Limited Suitability of EpCAM for Molecular Staging of Tumor Borders in Head and Neck Cancer

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Abstract. Background: The epithelial cell adhesion molecule (EpCAM) is expressed in most normal epithelia, but is absent from squamous stratified epithelia. However, a de novo expression can be observed in squamous epithelia during carcinogenesis. Materials and Methods: In order to evaluate EpCAM as a molecular marker to indicate borders of high risk for the development of local recurrences, its expression was examined in the marginal zone of malignancies. Specimens of squamous cell carcinoma of the head and neck (SCCHN), of the histologically tumor-free defined resection margin and of healthy epithelia of 20 patients were examined by RT-PCR in order to identify the expression of EpCAM in these three different areas. Additionally, immunohistochemistry was performed on biopsies from 10 patients in order to confirm these findings and to investigate a potential correlation between EpCAM expression and the degree of dysplasia. Results: By RT-PCR, high expression of EpCAM was found in the tumor. An inverse correlation was observed between EpCAM expression and the distance from the tumor, with no expression being detectable in healthy oral mucosa. In 70% of the cases, EpCAM was expressed in the marginal zone, which had been defined as tumor-free by routine histopathological assessment. Additional immunohistology revealed no correlation between EpCAM expression and the grade of dysplasia. Conclusion: Our data provide evidence that EpCAM is restricted as a marker for redefining the real tumor margin by RT-PCR. To complement routine histology, immunohistochemical staining with EpCAM is limited due to its expression in hyperplastic tissue without dysplastic changes. Both observations limit the reliable use of EpCAM for the molecular definition of the critical tumor border and resection margins.

The standard treatment of squamous cell carcinoma of the head and neck (SCCHN) is surgical resection of the tumor in combination with radio- and chemotherapy. The patient's survival strongly depends on a complete resection of the tumor, including an appropriate distance to the tumor margin. Therefore, histopathological assessment of the surgical margins is routinely performed. Due to the selective choice of few sections during routine histological workup and disseminated tumor cells in the margin, early stages of metastasis can be missed. Recently, new efforts have been made to improve histological diagnosis by using molecular methods (1).

EpCAM is a transmembrane glycoprotein expressed on the basolateral surface of many human epithelia. EpCAM has a direct impact on the cell cycle and proliferation and the ability to rapidly up-regulate the proto-oncogenes c-myc and cyclin A/E (2). However, the exact biological function of the molecule is still unknown. In normal human tissues, EpCAM is chiefly expressed in adenoepithelia, whereas it is absent from normal squamous stratified epithelia, but strongly expressed on the surface of more than 90% of SCCHN and preneoplastic lesions (3). First insights into the regulation of EpCAM demonstrated that its expression is negatively regulated by TNFα through the activation of NF-kappaB (4, 5).

The molecular analysis of EpCAM expression in marginal zones close to the tumor border could help to detect remaining tumor cells, or highly dysplastic tissues, and to define tumor borders more precisely, hence helping to identify patients at high risk for the development of local recurrences.
Materials and Methods

Patients' tissue samples. Specimens of 20 patients with primary SCCHN (for details see Table I) were taken intraoperatively, snap-frozen in liquid nitrogen and stored at –80°C. For each patient, three samples were collected from different locations: (i) tumor, (ii) tissue from the marginal zone (<1 cm distance to the tumor margin) and (iii) obviously healthy epithelium. The specimens of the marginal zone were collected from the tumor-free resection margins as confirmed by routine histopathology. The healthy epithelium was taken from a different location in the upper aerodigestive tract.

RT-PCR. Reverse transcription of 1 μg RNA was performed in a 20 μl reaction volume containing 10 mM DTT, 40U RNase Inhibitor (Boehringer, Mannheim, Germany), 4 μl 5xRT reaction buffer, 100 pmol oligo dT15 primer, 5 mM dNTP and 100U Superscript™II reverse transcriptase (Life Technologies, MD, USA). The mixture was incubated at 42°C for 60 min and then chilled on ice. PCR was carried out in a 50 μl reaction volume containing 2 μl cDNA, 1.5 mM MgCl2, 5 μl 10x reaction buffer, 100 pmol of each primer, 0.2 mM final concentration of each dNTP and 1U Goldstar DNA-Polymerase (Eurogentec, Seraing, Belgium). A Biometra thermocycler (Biometra, Göttingen, Germany) was used for amplification: 5 min at 94°C followed by 30 cycles of: 40 sec at 94°C, 30 sec at 60°C, 50 sec at 72°C. DNA-polymerization was completed at 72°C for 10 min. The analysis of amplified fragments was performed by electrophoresis through a 2% agarose gel and ethidium bromide staining. Sequences for the upstream and downstream primers, yielding a 301-bp fragment, were GAAGGCTGAGATAAAGGAGATGGG and TTAACGAGTGAGTCCAAGTTCTGG, respectively (Pharmacia Biotech, Denmark) was used for detecting the primary antibody. The specific bound primary antibody was visualized by staining with 3-amino-9-ethylcarbazol (AEC). Cell nuclei were counterstained with hemalaun (Merck, Darmstadt, Germany). Positive controls were performed with the elongation factor EF1γ.

Immunohistochemistry. Immunohistochemical analysis of the EpCAM protein was performed on paraffin sections with an avidin-biotin complex immunoperoxidase technique (Dianova, Hamburg, Germany). To block unspecific binding, sections were incubated with horse serum for 10 min. After removing excess fluid, the slides were incubated with the monoclonal EpCAM-specific antibody C215 for 60 min. The APAAP-system (DAKO, Glostrup, Denmark) was used for detecting the primary antibody. The specifically bound primary antibody was visualized by staining with 3-amino-9-ethylcarbazol (AEC). Cell nuclei were counterstained with hemalaun (Merck, Darmstadt, Germany). All experiments were repeated three times on two different slides.

Morphometric data evaluation. All immunostaining was initially qualitatively evaluated by light microscopy, by two independent observers, for the quality and extent of the staining results (+weak, ++ medium, +++ strong). Discrepancies in the observations were solved in conference. In addition, a semi-quantitative morphometric analysis of the amount of EpCAM-positive marked cells (tumor tissue, marginal zone, healthy epithelium) was performed.

Results

The expression of EpCAM in SCCHN and adjacent tissue by RT-PCR. The extent of the EpCAM expression by RT-PCR was graded as strong (+++), medium (+++) or weak (+) (Table II). Tumor tissue: All tumor probes investigated (n=20) demonstrated an expression of EpCAM: in 12/20 cases a strong expression was found, while in 6/20 cases a medium and in 2/20 cases only a weak expression of EpCAM was detectable. Marginal zone: In 14 out of 20 patients an expression of EpCAM in the marginal zone could be observed, while there was no expression of the epithelial glycoprotein in the other 6 patients. The extent of the EpCAM expression was graded as medium in 6 cases and weak in 8 cases. None of the specimens of the marginal zone showed a strong EpCAM expression. In comparison to the expression extent of the correspondent tumor, the expression in the marginal zone showed in 19 cases a weaker reaction and only in 1 case the same as the tumor. Healthy epithelium: Only 8 out of the 20 specimens taken from healthy epithelium showed an expression of EpCAM. There was no EpCAM expression found in the remaining 12 specimens.

Comparing the extent of the EpCAM expression of tumor, marginal zone and healthy epithelium, a decreasing expression of EpCAM on increase of the distance to the tumor could be found in 17 patients (Figure 1).
from the marginal zone showed normal tissue except for 2 slides, which showed singular carcinoma cells. Tissue taken from healthy epithelium in the head and neck region showed normal not dysplastic tissue.

**Immunohistochemistry. Tumor tissue:** The slides were checked for staining intensity (+ weak, ++ medium, +++ strong) and for the amount of EpCAM-positive tumor cells (Table II). Nine out of 10 slides stained with the antibody C215 showed a positive reaction for EpCAM (Figure 2). Six of the 9 positive slides showed more than 80% of positive tumor cells: 1 of them a weak, 1 a medium and 4 a weak to medium reaction. One slide showed a weak to medium reaction in 70% of the carcinoma cells. The remaining 2 slides had only a weak reaction in less than 10% of the carcinoma cells. Marginal zone: Five out of 10 slides taken from the marginal zone of SCCHN stained positive with C215. Two of them had been initially identified as carcinoma tissue. Their staining intensity was identical to the equivalent tumor slides. One slide showed a positive staining due to glandular epithelium, while the positive reaction of the other 2 slides was due to basal cell hyperplasia. **Healthy epithelium:** There was no positive staining for C215 in 9 out of the 10 slides. The positive reaction was due to EpCAM expression in the glandular epithelium.

The expression of EpCAM in SCCHN and surrounding tissue comparing RT-PCR and immunohistochemistry. Tumor tissue: In 9 out of 10 specimens, screened by both methods, a corresponding expression of EpCAM was found. Only 1 slide, identified as carcinoma, stained negative with C215 in immunohistochemistry while the RT-PCR was positive. Marginal zone: In the marginal zone, 4 specimens showed a positive reaction in both methods. In 2 cases the specimens had been immunohistopathologically proven as carcinoma, while the other 2 specimens showed basal hyperplasia of the epithelium. Three cases showed a positive reaction in the RT-PCR, while the immunohistochemistry showed no

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**Table II. The expression of EpCAM in tumor (T), marginal zone (MZ) and healthy epithelium (HE) by RT-PCR and immunohistochemistry (IH). The extent of the EpCAM expression was graded as strong (+++), medium (++ or weak (+). By IH, the percentage of positively-stained tumor cells is indicated (%). (nd = not determined)**

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One case showed positive staining for C215 due to glandular and hyperplastic tissue, while the RT-PCR failed. Two specimens showed no positive reaction, either in the RT-PCR or in the immunohistochemistry.

Healthy epithelium: Five specimens showed a positive reaction in the RT-PCR, while the immunohistochemistry was negative for EpCAM. One showed an EpCAM expression, due to glandular tissue in the specimen, by both methods. The last 4 specimens showed missing expression both in RT-PCR and immunohistochemistry.

Correlation of the expression of EpCAM with the grade of dysplasia of the tissue. There was no correlation between the expression of EpCAM and the grade of dysplasia of the tissue. However, expression of this glycoprotein was already evident in the early stages of hyperplastic or dysplastic squamous epithelium.

Discussion

The current five-year survival rate of patients with progressed SCCHN is 50% or less, the poorest of all epithelial tumors (6, 7). The current standard therapy combines the surgical resection of the tumor with adjuvant radio- and chemotherapy. The survival rate is mainly predicted by the spread of tumor cells to locoregional lymph...
nodes and complete surgical tumor resection. Remaining highly dysplastic or malignant tissue in the resection margin often leads to early recurrence, with potentially reduced prognosis. Although a histopathological examination of frozen sections is routinely performed during surgery in order to ensure complete tumor removal, patients with SCCHN of advanced stage often suffer from a local or distant recurrence which occurs shortly after the primary therapy. The rate of local recurrence is reported to be 50% to 60% and distant metastases to be 15% to 25% (8). These recurrences often occur in patients who had their tumors completely removed, as evidenced by histopathology (9). Cook et al. (10) showed a local recurrence rate in 30% of patients with SCCHN staged III to IV after a complete tumor resection with resection margins histopathologically classified as tumor-free.

This poor outcome may be variously explained. First, according to the hypothesis of field carcinization by Slaughter et al. (11), every tissue exposed to carcinogens, like alcohol and tobacco, shows highly dysplastic or premalignant alterations, leading to a condemned mucosa syndrome (12). Alternatively, single tumor cells may remain in the body after surgical therapy, to become the origin of local recurrence and lymph node metastases within a short period of time (13-15). A 5-μm slide of a tumor of about 1 cm diameter represents only 1/2000 of this tumor (16). Therefore, routine histopathology may not be accurate enough to detect single disseminated tumor cells remaining in the resection border. Currently, new methods are being developed to improve the molecular or immunohistochemical diagnosis of tumor resection margins.

Monoclonal antibodies (mAbs) against tumor-associated antigens (TAA) could be a suitable tool for both diagnosis and therapy. To date, only a few mAbs have yielded sufficient specificity against TAA. EpCAM could, in principle, be a promising target molecule, as it is not expressed on healthy squamous epithelium but appears during carcinogenesis (17, 18). As already observed for SCCHN, cancer of the cervix and the bronchus (3), EpCAM expression is neither dependent on the grade of differentiation nor on the histological type (17). Moreover, in other types of malignant tumors, such as melanomas, lymphomas and sarcomas, no EpCAM expression could be found. Keratinocytes, hepatocytes, fibroblasts and blood cells also do not express EpCAM (17). In contrast, sweat glands have been found positive for EpCAM (18).

These observations have attracted attention to EpCAM as a possible new tumor marker. Due to its de novo synthesis during carcinogenesis and the need to accurately assess the surgical resection margins in order to decrease the risk of local recurrence, tumor, the marginal zone and healthy epithelium from tumor patients were examined for the expression of the glycoprotein using RT-PCR. All tumor specimens, independent of localization, grade of differentiation and TNM-stage, were positive for EpCAM. EpCAM expression was still identified in specimens of the marginal zone and of healthy epithelium classified as tumor-free. By enlarging the distance from the tumor, the expression of EpCAM decreased. A similar observation, that EpCAM is combined with a loss of markers for differentiation and an increased proliferation activity, has been described for dysplastic and malignant cervix tissue by Litvinov et al. (3).

Brennan et al. (1) confirmed a similar result by examining the resection margins of SCCHN for a p53-mutation. They could demonstrate that 52% of the patients showed a p53-mutation in the resection margins already histologically defined as tumor-free. The follow-up showed a local recurrence in 38% of these patients. In contrast, patients without a p53-mutation developed no recurrence. Therefore, the early stages of carcinoma could not be recognized in an appropriate manner using histopathological examination, thus demonstrating the benefit of molecular staging. Therefore, a p53-mutation could also be a possible marker for the molecular detection of early tumor stages. Unfortunately, only 50% of SCCHN show a p53-mutation. In correspondence to the results of the p53-mutation (17), we established there is also no correlation between the histologically confirmed grade of dysplasia and the extent of the expression of EpCAM by RT-PCR. Moreover, it could be shown that both basal epithelial hyperplasia and dysplastic tissue showed an expression of EpCAM, with a significant increase of expression due to the de novo synthesis during carcinogenesis. This would qualify EpCAM as an ideal tumor marker for SCCHN.

Nonetheless, our results showed that EpCAM has major drawbacks in the detection of critical resection margins. In the daily routine, EpCAM is restricted as a possible tumor marker because of the manifold described heterogeneous expression within the tumor. In some cases, the expression of EpCAM by RT-PCR could not be confirmed by immunohistochemical examinations, although this could be due to different probes analyzed. On the other hand, the amplification of EpCAM from glandular and hyperplastic tissue without signs of dysplasia would lead to false-positive results in the RT-PCR. The RT-PCR may amplify single cells, which can not be detected by immunohistochemistry. Perhaps, this is also due to the fact that EpCAM is differentially glycosylated in healthy tissue and tumor cells (19).

Without correlation with morphological parameters, false-positive results could lead to unnecessary extensive resections with subsequent consequences for the patient. Even in routine immunohistochemistry, the use of EpCAM is limited because of the positive reaction in hyperplastic tissue without dysplasia, requiring additional staining for dysplastic tissue.
Future studies must aim at defining one or a combination of several tumor markers to increase the specificity of molecular staging for the improved definition of tumor margins.

References


