Heterogeneity of Primary Site Biopsies in Head and Neck Squamous Cell Carcinoma

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Abstract. *Aim: There is no common standard defining how biopsies for translational research purposes should be performed. In our study, the impact of two different biopsy methods on the results of immunohistochemical staining of the samples for epidermal growth factor receptor (EGFR) and the proliferation antigen Ki-67 were evaluated. Patients and Methods: Twenty-four patients who underwent surgical treatment of their HNSCC tumour were included. From each surgically resected tumour, one superficial biopsy and one core-needle biopsy through the cross-section of the tumour were taken. As a positive control, a tissue slide through the primary tumour was made. Results: The analysis showed that neither the superficial nor the core biopsy expression of EGFR correlated significantly with that of the tumour. The analysis showed that the superficial biopsy expression of Ki-67 correlated significantly with that of tumour. Conclusion: Translational research projects based on biopsy tissues should be using whole surgical resection specimens of a tumour.*

Despite improvements in the treatment of locally advanced squamous cell carcinoma of the head and neck (SCCHN) with multimodal therapy strategies, 5-year survival for these patients remains less than 50% (1). In recent years, clinical, clinical, histopathological and various biomarkers have been studied extensively, on the one hand to identify patients with an unfavourable prognosis who have a need for a more aggressive therapy, and on the other hand to determine patients who would benefit from targeted therapy with new biological agents. One of the most intensely investigated biomarkers in SCCHN is the epidermal growth factor receptor (EGFR), which is overexpressed in more than 90% of SCCHN cases. Several studies indicated that EGFR overexpression is linked to poor prognosis (2-4), although there are also contradictory results (5). Against this background, anti-EGFR therapies were introduced into clinical practice and have already proven their efficacy. In particular, the anti-EGFR monoclonal antibody cetuximab was shown to prolong survival in both locally advanced and recurrent/metastatic SCCHN when added to platinum-based chemotherapy, without an increase in toxicity (6). Similar findings were described when cetuximab was given with radiotherapy simultaneously in comparison to radiotherapy alone (5). Nevertheless, not all patients benefit from targeted therapy and there are still uncertainties regarding the prognostic relevance of the various biomarkers. In order to evaluate the true value of biomarkers for prognosis and patient selection, however, large prospective trials with widely standardized procedures for performing the tumour biopsy and conducting molecular investigations are needed. In fact there is no standard defining how a biopsy for translational research purposes should be performed, with the consequence that results may differ significantly depending on the technique used.

In our study, we evaluated the impact of two different biopsy methods, a core-needle and a superficial biopsy, in comparison to a transverse section of the resected tumour on the results of immunohistochemical staining of the samples for EGFR and the proliferation antigen Ki-67. Both are unfavourable prognostic markers when overexpressed (2-4) and EGFR is also a target for cancer therapy (5, 7).

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Patients and Methods

The study was performed in accordance with the guidelines of the Helsinki Declaration of 1975, as revised in 1983, and the study protocol was approved by the local institutional review board (Ethics Committee of the Johann Wolfgang Goethe University of Frankfurt/Main).

From November 2007 to March 2009, 24 patients who underwent surgical treatment of their HNSCC primary tumour in our clinic were included in this study. The patients included one woman and

Table I. *Baseline demographics and disease characteristics of the 24 patient who underwent tumour surgery and were included in this study. All 24 patients had a locally advanced squamous cell carcinoma of the head and neck.*

Characteristic		No. of patients
Mean age, years (SD)		59.5
Gender	Male	23
	Female	1
Site	Oral cavity	2
	Oropharynx	12
	Hypopharynx	1
	Larynx	9
pT stage	T1	2
	T2	1
	T3	9
	T4	12
pN stage	N0	5
	N1	5
	N2	14
	N3	0
Disease stage	I	0
	II	0
	III	7
	IVa	15
	IVb	2

23 men, with age ranging from 44 to 80 years, with an average age of 61.7 years. Clinical and pathological characteristics of the 24 patients are shown in Table I. The main inclusion criteria were histologically proven primary SCCHN and a signed informed consent for the study. Before surgery was performed, each patient underwent computed tomography or magnet resonance tomography and an examination under general anaesthesia. Thus all patients had a histologically and radiologically confirmed SCCHN.

From each surgically resected tumour, two different types of biopsy were taken: one superficial biopsy and one core-needle biopsy through the cross-section of the primary tumour. As a positive control a tissue slide through the primary tumour was made. To decrease the source of errors, all biopsies were performed by only two different surgeons, who used the same technique.

The core biopsies were all taken with a 16-gauge needle 20 mm length and a diameter of 1.6 mm (Samatex Medical Technologies GmbH). The superficial biopsies had a size of 3 mm. The tissue slide through the primary tumour was taken by scalpel (Figure 5).

Study material. From November 2007 to March 2009, 72 formalin-fixed, paraffin-embedded specimens from 24 patients who were treated with primary surgery for SCCHN were obtained.

Antibodies. Immunohistochemical studies were performed using a mouse monoclonal IgG antibody against EGFR (Dako, Denmark) and a rabbit antibody against Ki-67. Both antibodies were diluted 1:100 in Dako Retrieval Solution. Positive control slides were made from known positive cases of the tonsils. Negative control slides were from the same tissue and were processed with the same immunostaining as the test slides but without the addition of the primary antibody.

Immunohistochemical staining. The tissue samples, fixed in 10% buffered formalin and embedded in paraffin, were cut into 5-μm sections and placed on precoated slides. After deparaffinization and rehydration, antigen retrieval was performed by microwaving with DAKO Target Retrieval solution (Dako). After the background staining was reduced, the primary antibodies were added and the slides incubated for 45 minutes at room temperature in a humidified chamber for EGFR and Ki-67. Secondary antibodies conjugated with streptavidin peroxidase were used. The slides were washed and the antibody complex was visualized by using Fuchsin+Substrate-Chromogen System (Dako). The nuclei were counterstained by hematoxylin.

Evaluation of staining. The positively stained cancer cells in 10 evenly subdivided fields per slide were counted. Altogether, 14,440 fields were evaluated.

Statistical analysis. For the statistical analysis, Friedman's nonparametrical analysis of variance together with multiple comparisons by Conover were used. The analysis was performed using the statistical software BiAS for Windows. The number of biopsies needed to obtain a reasonable precision has been calculated as described in the Results section.

Results

From all patients, it was possible to obtain sufficient tissue samples and all were immunohistologically stained for EGFR and Ki-67 expression.

The immunohistological analysis of the expression of EGFR showed that both the superficial and the core biopsy were significantly different from the representative transverse section through the tumour (Figures 1 and 2) (p=0.013 and p=0.036, respectively).

The analysis of the expression of the proliferation marker Ki-67 shows that the core biopsy was also significantly different from the transverse tumour section (p=0.008). However, the expression of Ki-67 in the superficial biopsy was not significantly different from that of the transverse tumour section, as shown by p=0.153 (Figures 3 and 4).

In the next step, for illustration purposes only, we employed the unit circle as a simplistic tumour model in order to give an impression of the relation between the sample size and the error of estimating the tumour size. The biopsy needle may randomly hit an orthogonal radius of the circle with mean μ and standard deviation σ. Assuming repeated hits to be independently uniformly distributed, the mean value of n hits results in a mean error range of $\mu \pm \sigma/\sqrt{n}$. For both limits of this error range the corresponding length of the bioptical specimen may be computed and in turn the lower and upper error boundaries of a tumour's cross sectional area. The difference of the latter boundaries reflects the error range of the biopsy. For error ranges of 5%, 10% or 20% of the true area, n=133, 33 or 8 biopsy specimens of a tumour are needed, respectively. Evidently, for a low number of specimens, one should be aware of a presumably

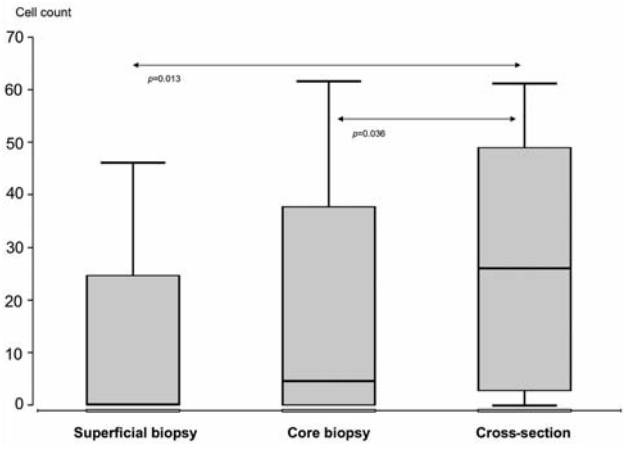

Figure 1. *EGRF expression in the superficial biopsies and the core biopsies compared with that in the cross-sections. The different biopsies were taken of 24 tumour preparations of head and neck cancer. There was a significant difference in the EGFR expression between the superficial biopsy and the cross-section through the tumour (p=0.013). The same was shown in the comparison between the EGFR expression of the core biopsy and that of the cross-section through the tumour (p=0.036).*

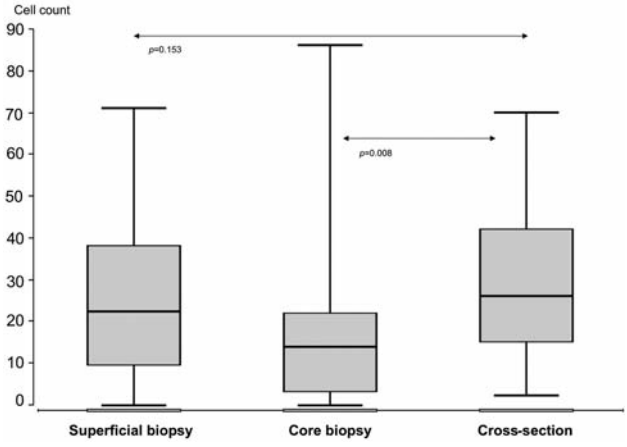

Figure 2. *The expression of Ki-67 in the superficial biopsies and the core biopsies compared with that in the cross-sections. The different biopsies were taken of 24 tumour preparations of head and neck cancer. There was no significant difference in the Ki-67 expression between the superficial biopsy and the cross-section through the tumour (p=0.153). There was a significant difference between the core biopsy and the cross-section in the Ki-67 expression.*

poor precision of estimating the true tumour area. Moreover, proceeding as described above, the true area will always be underestimated by a mean factor of about 0.75 resulting from the expected length of a specimen. With respect to the volume of a spherical model, considerably larger error ranges are to be expected.

Figure 3. *EGFR-positive tumour cells in the 24 superficial biopsies, 24 core biopsies and 24 tumour cross-sections.*

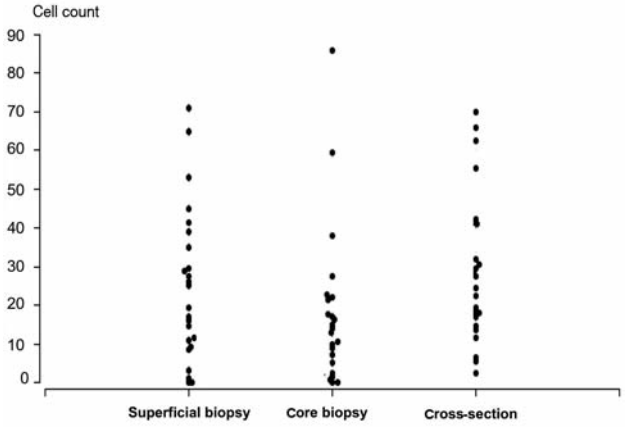

Figure 4. *Ki-67-positive tumour cells in the 24 superficial biopsies, 24 core biopsies and 24 tumour cross-sections.*

Discussion

Translational research programs represent an important tool to characterize the biological features of a tumour or haematological malignancy with the main aim of transferring the results into clinical practice. This includes the study of the biology of the disease to provide solid rationales for the development or improvement of new drugs or their combination and the evaluation of the biological effects of the drugs. In the last decade, with the development of molecular-targeted agents, more personalized treatment strategies have become available. Translational research helps to identify those patients who will most likely benefit from these newer targeted drugs. However, there are several uncertainties which possibly influence the reliability of these translational research efforts. In this study, we investigated the impact of

Figure 5. *Illustration of how the biopsies were taken. The core biopsy was taken using a 16-gauge needle with a length of 20 mm and a diameter of 1.6 mm. The superficial biopsies had a size of 3 mm.*

different biopsy methods, a superficial biopsy *versus* a core-needle biopsy, on the results of immunohistochemical studies of the obtained specimen when compared with a representative cross-section of the primary tumour. Because of anatomical limitations and possible functional impairment, radical surgery cannot be performed in all patients with SCCHN. Especially for these cases, validated biopsy methods are needed for primary diagnosis and translational research projects. But the anatomical limitations of head and neck cancer are not only a general problem in the cancer therapy of head and neck cancer, they also limit the possibilities of taking biopsies and this narrows the numbers and the size of biopsies. Even a small superficial biopsy of the larynx can cause a loss of function and dysphonia.

We found that neither the results of the core-needle nor superficial biopsy correlated with the results of the cross-section of the surgical resected tumour when immunohistochemically stained for EGFR. The same was true for the comparison of Ki-67 expression between core-needle biopsy and cross-section of the tumour. On the other hand, the results for Ki-67 staining showed there to be a significant correlation of the superficial biopsy and the cross-section of the tumour. This difference may be due to the known heterogeneity of protein expression in SCCHN (8) and technical uncertainties when various biopsy methods are used.

Takes *et al*. also found no correlation between immunohistochemical scoring for p53, Rb protein, E-cadherin, epithelial cell adhesion molecule (Ep-CAM), desmoplakin1 and cortactin on biopsy and resection material in SCCHN (9). Similar results were reported from a study validating tissue array technology in SCCHN. Three core biopsies of a tumour specimen and a full tissue section of the same tumour were immunohistochemically stained for cyclin-D1, Rb protein and EGFR. The authors used a weighted Cohen's κ coefficient as a measure of agreement between the two different samples and found only moderate agreement for Rb protein, a reasonable agreement for EGFR and substantial agreement for cyclin-D1. Therefore, the authors recommended using at least three, or better, four core biopsies for analyses (10). In our study, we calculated how many biopsies were needed to achieve concordance between the results of biopsy and cross-section of the tumour. At least eight biopsy samples were necessary for a correlation of 80%, 33 biopsies for a correlation of 90% and 133 for a correlation of 95%. As already mentioned, the anatomical limitations of the head and neck also limit the number of biopsies. An additional source of errors may originate from the staining technique, since immunohistochemical procedures are robust, versatile and sensitive methods for detection of specific molecules but provide only indirect evidence for the investigated target (11). Differences in staining conditions and techniques, antibodies and scoring systems may be responsible for conflicting results for many proteins especially in SCCHN (12-14).

With the emerging role of EGFR antibody therapy in the treatment of SCCHN, translational research projects have focused on the prognostic and predictive role of EGFR expression. The existing data are conflicting and this may be due to the uncertainties mentioned above: a) the heterogeneity of SCCHN in the tumour itself and in its metastases, b) the limitations of tissue samples obtained by biopsy, c) and the limitation of immunohistochemical methods. Even more specific examinations including the analysis of *EGFR* gene copy number with fluorescence *in situ* hybridization or the determination of *EGFR* mutations do not lead to reliable results. This is especially true in predicting which patients will benefit from targeted therapy (15, 16).

Conclusion

In summary, there are several reasons for the complexity of determining new prognostic and predictive factors in translational research projects. However, our study underlines the importance of obtaining representative tissue specimens with an adequate and thoroughly performed technique. Results of translational research projects, which are proven on biopsy tissues, should be valid when analyzing a complete cross-section of the resected tumour.

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