Tenascin-C in primary Merkel cell carcinoma

V Koljonen, T Jahkola, E Tukiainen, G Granroth, C Haeglund, T Böhling

Background/Aims: Merkel cell carcinoma (MCC) is a rare malignant cutaneous neuroendocrine tumour that mostly affects the elderly. It shows rapid progression of the primary tumour, together with a vertical growth pattern into the underlying subcutaneous tissue. Metastatic dissemination to regional lymph nodes is early and frequent. Tenascin-C (Tn-C) is a large extracellular matrix glycoprotein that is expressed in various benign and malignant processes. Expression of Tn-C is also associated with invasion and cellular proliferation, and is often downregulated in fully evolved advanced carcinomas. In previous studies, Tn-C expression correlated with prognosis in tumours of different origin.

Methods: Immunohistochemistry was used to investigate the expression of Tn-C in 25 MCC specimens and to evaluate the prognostic importance of this glycoprotein.

Results: Seventeen samples expressed Tn-C. Staining was mainly seen in the invasion borders and within the connective tissue septae inside the tumours. The expression of Tn-C correlated significantly with large tumour size. There was also frequent expression of Tn-C in primary tumours with metastatic dissemination. Most of the Tn-C negative samples were of small size.

Conclusions: Tn-C expression seems to increase with tumour size and malignant behaviour. Expression was slightly enhanced in tumours with high proliferative indices. Expression is seen mainly in areas of invasive growth and, in this respect, resembles that of other invasive tumours.

Materials and Methods

Our study comprised 25 patients treated for MCC between 1987 and 2003 at the department of plastic surgery, Helsinki University Central Hospital, and at Vaasa Central Hospital, Finland.

One primary tumour sample was collected from each of the 25 patients (25 samples). The diagnoses were confirmed with immunohistochemical analysis using antibodies to cytokeratin 20 and TTF-1, with TTF-1 being negative in all samples.

Abbreviations: ECM, extracellular matrix; MCC, Merkel cell carcinoma; Tn-C, tenascin-C; TTF-1, thyroid transcription factor 1
The greatest surface dimension, tumour size, was measured from haematoxylin and eosin stained slides and documented as < 2 cm or ≥ 2 cm. None of the patients received preoperative chemotherapy or radiotherapy. The clinical outcome was recorded from the patients' charts. The local ethics committee approved our study protocol.

Haematoxylin and eosin staining revealed that the typical histology of our MCC samples was that of dermal involvement with repeated extension to subcutaneous tissue. The tumours consisted of small blue cells with sparse cytoplasm; nuclei were medium sized and mitoses were abundant. Three of the tumours did not extend to the underlying subcutaneous tissue and were classified as superficial carcinomas.

The mean follow up time of the patients was 3.2 years (range, seven days to 11 years). Ten of the 25 patients died during follow up. Table 1 details the patient and tumour characteristics.

**Immunohistochemistry**

One 4 µm thick section was cut for each tumour from paraffin wax embedded blocks, dewaxed in xylene, and rehydrated in a series of graded alcohols. The sections were pretreated in trypsin for 30 minutes for antigen retrieval. They were then immersed in 0.6% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity, and in blocking solution (1.5/100 normal horse serum in phosphate buffered saline) for 15 minutes to block non-specific binding sites. Antihuman tenascin monoclonal antibody (clone DB7; Biohit Diagnostics Oy, Helsinki, Finland) was applied overnight at a dilution of 1/2000 in phosphate buffered saline containing 0.1% sodium azide and 0.5% bovine serum albumin at room temperature. All the sections were treated with biotinylated horse antimouse immunoglobulin (1/200 dilution; Vector Laboratories, Burlingame, California, USA); antibody binding sites were visualised with avidin–biotin–peroxidase complex ( Vectastain ABCComplex; Vector Laboratories) and 3-aminoperchloro (Vector Chemical Co, St Louis, Missouri, USA). Counterstaining was performed with Mayer's haematoxylin. Red/brown extracellular staining was considered positive.

The results were scored by three independent researchers (TB, TJ, and VK) unaware of the patient's status. The positive controls used throughout the staining procedure were formalin fixed, paraffin wax embedded sections of breast cancer. The negative controls consisted of omitting the primary antibody.

One section of each tumour was analysed, and the staining pattern was recorded. For quantification, the staining area was observed, and staining intensity was scored as low (+), moderate (++), or high (+++).

**Statistical analysis**

Statistical analysis was performed with NCSS 2000 software. The χ² test was used to analyse the correlation between clinical data and Tn-C staining. p Values < 0.05 were considered significant.

**RESULTS**

Seventeen of the 25 samples stained positive for Tn-C. The staining intensity was high in nine, moderate in four, and low in four samples.

Staining was patchy and occurred mainly in tumour borders, especially towards the subcutaneous fat. Apart from lining the vascular structure, Tn-C expression was virtually absent within the tumour tissue. In nine of the tumour samples, the connective tissue septae were stained. Staining tended to be stronger around the septae than at the tumour borders. No cytoplasmic staining was seen within the tumour cells. The staining was continuous, with a flame-like pattern, and had extensions to the surrounding tissues. Samples that contained normal skin showed moderate staining in the dermo–epidermal junction (fig 1A–C).

Interestingly, six of the eight negative tumour samples were of small size. Their size varied from 1 to 2 cm. Four of the eight negative cases were only 1 cm in diameter.

There was a significant correlation between Tn-C expression and tumour size. A correlation was established with large tumour size (≥ 2 cm; p = 0.032), but not with age, sex, tumour localisation, invasion, recurrence, or metastasis. We also found enhanced expression of Tn-C in tumours that developed metastases (82%; p = 0.2), although this was not significant.

Table 2 presents the correlations between Tn-C expression, tumour size, and tumours that metastasised.

**DISCUSSION**

In our series, about two thirds of the MCCs studied expressed Tn-C. Of the 17 positive samples, nine showed strong staining intensity, four showed moderate intensity, and four showed

| Table 2 Correlation between tenasin C (Tn-C) expression, tumour size, and tumours that metastasised |
|---------------------------------------------|------------------|------------------|------------------|------------------|
| Size < 2 cm | Size ≥ 2 cm | Non-metastatic tumours | Metastatic tumours |
| No. of samples | 11 | 14 | 14 | 11 |
| Tn-C negative | 6 (55%) | 2 (14%) | 6 (43%) | 2 (18%) |
| Tn-C positive | 5 (45%) | 12 (86%) | 8 (57%) | 9 (82%) |
| Staining intensity | 1 | 3 | 1 | 4 |
| Low | 1 | 3 | 2 | 2 |
| Moderate | 3 | 6 | 5 | 3 |
low staining intensity. A significant correlation was found between large tumour size and Tn-C positivity ($p = 0.032$).

Eight samples in our study did not express Tn-C. Most of them were small tumours, less than 2 cm in diameter. As a result of sample collecting bias, the proportion of negative samples might be even lower. We submitted only one slide for each tumour to immunohistochemical analysis, so that because Tn-C expression was focal, and most of our tumours were $\geq 2$ cm, we may have selected a tumour segment that showed no expression.

Detection of Tn-C does not reveal the malignant potential of an individual tumour directly; rather, it shows the capacity of the tumour to invade and the capacity of the cells to proliferate. Enhanced Tn-C expression has been shown in association with wound healing and in inflammatory processes in proliferating cells in the skin.27 28

We found that Tn-C expression, whether found in the invasion fronts or fibrous septae within the tumour tissue, correlated with larger tumour size. Large tumours are inclined to metastasise, resulting in poor overall survival of patients.26 Here, Tn-C expression was not evident in over half (six of 11) of the small tumours, whereas only two of the 14 large tumours were Tn-C negative.

In our samples, Tn-C was principally expressed at the invasive tumour front and extended into the underlying subcutaneous fat, in agreement with that found in other invasive malignant tumours.

"We noted a relation between tenascin-C expression and metastasis of the primary tumour"

There was no distinct staining pattern in three superficial carcinomas, which showed high, moderate, and low expression of Tn-C, respectively. In nine samples, the expression was found in the fibrous septae, where the tumour was invading the surrounding dermal matrix. Although the stromal pattern of staining has previously been correlated with a better prognosis,29 this was not the case in our study series. We also noted a relation between Tn-C expression and metastasis of the primary tumour. This may be a common staining pattern in neuroendocrine carcinomas, such as small cell lung carcinomas.29

A correlation between cell proliferation, measured by Ki-67, and Tn-C expression has been reported in breast cancer.30 As an aggressive neoplasm, MCC shows high proliferative indices. We have shown pronounced overexpression of cyclin A in primary MCC.31 Cyclin A is a cell cycle enhancer, the immunohistochemical expression of which has been used to detect proliferative cells. Moreover, its expression is a valuable prognostic factor in different types of carcinomas and sarcomas. Although we established overexpression in individual tumours and a slight tendency towards cyclin A accumulation in large tumours and in primary tumours with metastatic potential, this was not significant. Table 3 summarises Tn-C expression in 21 MCC samples with cyclin A expression within the same patient population. Tn-C expression correlated with high cyclin A expression (16 of 21 samples) and negative Tn-C expression with low (< 20%) cyclin A expression (13 of 21 samples).

Our samples showed high intensity staining around the vascular structures within tumours. Previously, Kim et al have found enhanced immunoreactivity of Tn-C in tumour vessels and even greater expression in high grade tumours.32

<table>
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<th>Table 3 Correlations between tenascin C (Tn-C) and cyclin A expression in 23 primary Merkel cell carcinomas</th>
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<tr>
<td>Cyclin A &lt; 20%</td>
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<tr>
<td>Tn-C negative</td>
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<tr>
<td>5 (63%)</td>
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<td>6 (37%)</td>
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TN-C controls the process of neoangiogenesis by regulating expression of the vascular endothelial growth factor, which activates the endothelial cells in mature blood vessels to sprout new capillaries.3

We performed an immunohistochemical study for TN-C in one lymph node metastasis of MCC. In this sample, staining intensity was moderate to high and was seen only in the septae (fig 1D). Of interest was the fact that expression of TN-C was low in the primary tumour. Our samples containing normal skin showed moderate TN-C expression in the dermo–epidermal junction. This finding is in line with the results of Schalkwijk et al.10

In conclusion, the expression of TN-C in this series of MCC samples appeared to increase with tumour size, malignant behaviour, and proliferation. Expression was highest in areas of invasive growth and, in this respect, resembles that of other invasive tumours.

Authors’ affiliations
V Koljonen, T Jahkola, E Tukiainen, Department of Plastic Surgery, Helsinki University Hospital, Helsinki, 00029 HUS, Finland
G Granroth, Helsinki University Hospital, Galleri D, PL 32, PO Box 56, 00029 HUS, Finland
C Haglund, Gastroenterological and General Surgery, Helsinki University Hospital

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