ORIGINAL ARTICLE

Pitfalls in immunohistochemical assessment of EGFR expression in soft tissue sarcomas

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Background: New targeted cancer treatments acting against growth factor receptors such as the epidermal growth factor receptor (EGFR) necessitate selecting patients for treatment with these drugs. Besides carcinomas, soft tissue sarcomas (STS) express EGFR and might thereby be a promising target for this new therapeutic strategy.

Objective: To test and compare different EGFR antibodies to determine the frequency of EGFR expression in STS.

Methods: 302 consecutive specimens of STS were examined using the tissue microarray technique. EGFR expression levels were assessed by immunohistochemistry using five different commercially available antibodies. Gene amplification status was measured by fluorescence in situ hybridisation (FISH). Immunoreactivity and amplification status were correlated with clinicopathological features and follow up data available in 163 cases.

Results: EGFR expression frequency ranged between 0.3% and 52.9%, depending on the antibody and scoring method used. In all, 3.5% of the tumours showed *egfr* gene amplification by FISH, which correlated with EGFR expression for three antibodies. Only one antibody had independent prognostic value in multivariate analysis and correlated with an unfavourable outcome; *egfr* gene amplification status showed no correlation with clinical features.

Conclusions: Frequency of EGFR immunopositivity in STS strongly depends on the antibody used, and only one of five antibodies tested predicted an unfavourable clinical outcome. This indicates that choice of primary antibody and scoring system have a substantial impact on the determination of EGFR immunoreactivity.

Soft tissue sarcomas of the extremities account for less than 1% of all malignancies in adults.¹ For the vast majority, complete surgical excision is the only curative approach.² Tremendous efforts have been made to gain a better understanding of the molecular pathogenesis of these tumours.^{3–4} In some entities this has already led to the development of alternate treatment strategies, targeted against specific gene products.^{5–6} These new “targeted cancer drugs” generally block the growth and spread of cancer cells by interfering with specific molecules involved in carcinogenesis and tumour growth. The epidermal growth factor receptor (EGFR) represents a potential target (table 1), and anti-EGFR drugs are already in use for the treatment of various carcinomas.^{14–19}

From a molecular point of view, several clues also point to EGFR as a potential target in soft tissue sarcomas.^{7–20–23} Treatment of sarcoma cells with EGF in vitro resulted in a more than fivefold increase in DNA synthesis and mitogenesis,²⁴ giving rise to proliferation and growth of sarcoma cells.²⁵ The transfection of sarcoma cell lines with antisense *egfr* DNA significantly impaired their proliferative ability.²⁶ The application of imatinib mesylate (Gleevec) in gastrointestinal stromal tumours (GIST) and dermatofibrosarcoma protuberans has already shown a selective blockade of the tyrosine kinases c-abl, PDGFR, and c-kit, with significant improvement in the clinical outcome.^{27–29}

However, the use of these new substances generally demands a reliable prediction of patients who might experience clinical benefit.^{30–32} As for c-*erbB2* or c-kit, it will therefore be necessary to provide reliable tools for determining EGFR status, including the optimal immunohistochemical detection of EGFR and the putative underlying genetic mutations.

The main focus of this study was therefore to test and compare a set of 5 different EGFR antibodies in order to determine the frequency of EGFR expression in a series of 302 unselected soft tissue sarcomas. The immunohistochemical data were compared with clinicopathological features and *egfr* gene amplification status measured by FISH analysis.

METHODS

Samples

In all, 302 specimens of malignant soft tissue tumours from 287 patients were retrieved from the files of the Institute of Pathology, University of Münster. All consecutive cases of malignant soft tissue tumour of the trunk and limbs received between 1988 and 2000 were included. Biopsy specimens and cases of Ewing sarcoma were disregarded. The collection consisted mainly of primary tumour tissue samples (97%), but also included six recurrent tumour specimens and three metastases. All patients were treated according to the same surgical protocol. Tumour specimens were used for investigation after informed consent had been obtained. The use of tumour tissue was also approved by the local ethics committee. The samples were formaldehyde fixed and embedded in paraffin. Specimens were classified according to standard protocols.^{4–33} The most common entities encountered were malignant fibrous histiocytoma, liposarcoma, and leiomyosarcoma. The frequencies of other tumour entities are listed in table 2. Fourteen per cent of tumours were classified as grade 1, 30% as grade 2, and 55% as grade 3; 73% had a

Abbreviations: EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridisation; STS, soft tissue sarcoma; TMA, tissue microarray

Table 1 Frequency of EGFR expression and amplification in soft tissue sarcomas according to recent studies

Frequency of expression	n	Tumour entity	Reference	Clone
51.4%	35	Different types of sarcoma	Gusterson <i>et al</i> , 1985 ⁷	Murine MAb EGF-R1
22%	28	Different types of sarcoma	Perosio and Brooks, 1989 ⁸	A431 human SCC Wistar
49%	29	Different types of sarcoma	Nielsen <i>et al</i> , 2003 ⁹	2-18C9
34%	29	Different types of sarcoma	Nielsen <i>et al</i> , 2003 ⁹	31G7
88%	8	Follicular dendritic cell sarcoma	Sun <i>et al</i> , 2003 ¹⁰	
70%	17	Synovial sarcoma	Barbashina <i>et al</i> , 2002 ¹¹	DAKO H11
90%	42	Synovial sarcoma	Nielsen <i>et al</i> , 2003 ⁹	2-18C9
67%	42	Synovial sarcoma	Nielsen <i>et al</i> , 2003 ⁹	31G7
60%	281	Different types of sarcoma	Sato <i>et al</i> , 2005 ¹²	2-18C9
49%	43	RNA expression	Duda <i>et al</i> , 1993 ¹³	
1.7%	117	egfr amplification	Duda <i>et al</i> , 1993 ¹³	

Table 2 Frequencies of included soft tissue tumour entities

Angiosarcoma	7	2.3%
Fibrosarcoma	16	5.3%
Haemangioendothelioma	4	1.3%
Malignant fibrous histiocytoma	70	23.2%
Leiomyosarcoma	36	11.9%
Liposarcoma	44	14.6%
Neurogenic sarcoma	9	3%
Rhabdomyosarcoma	32	10.6%
Malignant peripheral nerve sheath tumour	24	7.9%
Synovial sarcoma	34	11.3%
Sarcoma NOS	7	2.3%
Other	19	6.3%
	(n = 302)	

NOS, not otherwise specified.

diameter of more than 5 cm, 27% a diameter of 5 cm or less. Clinical follow up data were available in 163 cases. The mean follow up time was 46 months (range 3 to 235). Mean age at diagnosis was 47 years (range 2 to 87), with a standard deviation of 19.2 years. Thirty four per cent of patients developed local recurrence, 40.9% developed metastases, and 34.6% died of the disease. Seventeen patients (5.9%) had metastases on initial presentation. Of the patients with available clinical data, all had surgical resection of the tumour, 46.9% were treated with additional radiotherapy, 35.2% with additional chemotherapy (different protocols), and 8.6% and 14.8% received neoadjuvant radiotherapy or chemotherapy, respectively.

Tissue microarray construction

A tissue microarray (TMA) was composed,^{34 35} consisting of more than 600 cores with a diameter of 0.6 mm each and a distance of 0.2 mm. To locate representative tumour areas,

haematoxylin and eosin stained sections were prepared from each original tumour block. Two cores per specimen were punched out using a dedicated TMA instrument (Beecher Instruments, Silver Spring, Maryland, USA).

Immunohistochemistry

Five primary antibodies (table 3) were used for immunohistochemical evaluation of EGFR expression. Extensive testing was conducted using consecutive sections of a squamous cell carcinoma as positive control to determine where possible the optimal pretreatment, dilution, and antibody detection system of primary antibodies (table 3). Procedures were modified until high concordance was achieved among the different antibodies. Benign tumours and non-neoplastic tissues such as skin, included in the TMA, were used as additional positive and negative controls.

Expression was graded from 0 to 3 according to the DakoScore for HER-2/*neu*. For further statistical analysis, cases with a score of >0 were regarded as showing immunopositivity. Because of the use of a TMA, the reviewing pathologists were fully blinded to the clinical status of the patient.

Fluorescence in situ hybridisation

The probe for *egfr* detection was derived from homo sapiens PAC clone containing the whole *egfr* gene (GenBank accession No AC006977). The procedure was carried out as previously published.³⁶ For each core, 20 non-overlapping intact tumour cell nuclei were selected for scoring, as previously reported.³⁷ The cut off frequency for amplification was defined as four signals per nucleus.

Statistical analysis

Statistical analysis and tests were undertaken using SPSS Version 11.5.1. Correlations between EGFR expression, amplification, and clinical variables were tested with cross

Table 3 Overview of characteristics and tissue processing issues of 5 different antibodies to EGFR applied to soft tissue sarcomas

Antibody	Species	Clone	Dilution	Pretreatment	Detection	Specificity
DAKO	Mouse monoclonal	H11L	*1:200, 25' RT	Proteinase K 10'	LSAB/AP	Wild-type human EGFR, EGFRvIII
DCS	Mouse monoclonal	111.6	†Prediluted, 25' RT	Steamer 30'	LSAB/AP	Extracellular domain of human EGFR
Novocastra	Mouse monoclonal	EGFR.113	1:20 o/n, 4°C	Citrate buffer, steamer 20'	ABC	External domain of human EGFR
Ventana	Mouse monoclonal	3C6	†Prediluted, 32' 37"	Protease, 18' 37"	LSAB/AP	Extracellular domain of human EGFR wild type and EGFR vIII
Zymed	Mouse monoclonal	31G7	*1:100, 25' RT	Proteinase K 10'	LSAB/AP	Extracellular domain of human EGFR

*Using a DAKO Autostainer instrument.

†Using the Ventana Benchmark Instrument.

tables applying the χ^2 test,² and correlation analysis was done according to Kendall (Tau b). For survival analysis, Kaplan-Meier analysis, log rank tests, and multivariate survival analysis according to Cox's regression model were used.

RESULTS

Immunohistochemistry

Immunoreactivity for EGFR was seen along the membranes and in the cytoplasm of soft tissue sarcoma cells. Depending on the antibody used, certain variations were observed: while the DCS and Novocastra antibodies preferably stained the cytoplasm of tumour cells, the DAKO, Ventana, and Zymed antibodies showed a more accentuated membranous reactivity. However, no nuclear staining could be observed with any of the antibodies.

There were striking differences in the frequency of EGFR immunoreactivity in the tumours, depending on the primary antibody used: a score >1 was seen in six tumours (2.1%) with the DAKO antibody, in one (0.3%) with DCS, in three (1%) with Novocastra, in 92 (30.5%) with Ventana, and in 34 (11.6%) with Zymed (fig 1). A mild to strong immunoreaction (score >0) was seen in 49 tumours (17%) with DAKO, in six (2.1%) with DCS, in 13 (4.5%) with Novocastra, in 156 (52.9%) with Ventana, and in 100 (34.1%) with Zymed (fig 1). Despite these discrepancies, there were significant correlations between all the antibodies when tumours with a score of >0 were regarded as positive.

The frequency of EGFR expression varied among different histological subtypes depending on the antibody used. Figure 2 illustrates the relative frequencies of the most common histological subtypes.

Fluorescence in situ hybridisation

Using FISH, 283 samples could be analysed. Ten of these (3.5%, three malignant fibrous histiocytomas, two rhabdomyosarcomas, and one each of angiosarcoma, myofibrosarcoma, leiomyosarcoma, liposarcoma, and schwannoma) showed amplification of the *egfr* gene, mostly high level amplifications. Interestingly, amplification status of the tumour cells was quite inhomogeneous. Next to regions with a regular number of signals per nucleus, there were areas with clear high or low level amplifications (fig 3).

Correlations

When samples were regarded as immunopositive for EGFR with an immunohistochemical score of >0 , EGFR immunoreactivity with Ventana's and Zymed's antibodies correlated with tumour grade (both $p<0.001$). Novocastra's antibody even correlated with development of metastasis ($p=0.019$) as well as with an unfavourable clinical outcome ("died of the disease") ($p=0.035$). Log rank testing showed a significant ($p=0.036$) correlation with worse survival (fig 4). In multivariate analysis entering tumour size, grade,

Figure 1 Percentage of sarcomas showing epidermal growth factor receptor (EGFR) immunopositivity depending on antibody and scoring method (>0 , >1).

Figure 2 Frequency of epidermal growth factor receptor (EGFR) immunopositivity in different histological sarcoma subtypes depending on the antibody used (only the four most common entities are shown).

and the five EGFR antibodies, only the Novocastra antibody was selected, and none of the other variables had additional prognostic value.

Figure 3 Fluorescence images showing sarcoma cells with one to two *egfr* gene copies each (upper panel) and with strong whole gene amplification of *egfr* (lower panel).

Figure 4 Kaplan-Meier diagram showing worse overall survival of patients with epidermal growth factor receptor (EGFR) immunopositivity, as detected with Novocastra's antibody.

When considering cases with a score of >1 as positive for EGFR, no correlation with clinical features was found except between Ventana's antibody and tumour grade ($p = 0.001$). Figure 4 shows the Kaplan-Meier survival diagram for Novocastra antibody.

No correlation between *egfr* amplification status and clinical features (overall survival, local recurrence-free survival, metastasis-free survival), tumour grade, or size could be found. A good correlation ($p < 0.01$ for all) was, however, present with EGFR immunoreactivity assessed by the Novocastra, Ventana, and Zymed antibodies, but none when assessed by the other antibodies. Figure 5 shows representative staining results for the five antibodies in the same sarcoma in comparison with the positive control.

Novocastra's antibody mostly stained larger tumours (six tumours >5 cm, three <5 cm), one half each grade 2 and 3. Two thirds were located in the extremities, only one third were excised from the trunk. Only one of the positive tumours featured metastases initially.

DISCUSSION

New molecular targeted treatments require a reliable prediction in order to optimise the therapeutic response and reduce adverse side effects. In this context it is obvious that a set of technical tools including immunohistochemistry, gene dosage measurements, and mutation analysis is mandatory, especially with regard to anti-EGFR targeted treatments in lung and other epithelial cancers.^{31 32}

Compared with carcinomas, knowledge of EGFR in soft tissue sarcomas is sparse. However, experimental results have

given clear hints that blockade of EGFR mediated pathways might also be of clinical benefit in soft tissue sarcomas.^{7 20-26}

Assessment of EGFR expression by immunohistochemistry is not straightforward, though the antibodies used for the present study are widely used and commercially available. The frequency of EGFR immunoreactivity in soft tissue sarcomas showed wide variability, ranging from 0.3% to 52.9%. Using tissue microarrays, inter-experiment variation could be reduced. Nevertheless, our results show that the routine use of these antibodies for predictive purposes in soft tissue sarcoma requires initial definition of specificity and sensitivity levels.

The interpretation of our findings is complicated by numerous unanswered questions such as the differing frequencies of immunoreactivity depending on the antibodies and scoring schemes used. On top of that, different antibodies show mixed accentuated cytoplasmic and membranous staining patterns. One could argue on the one hand that only a membranous localisation of EGFR indicates a biologically intact receptor and allows response to targeted treatment (as for c-erbB2); on the other hand, one might speculate that intracellular immunoreactivity reflects a high intracellular content and turnover of EGFR. After ligand binding and dimerisation with other members of the erb family, EGFR is internalised and located in the cytoplasm. It is not yet clear if this reflects a biologically and clinically relevant status. However, the predominant intracytoplasmic staining pattern of c-kit in gastrointestinal stromal tumours indicates that this may be the case.³⁸ Cytoplasmic detection of c-kit generally provides evidence for the presence of activating mutations as the underlying cause of the overexpression and is regarded as sufficient to justify targeted treatment.

We wanted to shed some light on the value of different antibodies by studying variables such as outcome and the correlations between protein expression and gene amplifications. A significant correlation between EGFR immunopositivity and overall survival, as well as occurrence of metastases in multivariate analysis, could be seen for only one antibody (Novocastra). The interpretation of this result is nevertheless problematic. Our tumour collection was rather heterogeneous, and in fact only two of the five antibodies tested showed associations between tumour grade and EGFR immunopositivity. In contrast to other studies¹³ we were able to demonstrate a significant correlation of EGFR immunopositivity and *egfr* amplifications with three of the five antibodies used, while *egfr* amplifications were detected in only 3.5% of cases by fluorescent in situ hybridisation and did not correlate with any clinical feature. It has to be admitted that the absolute frequency of *egfr* amplifications may be underestimated in TMA because of their potentially heterogeneous distribution in soft tissue sarcomas. On the other side, the use of TMAs allows direct comparison between gene amplifications and EGFR expression in the same tumour area, thus increasing comparability. In spite of the correlations between gene amplification and protein overexpression, a significant number of sarcomas did not show any amplification despite protein overexpression. This further supports the thesis that overexpression of EGFR is not driven by whole gene amplifications alone, as we were previously able to show for breast carcinoma.³⁶

In conclusion, the immunohistochemical detection of EGFR as a screening technique is restricted by various pitfalls. The consequence might be that with increasing knowledge of the role of EGFR in soft tissue sarcomas, other indices will complement or even substitute EGFR immunohistochemistry. Activating mutations in non-small-cell lung cancer and gastrointestinal stroma tumours have already shown the power of molecular approaches. Nevertheless, our results clearly indicate that immunohistochemical detection

Figure 5 Photomicrographs depicting positive controls (left side) and one sarcoma tumour specimen (right side) stained with five different EGFR antibodies (A, DCS; B, DAKO; C, Novocastra; D, Ventana; E, Zymed).

Take-home messages

- Frequency of EGFR immunopositivity in soft tissue sarcomas depends on the antibody used.
- This indicates that choice of primary antibody and scoring system have a substantial impact on the determination of EGFR immunoreactivity.

of EGFR without detailed knowledge of the underlying molecular changes remains problematic and requires further intense research in the field.

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