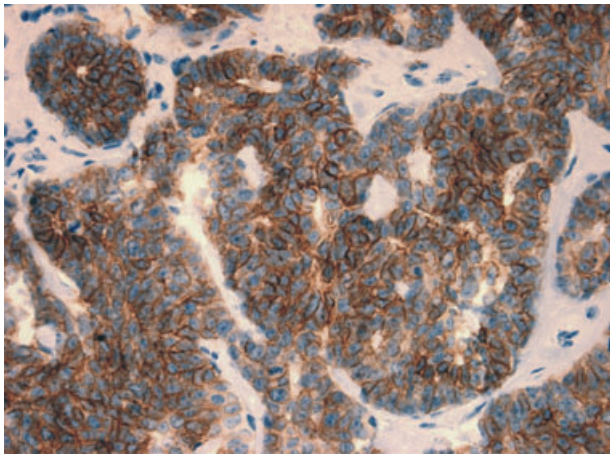

Figure 1 KIT-expression in an adenoid cystic carcinoma, colour intensity 3+ (KIT ×200).

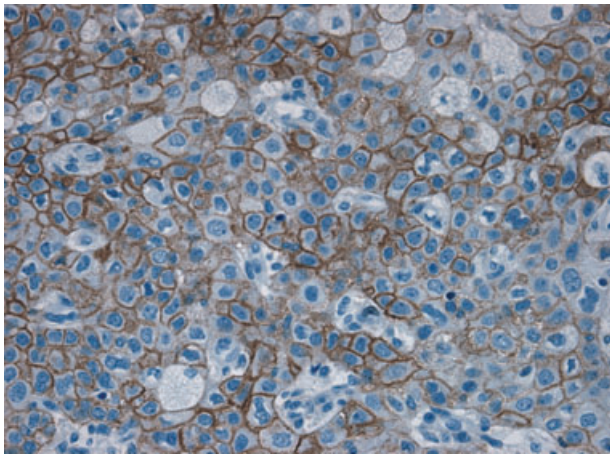

Figure 2 EGFR-expression in a mucoepidermoid carcinoma, colour intensity 3+ (EGFR ×200).

sequencing, genomic DNA was extracted from paraffin embedded tissue and purified by QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) was performed according to Nagata et al. (25). The amplified 328 base pair PCR product was isolated from agarose gel by SpinX (Coster). Sequencing reactions were performed using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analysed in an ABI3100 (Applied Biosystems).

The results of the immunohistochemical investigations were registered in a database. The database and analysis system Medlog was used for registering and statistical calculations. For analysis of categorical data, the χ^2-test with correction was used. Two-sided *P*-values < 0.05 were considered significant. Survival curves were calculated using the Kaplan–Meier method and compared to the Mantel–Haenszel log-rank test.

Results

Expression of KIT and EGFR in relation to histology appears from Table 1. According to the definitions described above, the total material (73 patients) revealed 18 (25%) KIT-positive and 58 (79%) EGFR-positive tumours. Only one of 20 acinic cell carcinomas, one of eight adenocarcinomas not otherwise specified (NOS), one of six squamous cell carcinomas, and none of 11 malignant mixed tumours were KIT-positive. Two of nine mucoepidermoid carcinomas and 12 of 13 adenoid cystic carcinomas were KIT-positive (92%). KIT was not expressed in normal salivary gland tissue.

Table 2 shows the expression of KIT and EGFR in relation to histological grade according to Therkildsen et al. (26), median tumour size, lymph node status and tumour stage (UICC 1992). No significant differences were found between the groups. Five-year disease specific survival (DSS) and crude survival (CS) rates according to KIT- and EGFR-expression are shown in Table 3. Kaplan–Meier plots showing DSS according to KIT and EGFR are presented in Fig. 3 and 4. No significant differences between the groups were found.

The 12 KIT-positive adenoid cystic carcinomas were subjected to mutational analysis of codon 816. The

Table 2 Expression of KIT and EGFR in relation to histological grade, tumour size, lymph node metastases and staging

	KIT+ [n (%)]	*KIT– [n (%)]*	*P-value*	*EGFR+ [n (%)]*	*EGFR– [n (%)]*	*P-value*
Grading						
Low	3 (17)	25 (45)	NS	21 (36)	7 (47)	N.S.
Intermediate	14 (78)	21 (38)	NS	29 (50)	6 (40)	NS
High	1 (6)	9 (16)	NS	8 (14)	2 (13)	NS
Median tumour size (mm)	30	33	NS	30	40	NS
Lymph node metastasis						
N0	16 (89)	38 (69)	NS	45 (78)	9 (60)	NS
N+	2 (11)	14 (25)	NS	12 (21)	4 (27)	NS
NX		3 (5)	NS	1 (2)	2 (13)	NS
Stage (UICC 1992)						
I	6 (40)	11 (61)	NS	27 (47)	6 (40)	NS
II	2 (13)	3 (17)	NS	9 (16)	2 (13)	NS
III	1 (7)			5 (9)	1 (7)	NS
IV	6 (40)	4 (22)	NS	16 (28)	6 (40)	NS
No staging				1 (2)		

NS, not significant ($P \geq 0.05$).

Table 3 Five-year survival of 73 patients with primary parotid carcinoma according to KIT- and EGFR-expression

Immunohistochemical marker	*DSS (%)*	*SE (%)*	*CS (%)*	*SE (%)*
KIT-positive	62	12.4	41	14.8
KIT-negative	76	6.2	53	6.9
EGFR-positive	71	6.6	49	7.0
EGFR-negative	73	11.7	59	12.9

DSS, disease-specific survival; CS, crude survival; SE, standard error.

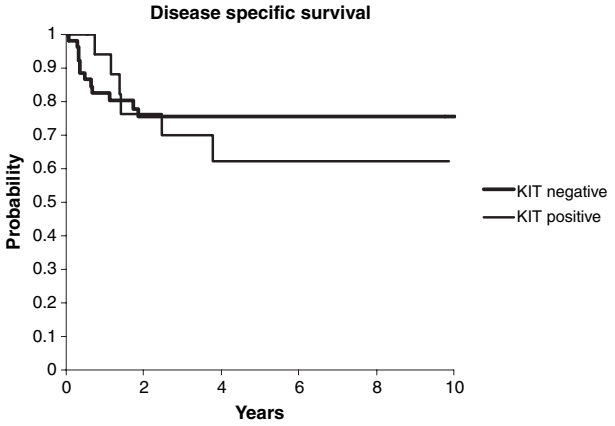

Figure 3 Disease-specific survival of 73 patients with primary parotid carcinoma according to the expression of KIT.

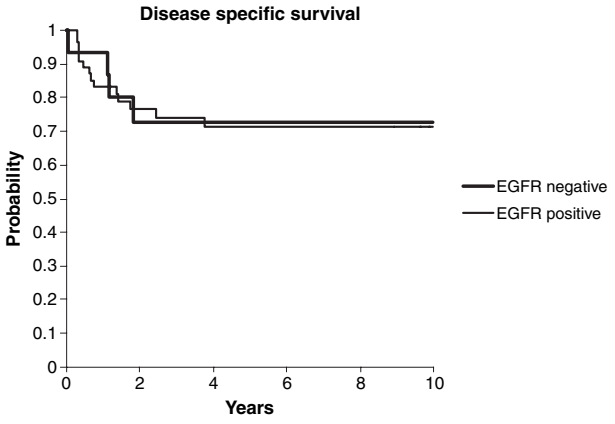

Figure 4 Disease-specific survival of 73 patients with primary parotid carcinoma according to the expression of EGFR.

quality of the DNA in four of the samples was too poor for the analysis. The DNA sequence of the remaining eight samples revealed no mutations.

Discussion

We found a high percentage of KIT-positive adenoid cystic carcinomas (92%) which is in agreement with other studies (8, 9, 18–21). The only negative case was a solid adenoid cystic carcinoma and as some studies have indicated a correlation between higher grade of malignancy and the number of KIT-positive tumour cells (8, 18, 19), we examined KIT-expression in three solid adenoid cystic carcinomas (not included in the present series). They were all KIT-positive with a colour intensity of 3+ in over 50% of the tumour cells. Poor fixation or diagnostic failure may be an explanation of the negative staining. Holst et al. (8) found 90% KIT-positive tumours of 30 adenoid cystic carcinomas. Jeng et al. (9) found positive KIT-expression in 80% of 25 adenoid cystic carcinomas, in six of six lymphoepithelioma-like carcinomas and in two of two myoepithelial carcinomas while the remaining 46 salivary gland tumours, which covered a broad histological spectrum were all negative. In other studies of adenoid cystic carcinoma KIT-expression was found to be 100% (18, 19, 21).

Mino et al. (20) used two different immunohistochemical methods (H300 and A4502) to study the KIT-expression in 66 adenoid cystic carcinomas and found 82% and 89% positive tumours respectively. Ninety-four percentage expressed at least one of two antibodies. In the remaining 98 salivary gland neoplasies – both malignant and benign, they found 16% were positive for at least one antibody while only 8% were positive for both antibodies. Generally, it seems that adenoid cystic carcinomas are KIT-positive though different factors may affect the results.

Several authors have examined KIT-expression in polymorph low-grade adenocarcinomas (PLGA) (18–20) and some argue for KIT as a marker to differentiate between this tumour and adenoid cystic carcinoma. Our material contains no PLGA, since they appear to be very rare in the parotid gland. All bidirectional differentiated tumours express KIT in varying degrees, that is why the antibody is not sufficient as the only marker in differentiation between these tumours, whereas KIT in a panel with other antibodies can be helpful in distinguishing tumours morphologically similar to adenoid cystic carcinoma. All 11 carcinomas in pleomorphic adenoma were negative and so were 19 of 20 acinic cell carcinomas.

The EGFR-expression in our material was generally high. We found that 79% were EGFR-positive. Seventy-seven percentage (10 of 13) of adenoid cystic carcinomas and 78% (seven of nine) of mucoepidermoid carcinomas were positive. Seventy percentage (14 of 20) of acinic cell carcinomas, the six squamous cell carcinomas, and the three salivary duct carcinomas were positive.

Few studies deal with the EGFR-expression in salivary gland tumours. The results of these studies are very divergent. The earliest studies found practically no expression (22). Katopodi et al. (14) found positive EGFR-expression in 56% mucoepidermoid carcinomas, 36% adenoid cystic carcinomas, and 22% adenocarcinomas NOS. Vered et al. (22) found negative EGFR-expression in four of 27 adenoid cystic carcinomas. The remaining 23 were scored by multiplying the number of positive coloured tumour cells by the colour intensity. This gave the results between 0.1 and 2.0. A score of 1.0 equals a weak colouring. Seventeen of the adenoid cystic carcinomas had a score under 0.5. This system of scoring makes a

comparison with our study difficult. Gibbons et al. (23) found positive EGFR- expression in about 70% mucoepidermoid carcinomas. Five of six adenoid cystic carcinomas were negative. Chen et al. (24) investigated the ligand of EGFR in 40 adenoid cystic carcinomas and found 35% positive. It seems that our samples have a higher EGFR- expression in comparison with those of the earlier studies. We found no significance between the EGFR- or the KIT-expression and the malignancy grade, tumour size, lymph node status or tumour stage. In keeping with this, no connection was found between expression of the two antigens investigated and survival. The Kaplan–Meier curve for KIT initially shows better survival for KIT-positive patients. After approximately 3 years the curves cross and the survival for KIT-positive patients obviously decreases in comparison with the KIT-negative patients. This can be due to adenoid cystic carcinomas comprising a big part of the KIT-positive tumours (67%) and that the well-known clinical course with late mortality breaks through. The survival analysis indicate that expression of KIT and EGFR is not qualified as a prognostic indicator for patients with primary parotid cancer.

One of the purposes of this study was to analyse, whether a mutation was present in codon 816 of the *c-kit* gene in KIT-positive adenoid cystic carcinomas. Mutation analysis was successful in eight of 12 KIT-positive adenoid cystic carcinomas and showed no mutation in codon 816 of the *c-kit* gene in any of the samples. The DNA in the remaining four samples was too degraded for PCR and sequencing. Holst et al. (8) and Jeng et al. (9) likewise found no mutation in codon 816. These discoveries led to a phase II investigation in Princess Margaret Hospital in Chicago (27). Sixteen patients with non-resectable or disseminated KIT-positive adenoid cystic carcinomas were treated with Imatinib and the results were published in January 2005. They found no clinical effect of Imatinib in these patients with advanced adenoid cystic carcinomas. Another phase II investigation in Beaujon University Hospital, France was published in September 2005 (28). They included eight patients all with documented KIT-positive adenoid cystic carcinoma in progression and pulmonary metastasis and treated them with Imatinib with more promising results.

Still, further and larger studies are needed in order to conclude on the effect of Imatinib in adenoid cystic carcinoma. The pathogenesis is still unknown and there is a need of new and better KIT-inhibitors to improve the treatment and prognosis of these patients.

Conclusion

It is concluded that KIT is extensively expressed in adenoid cystic carcinomas and that high EGFR-expression is found in all histological subtypes of parotid carcinomas.

Further, this study indicates that KIT- and EGFR-expression is not significantly correlated to histological grade or survival in parotid carcinomas. Finally, the KIT-positive adenoid cystic carcinomas do not have mutations in codon 816 of the *c-kit* gene.

Conflicts of interest

The Regional Scientific Ethical Committee has been informed about the study. There are no potential conflicts of interest.

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