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EGFR and PCNA Expression in Oral Squamous Cell Carcinomas—a Valuable Tool in Estimating the Patient's Prognosis

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We investigated 100 cases of oral squamous cell carcinomas immunohistologically with respect to the expression of the epidermal growth factor receptor (EGFR) and the proliferating cell nuclear antigen (PCNA). The results were correlated with a new malignancy grading of the invasive tumour areas and the clinical outcome of the patients to estimate the individual prognosis. In conclusion, the amount of antigen expression of both antigens increases with the increasing grade of malignancy of the oral squamous cell carcinoma. Furthermore, there is a statistically significant correlation between the amount of antigen expression and the patient's prognosis. An overexpression of EGFR and PCNA is associated with a short survival of the patient. Both antigens detect relevant tumour biological parameters and are worthy factors in estimating the individual prognosis in patients suffering from oral squamous cell carcinomas.
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INTRODUCTION

ORAL SQUAMOUS cell carcinomas are clinically the most significant malignant neoplasms in the oral region. At present, about 7000 patients are afflicted in Germany every year. According to new epidemiological investigations, the incidence and mortality has been increasing continuously in the past few years [1].

The estimation of a patient's prognosis is of great importance as the selection of an individual therapy is based upon it. New immunohistologically detectable factors like oncogene products and growth factors have been introduced to complete the generally accepted clinical and classical morphological factors of prognosis such as staging and grading (especially grading of the invasive areas). These new factors, for example oncogene products or proliferation associated antigens, should allow getting a better estimation of the tumour biology and the patient's prognosis.

The following study deals with the question of the prognostical value of the expression of the epidermal growth factor receptor (EGFR), which is a product of the *erb* b oncogene, and the expression of the proliferating cell nuclar antigen (PCNA), which is a helper protein of the DNA polymerase delta [2], in oral squamous cell carcinomas.

MATERIALS AND METHODS

We investigated 100 patients with a primary squamous cell carcinoma of the oral cavity who were operated on and experienced a clinical follow-up of more than 5 years. Data concerning macroscopical tumour aspects and stage have not been included in the present study because we focussed on the biopsy specimens situation (for details see ref. 3).

All pathohistological and immunohistological preparations were done on paraffin sections of 5 μm thickness by an experienced technician. The sections were cut with a Reichert microtome, specimens of inferior quality were separated.

The pathohistological grading of the tumours (grade 1–3) was done on haematoxylin eosin stained sections according to the investigations of Bryne *et al.* [4]. This grading system, which is a modification of the malignancy grading recommended by Anneroth *et al.* [5], is established selectively at the tumour front (only the most anaplastic fields in the most invasive areas). It especially takes into account features of the tumour cell, i.e. the amount of keratinisation, the nuclear polymorphism, the number of mitoses, and features of relationship between tumour cells and connective tissue, i.e. pattern of invasion and leucocyte infiltration. These features were scored and summed up in three grading steps (grades 1–3).

The immunohistological investigations were prepared with the avidin biotin method using peroxidase as a marker enzyme and diaminobencidine as a chromogen [6]. Endogeneous biotin activity was blocked by 3% H_2O_2 preincubation for 10 min. The following primary monoclonal antibodies were used: anti EGFR (mab, batch number 113900102, Cat. 20050), E. Merk, Darmstadt, F.R.G., dilution 1:50, anti PCNA (mab, batch number 011A), Boehringer, Mannheim, F.R.G., dilution 1:20. The staining procedure included a series of obvious controls, i.e. absence of primary or secondary antibody (biotinylated sheep antimouse antibody, Dako, Hamburg, F.R.G., 1:50), substitution of the primary antibody with a non-specific antibody, and positive controls for the detection system.

The analysis of the EGFR staining reaction was done with respect to qualitative (staining intensity) and quantitative (number of positively stained tumour cells) aspects refering to the antigen expression in normal oral squamous epithelium. The quantitative approach was analogous to the staining score

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of oestrogen receptors in mammary carcinomas as proposed by Remmele and Stegner [7], i.e. the product of the amount of stained cells (1 pt ≤ 25% stained cells, 2 pts = 26–50% stained cells, 3 pts = 51–75% stained cells, 4 pts ≥ 76% stained cells) and staining intensity (1 pt = weak, 2 pts = medium, 3 pts = strong, 4 pts = maximal). This resulted in an EGFR expression score ranging from 1 to 16 points. With regard to the aspired Kaplan–Meier analysis these score points were divided into three groups (group I = low expression ≤ 8 pts, group II = intermediate expression ≥ 8 and < 12 pts, group III = maximum expression > 12 pts).

The analysis of the PCNA expression used the percentage of positively stained tumour cells and distinguished between four groups (group I < 25% stained cells, group II = 26–50% stained cells, group III 51–75% stained cells, group IV > 76% stained cells).

EGFR and PCNA analysis was carried out at the leading margin of the tumours and 9–32 (mean 20.3) areas (size 0.89 mm^2) were counted.

The statistical analysis was done with the SAS program package (SAS Institute Inc, North Carolina) including the Wilcoxon test. The Kaplan and Meier test (PROC LIFETEST) and the COX regression analysis (PROG PHGLM) was choosen to estimate the prognostic value.

The microscopical analysis and photo documentation was made with a Leitz DM microscope.

RESULTS

With respect to the patho-anatomical grading of the tumour front, there are 24 cases of grade 1 carcinomas, 43 cases of grade 2 carcinomas and 33 cases of grade 3 carcinomas. Grade 1 carcinomas exhibit a largely closed tumour front with little atypia and intensive keratinisation. Grade 3 carcinomas show a massive splitting of the tumour front, single tumour cell invasion, and, as a rule, higher atypia and loss of keratinisation. Grade 2 carcinomas take on a middle position with plump infiltrating tumour trabeculae (Fig. 1a–c).

In the EGFR analysis, there are 26 cases (group I) with a low antigen expression (< 8 score points), 50 cases (group II) with medium (> 8 and < 12 score points), and 24 cases (group III) with maximum antigen expression (> 12 score points). The staining reaction for the EGF-receptor is located strictly on the cell membrane. In grade 1 carcinomas there is only a reaction in the basal layer of the epithelium, while in grade 2 carcinomas many tumour cells are stained intensively. An overexpression of the antigen is found in grade 3 carcinomas on nearly all tumour cells (Fig. 2a–c) especially in the tumour front areas.

The proliferation characteristics (PCNA expression) of the squamous cell carcinomas investigated suggest 14 cases in group I (< 25% stained cells), 42 cases in group II (26–50% stained cells), 34 cases in group III (51–75% stained cells),

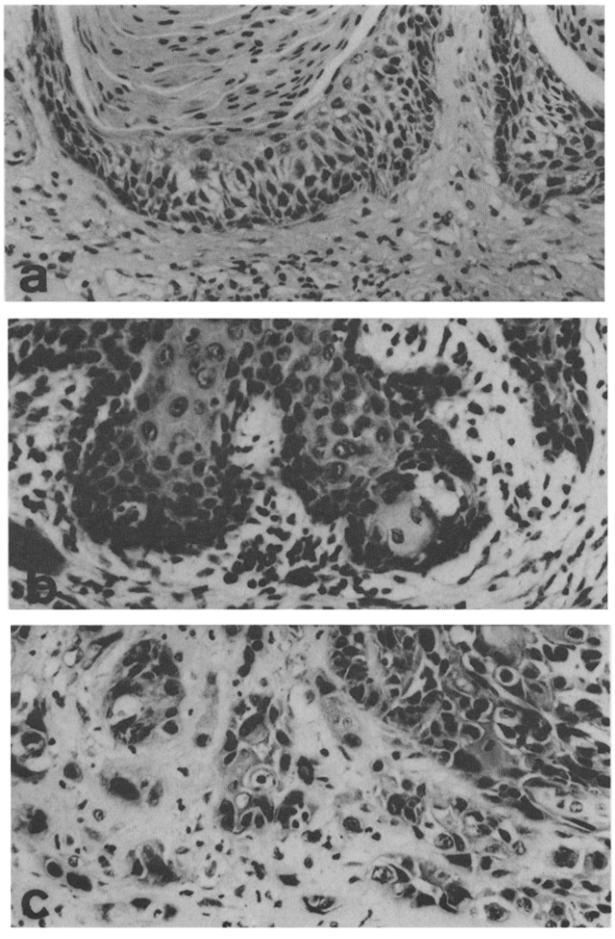

Fig. 1. (a) Oral squamous cell carcinoma, grade 1, tumour front areas (haematoxylin and eosin). (b) Oral squamous cell carcinoma, grade 2, tumour front areas (haematoxylin and eosin). (c) Oral squamous cell carcinoma, grade 3, tumour front areas (haematoxylin and eosin).

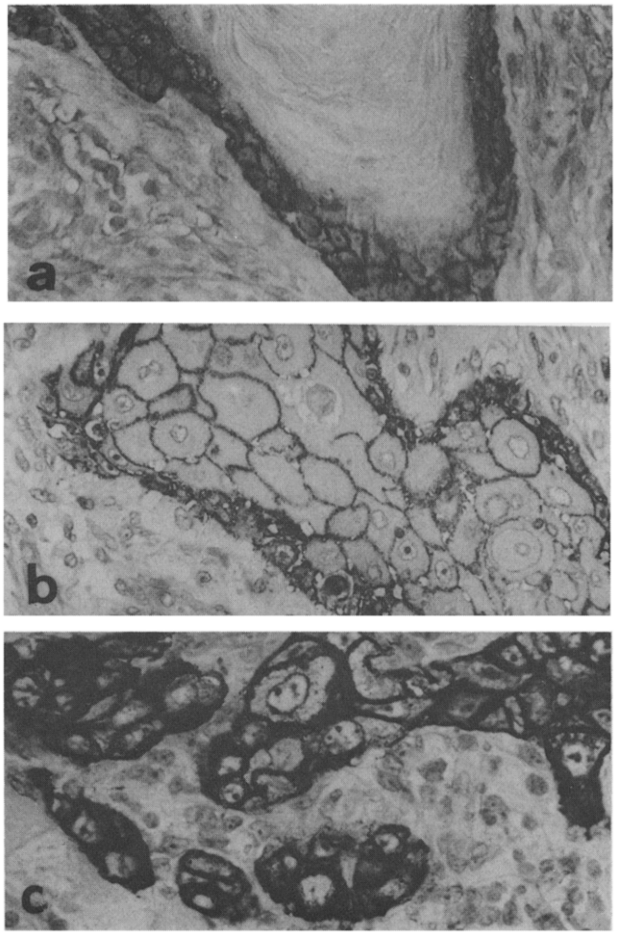

Fig. 2. (a) Oral squamous cell carcinoma, grade 1, tumour front areas, EGFR expression (POX). (b) Oral squamous cell carcinoma, grade 2, tumour front areas, EGFR expression (POX). (c) Oral squamous cell carcinoma, grade 3, tumour front areas, EGFR expression (POX).

and 10 cases in group IV (>75% stained cells). Taking the pathoanatomical grading according to Bryne *et al.* [4] as a basis, there is a minimal proliferation in the grade 1 carcinomas, a moderate proliferation in the grade 2 carcinomas, and a maximum proliferation in the grade 3 carcinomas. The staining reaction is found to be gradually different, microgranular, and brown coloured in the tumour cell nuclei (Fig. 3a–c).

Comparing the results of the tumour front grading and the EGFR expression, there is an increase in the EGFR expression (increase in score points) with the increasing grade of malignancy (Fig. 4). The medium values of the EGFR score points rise from 8.2 in cases of grade 1 carcinomas to 10.0 in cases of grade 2 carcinomas up to 13.7 in cases of grade 3 carcinomas. The differences between all tumour grades is of statistical significance (all P values $\leqslant 0.0176$).

Similar characteristics as found with the EGFR expression can be observed with the nuclear PCNA expression (Fig. 5). There is an increase in the percentage expression rate of PCNA from 40% in cases of grade 1 carcinomas to 58% in cases of grade 2 carcinomas up to 78% in cases of grade 3 carcinomas. The results are statistically significant (all P values < 0.0005).

The survival curves of the Kaplan–Meier analysis indicate, that the survival rate of the patients is lower when the EGFR expression is high (Fig. 6). The survival curve of the group of cancers with low EGFR expression (group I $\leqslant 8$ score points) runs very flat and exhibits a 5 year survival rate of 96%. The survival curve of the cases with intermediate EGFR expression (group II $\geqslant 8$ and <12 score points) runs much steeper presenting a 5-year survival rate of 57%. The survival curve of patients with maximum EGFR expression on the tumour cells (group III $\geqslant 12$ score points) is the steepest with a 5-year survival rate of 29%. The results of the COX regression analysis demonstrate that the survival time of the patients correlates highly with the EGFR expression ($\chi^2 = 16.3$; $P = 0.0001$).

Looking at the survival curves of the Kaplan–Meier analysis with respect to the proliferation rate of the tumours, one can see a reduction in the survival time with increasing PCNA expression (Fig. 7). The survival curves of groups I (<25% PCNA positive tumour cells) and II (26–50% PCNA positive tumour cells) run on a similar level in a very flat manner. This leads to a 5 year survival rate of 92% for group I carcinomas and 86% for group II carcinomas. The survival curves of group III (51–75% PCNA positive tumour cells) and group IV (>75% PCNA positive tumour cells) run at an identical level but compared to the ones mentioned earlier they are much steeper. The mean 5-year survival rate amounts to 28% in group III and 20% in group IV. The results of the COX regression analysis indicate that there is an excellent correlation between the survival rate of the patients and the amount of PCNA positive tumour cells ($\chi^2 = 22.4$; $P = 0.0001$).

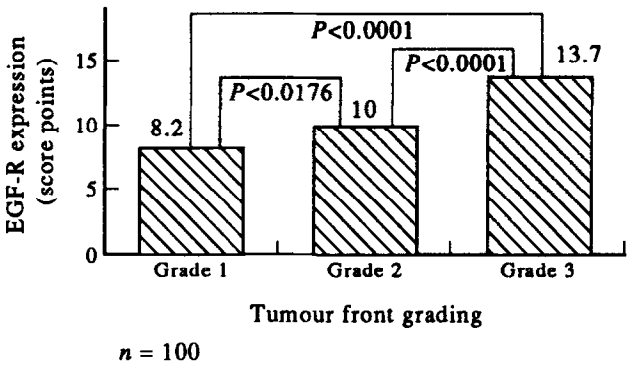

Fig. 4. Relation between EGFR expression (staining score) and tumour front grading in 100 oral squamous cell carcinomas.

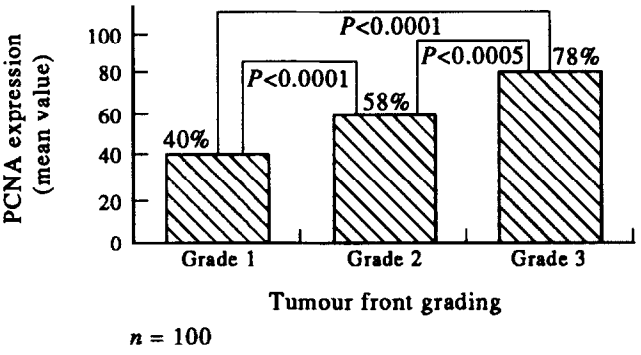

Fig. 5. Relation between PCNA expression (percentage of positive tumour cells) and tumour front grading in 100 oral squamous cell carcinomas.

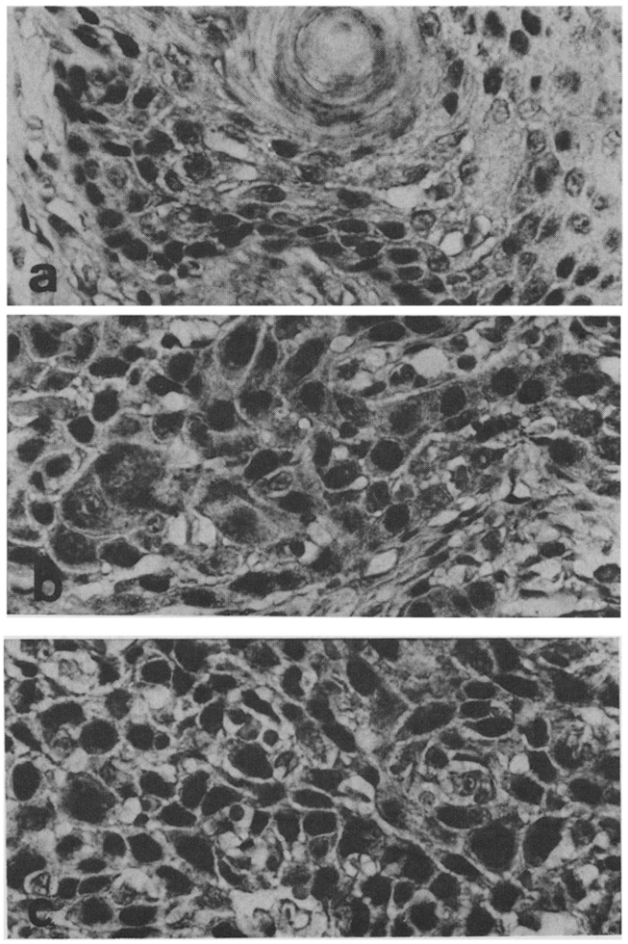

Fig. 3. (a) Oral squamous cell carcinoma, grade 1, tumour front areas, PCNA expression (POX). (b) Oral squamous cell carcinoma, grade 2, tumour front areas, PCNA expression (POX). (c) Oral squamous cell carcinoma, grade 3, tumour front areas, PCNA expression (POX).

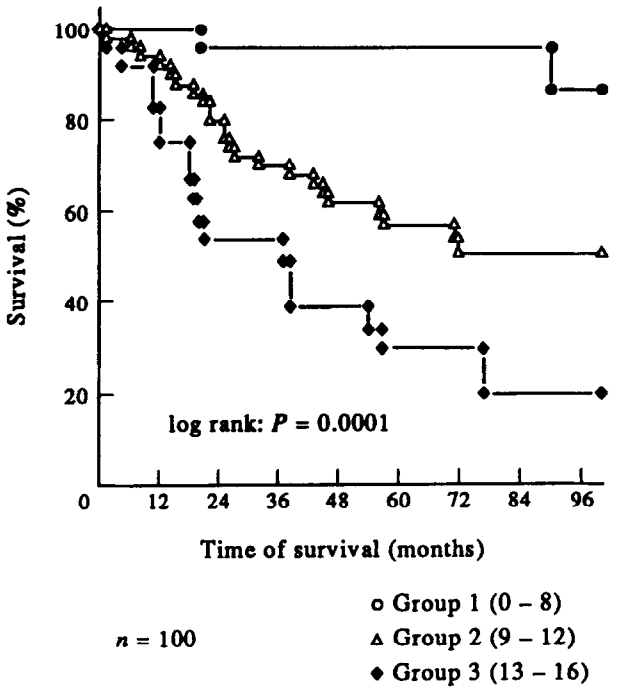

Fig. 6. Kaplan–Meier survival curves with regard to EGFR expression (score groups I–III) in 100 oral squamous cell carcinomas.

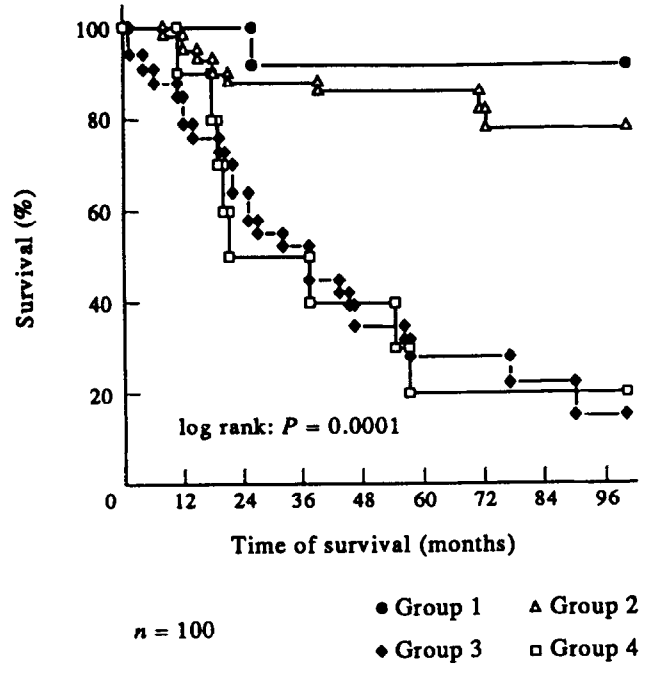

Fig. 7. Kaplan–Meier survival curves with regard to PCNA expression (groups I–IV) in 100 oral squamous cell carcinomas.

DISCUSSION

The estimation of prognosis of oral squamous carcinomas is of great importance for the choice of an individual concept of therapy. Clinical as well as morphological parameters of prognosis play an important role; for example, localisation and diameter of the tumour, tumour stage, age and sex of the patient, the prognostic index determining the therapy [8] as clinical parameters on one hand [9, 10], and different pathoanatomical grading systems [5, 11] and cellular features like the amount of tumour cell keratinisation and nuclear atypia as morphological parameters on the other hand [4, 12]. As there has been no satisfactory prognosis possible on this basis up to now [5], because the results varied from no to highly significant, it has been the aim to improve the estimation of prognosis with the aid of new prognostic indicators for some years. One important step was the new pathohistological malignancy grading of oral squamous cell carcinomas based on the analysis of the most invasive areas as proposed by Anneroth *et al.* [5] and modified by Bryne *et al.* in 1989 [4]. In our experience the latter modification proved to be the grading system of choice in a multivariate analysis besides tumour stage and metastatic spread [3].

In addition, functional factors (for instance adhesion molecules, oncogene products, immunological response modifiers and so forth) and growth rate characterising factors are of significance for the pathologist and they can easily be detected in paraffin sections with immunohistological methods. In particular, many of these factors are oncogene products essential for tumour development, tumour growth and tumour differentiation [13].

One of the important oncogene products in this field is the membraneous epidermal growth factor and its receptor (EGFR) which has already exhibited a good correlation to the tumour grading in carcinomas of the breast [14], bladder [15] and in oral squamous carcinomas [16]. In the present study of 100 oral squamous carcinomas all tumours were stained positively for EGFR with slight intratumoral variations of the membranous expression intensity, which confirms the observations of Partridge *et al.* [17]. The high incidence of EGFR expression in our study is in some contrast to the findings of other authors who reported on a cytoplasmic EGFR staining, only four positive squamous cell carcinomas out of 28 [18] and a 51% positivity [19]. We suppose that these differences might depend in part on the methods and used antibodies.

In this study there was an excellent correlation between the expression of EGFR and the modified form of the pathoanatomical grading system [4] too, which substantiates former findings [16, 20]. In addition, there is a good correlation between the expression of EGFR on the tumour cells and survival time as proven in the three different Kaplan–Meier curves and associated 5-year survival rates of 96%, 57% and 29%, respectively.

If we compare the proliferation associated antigens, the proliferating nuclear cell antigen (PCNA) working on paraffin sections in the past has gained more importance than the KI 67 antigen working on frozen sections (recently a new KI 67 antibody working on paraffin sections has also been available). PCNA is an auxiliary protein of DNA polymerase delta and according to Bravo and Frank [2] starts accumulating in the karyoplasm during the G1 phase of the cell cycle, finds its greatest expression in the S phase, and is reduced in the G2 and M phase. There are some publications in the literature about its occurrence in normal cells and haematological neoplasms [21], while only a few descriptions exist about its occurrence in solid tumours [22], especially squamous cell carcinomas [23–25]. In our study, we found an excellent correlation between the expression of PCNA and the pathoanatomical grading at the tumour front and the survival time of the patients. This is in contrast to the findings of Tsuji *et al.* [25] who found no correlation between increased PCNA expression, proliferation and worse tumour differentiation.

The Kaplan–Meier survival curves exhibit only two prognostic relevant groupings, one with a proliferation rate below 50% and the other one with a proliferation rate above 50% with regard to the choosen groups. The 5-year survival rate, on average, amounts to 89% (86–92%) for the first grouping and 24% (20–28%) for the second grouping which results in a clear bisection of the patients suffering from oral squamous carcinoma.

It appears that the immunohistological investigation of proliferation associated antigens like EGFR and PCNA on paraffin sections is able to detect important tumour biological aspects of oral squamous cell carcinomas [16, 26]. The investigated antigens seem to be better prognostic indicators than the clinically applied parameters, especially the prognostic index determining the therapy [8] which is widely spread in German-speaking countries. These data complete other established pathoanatomical parameters for instance tumour stage [3] and represent a worthy contribution to the characterisation of a tumour in an individual case. This is strengthened by the close correlation between the antigen expression in the tumour tissue and the patient's survival time. Another important advantage is that these markers can be easily used in routine diagnosis [24] and even on biopsy material prior to the analysis of resection material. The application of visual analytical systems can nowadays help the pathologists to evaluate the immunohistological preparations in a very fast and comfortable way.

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