Tissue Microarray Technology used to Assess EGFR Status in Human Meningiomas

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Abstract

Background Large immunohistochemical studies on formalin fixed and paraffin embedded tissues requires sectioning and processing of many tumors, which is both expensive and time-consuming. Tissue microarray (TMA) technology allows for more efficient investigation of a large number of cases. However, the credibility of this procedure is not fully clarified for EGFR expression in human meningiomas. **Aim** In this study we wanted to compare the immunostaining of EGFR on traditional whole tissue sections (WTS) with TMAs in these tumors. **Methods** Two TMA blocks and their corresponding WTSs encompassing a total number of 47 cases were investigated using an EGFR antibody. The expression levels were recorded as a staining index (SI) and results from the two immunostainings were compared. **Results** Significantly higher expression levels were found on WTSs compared with TMAs, however, the SIs were positively correlated. Further, some tumors regarded as negative on TMAs were positive on whole-tissue sections. Assessment of heterogeneous EGFR expression was also more indistinct on TMAs **Conclusion** The use of TMAs to assess EGFR receptor status in human meningiomas is encumbered with some degree of uncertainty, and thorough optimizing of the immunohistochemical procedure is required. With this in mind, TMA is an efficient method for immunohistochemical analyses of large tumor series.

Introduction

Meningiomas account for over one-third of tumors in the central nervous system and are the most common intracranial tumor in humans (1). Despite benign histology, they may grow to substantial size and have a tendency to recur even after gross-total surgery (2, 3). Identifying recurring cases is therefore an important issue to establish optimal follow-up and appropriate treatment for these patients.

Microscopy is the current gold standard for diagnosing human meningiomas and thus fundamental for prognostic considerations. However, biomarkers could facilitate the diagnostic process and potentially serve as prognostic markers. A possible target is the epidermal growth factor receptor (EGFR)/c-erb-B1, a cell-surface tyrosine receptor protein which initiates cellular responses such as proliferation and differentiation (4). This receptor protein is commonly expressed in human meningiomas (5-8), however, the prognostic value is still uncertain (9-11).

Formalin-fixed and paraffin embedded tissues are routinely used in immunohistochemical (IHC) studies to identify tumor-related proteins in both clinical and experimental settings. In large studies, whole-tissue sectioning is both expensive and time-consuming. On the other hand, tissue microarray (TMA) technology is an efficient method compensating for these inconveniences and allows examination of a large number of cases under standardized conditions. A disadvantage of TMA technology is that only focal areas of the tumor tissue are investigated and consequently heterogeneous protein expression may not be fully unveiled, leading to discrepant results between marker expressions on TMAs and whole tissue sections (WTS). This issue has previously been investigated by other research groups for different tumor entities and proteins, and both high (12-21) and variable or low (22-25) levels of concordance are reported. No such studies have to our knowledge been performed on meningioma tissues, and exploring the credibility of TMA to assess a potential biomarker such as EGFR is important. Here, we compare the immunostaining of this receptor protein on TMAs with WTS of meningioma tissue.

Material and methods

Specimen selection and initial investigation

The material in this study was collected in connection with a previous study originally comprising 196 adult patients (over 18 years old) operated for primary intracranial meningioma between 01.01.1991 and 31.12.2000 (26). Representative tumor tissue, which was defined as tumor cells lacking necrosis, hemorrhages and blood vessels, and with minimal connective tissue and calcific components, was chosen from HES-stained whole tissue sections (WTSs) of each tumor.

TMA construction

For TMA construction the semi-automated Alphelys Tissue Arrayer Minicore® 3, AH diagnostics, with TMA Designer2 software was used. Three tissue cylinders with 1000 μm diameters were extracted from each individual tumor. The spot spacing was set to 1600μm, which resulted in 600 μm gaps between the borders of each tissue cylinder. From the whole series of meningiomas a total of nine TMA blocks were constructed. In the current study, two of these TMA blocks were chosen at random for investigation with a total of 47 cases (**Fig 1**). The TMA blocks included three liver cylinders for orientation of the TMA and 24 individual tumors. One tumor was not found in the archives and was therefore excluded from analysis. The grades and subtypes are shown in **Table 1**.

Immunohistochemistry

Sections were cut from both TMA- and original paraffin blocks with a thickness of 4μm and put on Superfrost® Plus, Thermo Scientific glass slides. All sections were dried over night at 37°C and stored in a -20°C freezer. Sections were heated at 60°C for one hour before staining procedures, deparaffinized and pre-treated for antigen retrieval with PT Link (Dako) using pH 9 solution, and endogenous peroxidase activity was quenched using hydrogen peroxide for 10 minutes. Thereafter, sections were incubated for 60 minutes with an EGFR antibody (clone EGFR.113, mouse monoclonal Ab IgG2a, 1:10 dilution, Novocastra, Leica Biosystems) and then incubated for 30 minutes with the detection system Dako EnVision™ + HRP in a Dako AutostainerPlus. Diaminobenzidin (DAB) was used as chromogen (2 x 5 minutes) and hematoxylin as counterstain. EMA confirmed the presence of meningioma tissue. Normal human skin tissue and liver served as positive control, and in the negative control the primary antibody was excluded (**Fig. 2**). TMA and WTSs were analyzed using either Nikon Eclipse

50i or 80i microscopes. A staining index (SI), defined as the product of staining intensity and the proportion of tumor cells stained, was calculated for each case to assign numeric values to antibody reactivity (**Table 2**) (27). Both authors independently scored each case and were unaware of any case identifiers. Whole-tissue sections were assigned a temporary random ID to ensure that any links to TMA core identity were eliminated. Discrepancies in results between the investigators were discussed, and a consensus was reached before final scores were established.

Statistics

SPSS version 24.0 (SPSS Inc., Chicago, IL) was used for statistical analyses, and significant values were defined as $p < 0.05$. The non-parametric Spearman correlation was used to compare the SI scores between TMAs and WTSs. The Wilcoxon signed ranks test was used to check for significant differences between the two methods. Cohen's kappa was used to evaluate agreement between the methods. A percentage for cases with perfect agreement was also calculated, and this also included a comparison of dichotomized data according to high vs. low values with the median staining indexes as cutoff points (median or above vs. below median). The Kruskal-Wallis test was used to assess if the differences in SI between TMA and WTS was related to tumor subtype.

Ethics

The study was approved by the Regional Committees for Medical and Health Research Ethics (project number 4.2006.947).

Results

The EGFR expression levels were lower on TMAs than on WTS. The mean and median SIs for tumors on TMAs were 3.89 and 3.00, respectively, compared with mean and median values of 6.00 on WTS (**Table 3**). The Spearman correlation test showed a significant association between the SIs of WTS and TMAs (ρ=0.536, bootstrap CI (0.286 to 0.726), $p<0.001$), but the Wilcoxon signed ranks test showed that the SI-values for WTS were significantly higher than the SI for tumors on TMAs ($p<0.001$). The kappa-statistic was "slight" for continuous SI-values (0.168), and "fair" for dichotomized data (0.242), scored after Landis & Koch (28).

Only 29.8% of the tumors (14 cases) had perfect agreement between SI of the two methods. Eight cases which were evaluated as negative on the TMA sections, were positive on WTS. With dichotomized values (median as cut-off), 31/47 tumors (66%) were in agreement between the methods.

The EGFR expression on WTS was often heterogeneous, and it appeared that tissue cylinders occasionally were extracted from areas with seemingly low expression (**Fig. 3**). The differences in SI between the two methods did not vary significantly across tumor subtypes ($p=0.336$). In total, four TMA cores were lost during laboratory procedures, but from different tumors.

Discussion

In this study we compared the immunostaining of EGFR in meningioma tissue in TMAs with traditional WTS and found significantly higher expression levels on WTS. Further, heterogeneous EGFR expression on WTS was to a lesser extent seen in TMAs, and some positive cases on WTS were negative on TMAs.

The reasons for the discrepancies between the two methods are not obvious. It could be due to tissue cores being caught from areas of low EGFR expression or related to the intrinsic properties of the immunohistochemical procedures including antibody incubation, working dilution, chromogen reaction, antigenicity, and area/amount of tissue available. Hence, thorough optimization of the immunohistochemical procedure of TMAs is therefore required with special emphasis on the abovementioned factors as well as the use of proper controls. In our experiments human skin and liver biopsies were used as positive controls on WTSs and TMAs, respectively. In retrospect, both skin and liver samples should have been included in our TMAs to ensure unbiased evaluation. However, liver tissue is distinctly EGFR immunoreactive according to the literature, as observed in our analyses (29). In sum, the immunohistochemical procedure on WTS cannot automatically be applied on TMAs, and separate testing should be considered. Furthermore, we observed some false-negative cases on TMAs which can be a result of heterogeneous EGFR labeling, a phenomenon that has been observed in other cancers as well (15, 19, 23) and for p53 in breast cancer (13). Since the available area of tumor tissue in TMAs is lower than in WTS, it is reasonable that assessment of distribution of EGFR in meningioma tissue differs in these two methods. Using more than three tissue cores from each tumor could perhaps compensate for tissue heterogeneity and improve concordance between TMA and WTS. Neither can it be excluded that different section depth between TMAs and WTS could be influential on the EGFR expression pattern. We tried, however, to compensate for this by regular HE-staining of the TMA sections to ensure similar tumor histology. It should be commented that WTS are usually considered representative of the tumor bulk (30, 31), however, heterogeneity and focal lesions may be found at different levels in paraffin blocks of WTS as well. Finally, the general lower intensity of immunolabelling on TMAs may also explain why some positive cases were “lost” on TMAs.

Despite considerable differences in levels of immunostaining, the SI-values were significantly

correlated. These results are likely due to some systematic error related to methodology rather than subjective assessment. Our findings indicate that morphology alone may not explain this issue, as differences in antibody expression between methods was fairly evenly present in tumor subtypes. The current findings are probably not a result of poor tissue sampling, but more likely due to actual heterogeneous EGFR distribution in the tissues.

The level of agreement between TMA and WTS was low (κ=0.168), even when values were dichotomized (κ=0.242). Similar findings have been seen in squamous cell head and neck cancer (25), yet others found high levels of agreement in other cancers (20). Lower levels of agreement have also been found for the marker caspase-3 with dichotomized values in vulvar cancer (κ=0.40), and it was observed that all discordant cases were negative on TMAs and positive on WTS (24), similar to our results on EGFR. However, the level of agreement of EGFR (among other markers) in vulvar cancer was quite good (κ=0.68), and positive cases on TMA were sometimes negative on WTS (24). This is contradictory to our observations, and underlines the importance of biomarker validation studies for different tumor entities. An interesting finding by Chen et al. was that the moderate kappa-scores in their study did not improve when the homogenously staining tumors were analyzed individually (25). Finally, the poor concordance between TMAs and WTS in the current study as well as discrepancies with results in other tumors, may be due to sampling errors or suboptimal scoring system, as the use of different systems may change agreement (kappa) between the methods (19, 24).

In conclusion, immunostaining on TMAs compared with WTS has some elements of uncertainty that have to be taken into consideration. For instance, adequate testing of the immunohistochemical protocol for TMAs with relevant controls is important. Further, TMA is seemingly not optimal for assessment of heterogeneous expression and as such is more applicable in research than in diagnostics. It also seems important with large sample series to achieve reliable data (14, 32).

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Tables

Table 1. Tumor grades and subtypes.

Subtype	Frequency	Percent (%)
Grade I total	31	66.0
Meningothelial	4	8.5
Fibrous	3	6.4
Transitional	23	48.9
Angiomatous	1	2.1
Grade II total	15	31.9
Atypical	14	29.8
Clear cell	1	2.1
Grade III total	1	2.1
Anaplastic	1	2.1
Total	47	100

Table 2. Staining index (SI) formula.

	0	**1**	**2**	**3**
Staining intensity	Absent	Weak	Moderate	Strong
Proportion of positive cells	None	<10%	10-50%	>50%

Staining index (SI) = staining intensity X proportion of positive cells

Table 3. Staining index (SI) results.

SI value	TMA frequency	TMA percent (%)	WTS frequency	WTS percent (%)
0	10	21.3	2	4.3
1	1	2.1	2	4.3
2	5	10.6	5	10.6
3	10	21.3	1	2.1
4	1	2.1	6	12.8
6	14	29.8	12	25.5
9	6	12.8	19	40.4
Total	47	100	47	100

TMA: tissue microarray

WTS: whole-tissue sections

Figures

Figure 1. Tissue microarray design. A TMA block with three liver cores to assist in orientation and tissue cylinders from meningioma specimens (three cylinders per tumor). **B** The TMA blocks used in the current study with corresponding sections stained with HES.

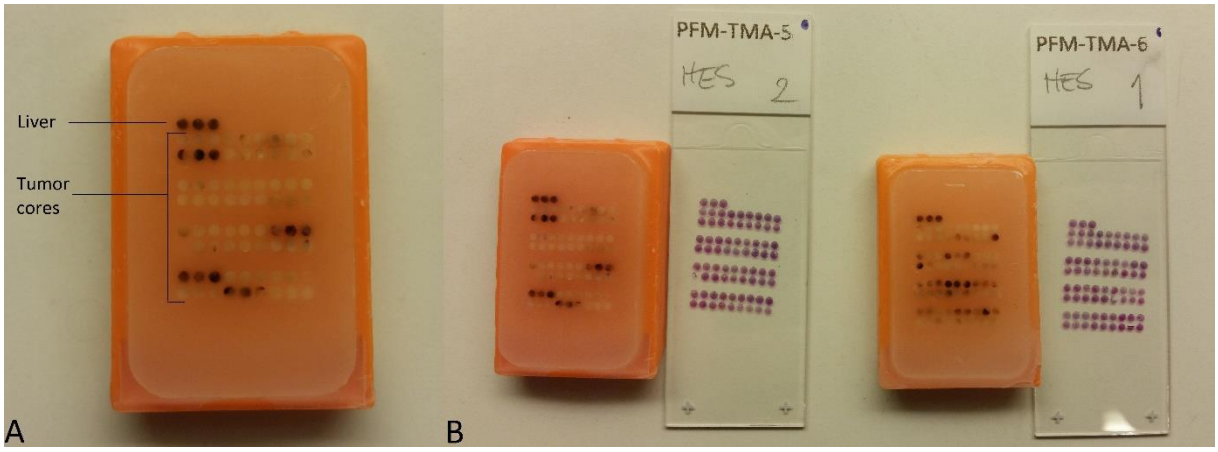

Figure 2. Controls, 200x magnification. A Normal liver sample showing strong EGFR expression. **B** Positive control from skin biopsy. **C** Negative control (skin).

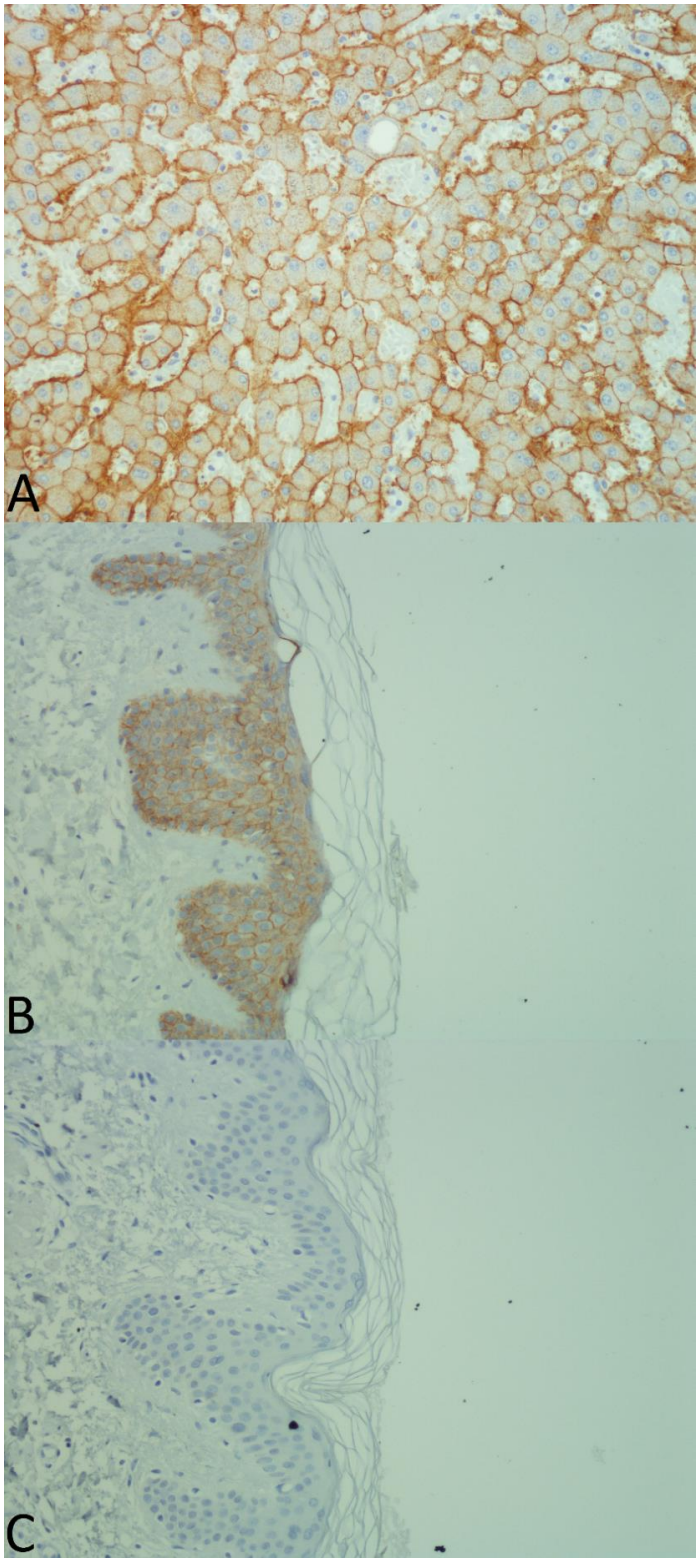

Figure 3. Heterogeneous EGFR expression patterns, 40x magnification. A Heterogeneous expression pattern in a meningioma specimen containing psammoma bodies. WTS SI: 9, TMA SI: 6. **B** The tissue core was extracted from an area with a meningothelial growth pattern, which showed lower expression than the adjacent fibrous tissue. WTS SI: 9, TMA SI: 0.