ORIGINAL ARTICLE

cDNA microarray analysis of cancer associated gene expression profiles in malignant peripheral nerve sheath tumours

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Background: Malignant peripheral nerve sheath tumour (MPNST) is a highly aggressive malignancy that arises within peripheral nerves, and is associated with poor prognosis. Little is known about the underlying biology of MPNST, especially the mechanisms involved in cell proliferation, invasion, or escape from apoptosis.

Aims: To identify genes differentially expressed in MPNST compared with benign tumours, such as neurofibromas and schwannomas, by means of cDNA microarray analysis.

Methods: Six MPNST cases and five benign cases (three schwannomas and two neurofibromas) were analysed.

Results: Six genes (keratin 18, survivin, tenascin C, adenosine deaminase, collagen type VIa3, and collagen type VIIa1) were significantly upregulated in MPNST, whereas one gene, insulin-like growth factor binding protein 6, was downregulated in MPNST. Survivin and tenascin C expression was validated by reverse transcription polymerase chain reaction. Immunohistochemistry confirmed upregulation of survivin in MPNST at the protein level in six of eight cases compared with benign tumours. Tenascin C was also expressed at the invasive front and tumorous stroma in all MPNST cases. MPNST cells expressed tenascin C in four of nine cases.

Conclusions: Survivin and tenascin C may be associated with the malignant potential of MPNST and could be considered as potential therapeutic targets.

Malignant peripheral nerve sheath tumours (MPNSTs) are highly aggressive malignancies that arise within peripheral nerves and are associated with a poor prognosis.¹ MPNSTs are frequently seen in patients with neurofibromatosis type 1 (NF1), who have increased Ras activity as a result of loss of the NF1 gene product, neurofibromin.^{2,3} Neurofibromin is expressed in Schwann cells, from which MPNSTs are thought to originate.⁴ However, complete inactivation of NF1 has also been found in benign neurofibromas, suggesting the presence of other genes that are associated with the malignant potential of MPNST. Skotheim *et al* showed that topoisomerase II α is upregulated in MPNST,⁵ but their study was performed using chromosome 17 specific cDNA microarrays; that is, the search was limited to chromosome 17, which contains NF1. In our study, we used cDNA microarrays, which cover a wide range of carcinoma associated genes, to identify genes differentially expressed in MPNST compared with benign peripheral nerve sheath tumours (BPNSTs)—neurofibroma and schwannoma. These differentially expressed genes may be associated with the high malignant potential of MPNST.

“Malignant peripheral nerve sheath tumours are highly aggressive malignancies that arise within peripheral nerves and are associated with a poor prognosis”

MATERIALS AND METHODS

Tumour samples

We studied 23 tumour tissue samples from 18 cases of peripheral nerve sheath tumour, comprising nine MPNSTs, five schwannomas, and four neurofibromas, diagnosed at the department of pathology, Fukuoka University, Japan

Table 1 Clinicopathological characteristics of the patients

Case	Site	Size (cm)	Age/sex	Neurofibromatosis
MPNST				
1	Shoulder	5.6	75/F	—
2	Face	5.0	84/M	—
3	Neck	4.0	46/M	—
4	Forearm	4	41/M	—
5	Femur	3	61/F	—
6	Femur	15	35/M	—
7	Axilla	10	78/M	—
8	Hip	8	43/F	+
9	Axilla	3.5	40/M	—
Schwannoma				
10	Chest wall	2.2	43/F	—
11	Femur	3	55/M	—
12	Forearm	6	52/M	—
13	Knee	4	49/M	—
14	Abdomen	4	49/F	—
Neurofibroma				
15	Chest wall	5	25/F	—
16	Forearm	2	50/M	—
17	Femur	6	59/F	+
18	Neck	1	69/F	+

MPNST, malignant peripheral nerve sheath tumour.

(table 1). The median age of the patients with MPNST, schwannoma, and neurofibroma was 46, 49, and 54.5 years, respectively. Three patients (one with MPNST, two with

Abbreviations: BPNST, benign peripheral nerve sheath tumour; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; MPNST, malignant peripheral nerve sheath tumour; NF1, neurofibromatosis type 1; RT-PCR, reverse transcription polymerase chain reaction; TBS, Tris buffered saline

neurofibroma) had previously been diagnosed as having neurofibromatosis. The diagnosis was performed by two specialist pathologists (IH and NK), based on the World Health Organisation classification.⁶

Tumour tissue was snap frozen immediately after surgical excision and stored at -80°C . For each tumour, frozen sections were haematoxylin and eosin stained to confirm a tumour cell content of $> 80\%$. Formalin fixed, paraffin wax embedded sections were used for diagnosis and immunostaining.

RNA isolation

Total RNA was extracted and isolated from tumour tissue by the guanidinium thiocyanate/phenol/chloroform method using the Total RNA separator kit (Clontech, Palo Alto, California, USA). The quality of RNA was analysed by electrophoresis, and 11 samples (from six patients with MPNST (cases 1–6) and five with BPNST (cases 10–12, 15 and 16)) were deemed to be useful for microarray analysis.

Microarray procedures

Total RNA ($5\text{ }\mu\text{g}$) from each sample was directly labelled with cyanine 3 conjugated dUTP (Cy3), whereas $5\text{ }\mu\text{g}$ of the Universal Human Reference RNA (Stratagene, La Jolla,

California, USA) was labelled with cyanine 5 conjugated dUTP (Cy5) as a reference. Hybridisations were performed as described previously.⁷ For all microarray studies, we used Cancer Chips version 4.0 (Takara Bio, Otsu, Japan) (886 genes), which are composed of 588 human genes related to cancer and 298 cDNA fragments shown to be upregulated in cancer tissue compared with normal tissue. A complete list of the genes and controls included on the slides is available on the Takara website (<http://www.takara-bio.co.jp>), in addition to details on the process of preparing the DNA for spotting and preparation of the slides.

After washing, the slides were scanned using a Scan Array 4000 scanner (GSI Lumonics, Boston, Massachusetts, USA). Images were analysed with QuantArray (GSI Lumonics).

Data analysis and clustering

Data obtained from each hybridisation were stored in a database for analysis. The Cy3 to Cy5 ratios were normalised to the median ratio value of all of the spots in the array. After normalisation, spots with intensities for both channels of less than 2.0 times the local background were discarded. The Student's *t* test was used to identify MPNST specific genes, in comparisons between MPNST and BPNST. Genes that were more than twofold differentially expressed in MPNST compared with BPNST were categorised as significantly differentially expressed genes. A more precise statistical evaluation of these genes was carried out by adding the permutation test for multiple comparisons. Hierarchical clustering was applied to both axes using the weighted pair group method with centroid average as implemented in J-Express (<http://www.molmine.com>, Dr MolMine; contact@molmine.com).⁷ We performed microarray assays twice in two cases to confirm the reproducibility of the microarrays. Both cases showed the same clusters in hierarchical clustering in two assays.

RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed as described previously.⁸ The sequences of primers for survivin,⁹ tenascin C,¹⁰ and β actin⁹ as a control have been published previously.

Immunohistochemistry

We used monoclonal antibodies to human survivin (Santa Cruz Biotechnology, Santa Cruz, California, USA) and tenascin C (Affiniti Research Products, Mamhead, UK). Immunostaining of frozen (for survivin) and formalin fixed, paraffin wax embedded tissue sections (for tenascin C) was performed using a biotin–streptavidin method. Briefly, sections were dewaxed, rehydrated in serial alcohol dilutions, and washed in Tris buffered saline (TBS), pH 7.6. After non-specific sites were blocked with 3% bovine serum albumin and 1% non-fat dry milk in TBS for 30 minutes at room temperature, the sections were incubated with the primary antibody overnight at 4°C . The sections were then washed in TBS, and incubated with biotinylated horse antimouse IgG (Vector Laboratories, Burlingame, California, USA) for 30 minutes at room temperature, followed by streptavidin conjugated to alkaline phosphatase (Dako Corporation, Carpinteria, California, USA) for another 30 minutes. The reaction was revealed with naphthol AS-BI phosphate (Sigma Chemical Co, St Louis, Missouri, USA) in 100 ml of 0.2M TBS (pH 8.2) containing 4% hydrochloric acid and 4% nitric acid, and counterstained with Mayer's haematoxylin or methyl green.

The immunohistochemical specificity of the antibody was confirmed by two types of negative controls: substituting mouse non-immune IgG for the primary antibody and omitting the primary antibody in the staining protocol.

Figure 1 Expression profiles of malignant peripheral nerve sheath tumours (MPNSTs) and benign peripheral nerve sheath tumours (BPNSTs). Hierarchical clustering analysis using 68 genes that are differentially expressed in MPNST compared with BPNST (Student's *t* test, $p < 0.05$) completely separated MPNST cases from BPNST cases.

Table 2 Genes that are differentially expressed in MPNST and BPNST

Gene	p Value*	p Value†	MPNST:BPNT‡
Genes upregulated in MPNST			
Keratin 18	0.0078	0.0065	3.3
Survivin (baculoviral IAP repeat containing 5)	0.0023	0.0043	3
Tenascin C (hexabrachion, cytactin)	0.013	0.017	2.8
Adenosine deaminase	0.0018	0.0043	2.4
Collagen, type VI, $\alpha 3$	0.0083	0.024	2.2
Collagen, type VII, $\alpha 1$ (epidermolysis bullosa, dystrophic, dominant, and recessive)	0.012	0.0087	2.2
Downregulated in MPNST			
IGFBP6	0.0037	0.0022	0.49

*Derived from the *t* test; †derived from the permutation test; ‡average.

BPNT, benign peripheral nerve sheath tumour; IGFBP6, insulin-like growth factor binding protein 6; MPNST, malignant peripheral nerve sheath tumour.

Staining results were evaluated semiquantitatively by two independent observers. Staining for survivin and tenascin C in tumour cells or tumour stroma was semiquantitatively classified into four groups, as follows: –, no positive cellular or extracellular immunostaining; 1+, < 25% of tumour cells or intratumorous stroma positive; 2+, 40–70% of tumour cells or intratumorous stroma positive; and 3+, > 80% of tumour cells or intratumorous stroma positive.

RESULTS

Homogeneous gene expression profile in MPNST

Six MPNST cases and five BPNST cases were analysed by cDNA microarray technology. The unsupervised hierarchical clustering showed that MPNST cases were an almost homogeneous group with similar expression patterns (fig 1A). To confirm the reproducibility of the technique, we repeated the hybridisation procedure in four of the samples and analysed them together. Different hybridisations corresponding to the same patient clustered together, showing that the expression profiles obtained were reproducible.

Applying the Student's *t* test ($p < 0.05$), we identified a total of 68 probe sets out of 884 where expression differed between MPNST and BPNST. The expression profile of each sample using these 68 genes is shown in fig 1B.

Differentially expressed genes in MPNST

We selected seven of the above 68 genes where expression differed in MPNST by more than twofold compared with BPNST (table 2). The upregulated genes were keratin 18, survivin, tenascin C, adenosine deaminase, collagen type VI $\alpha 3$, and collagen type VII $\alpha 1$, whereas insulin-like growth factor binding protein 6 (IGFBP6) was downregulated in MPNST compared with BPNST. Using the permutation test for multiple comparisons these genes were significantly differentially expressed.

Validation of the expression of survivin and tenascin C by RT-PCR and immunohistochemistry

Survivin and tenascin C were selected for further analysis because these genes had not been analysed previously in MPNST, but have been reported to play important roles in various malignant tumours.^{11 12} Table 3 details the mRNA expression ratios obtained by cDNA microarray analysis. There was a tendency for MPNSTs, which had high survivin and tenascin C mRNA expression, to have stronger protein expression than BPNSTs, which had low mRNA expression.

It was possible to analyse mRNA expression by RT-PCR in seven cases (almost all of the mRNA isolated from four cases (6, 11, 15, and 16) was used for cDNA microarray analysis). RT-PCR detected survivin (four cases) and tenascin C (three

Table 3 Immunohistochemistry results

Case	Survivin (expression)*	Survivin (RT-PCR)	Survivin (IHC)	Tenascin C (expression)*	Tenascin C (RT-PCR)	Tenascin C (tumour centre; IHC)	Tenascin C (invasive front, collagen; IHC)
MPNST							
1	1.46	–	3+	0.82	–	–	+
2	1.2	+	+	0.34	+	+	3+
3	0.78	+	+	0.53	–	–	2+
4	0.97	+	+	1.08	+	–	2+
5	1.61	+	2+	1.01	+	+	2+
6	1.04	ND	ND	1.26	ND	–	+
7	ND	ND	–	ND	ND	2+	3+
8	ND	ND	+	ND	ND	–	2+
9	ND	ND	–	ND	ND	2+	Not determined†
Schwannoma							
10	0.59	–	–	0.26	–	–	+
11	0.44	ND	+	0.35	ND	–	+
12	0.26	–	–	0.29	–	–	2+
13	ND	ND	–	ND	ND	–	–
14	ND	ND	–	ND	ND	–	2+
Neurofibroma							
15	0.58	ND	–	0.21	ND	–	2+
16	0.46	ND	ND	0.4	ND	–	+
17	ND	ND	+	ND	ND	–	–
18	ND	ND	–	ND	ND	–	–

*The ratio between the samples and control human RNA (Universal Human Reference RNA; Stratagene, La Jolla, California, USA); †evaluation was impossible because only tumour tissue was excised and no invasive front or stromal tissue were seen.

IHC, immunohistochemistry; MPNST, malignant peripheral nerve sheath tumour; ND, not done; RT-PCR, reverse transcription polymerase chain reaction.

Figure 2 Reverse transcription polymerase analysis for tenascin C. Representative results of positive malignant peripheral nerve sheath tumours (MPNSTs; cases 2, 4, and 5) and negative benign peripheral nerve sheath tumours (BPNSTs; cases 10 and 12). Case 2 was used as a positive control (because this case showed the highest expression in microarray analysis). Bands are visible between 300 bp and 400 bp, compatible with previous reports (344 bp). N, negative control (water).

cases) expression in five MPNSTs, whereas none of the BPNSTs expressed survivin (fig 2). Despite the small number of samples, these findings agree with the cDNA microarray results.

Immunohistochemistry detected upregulation of survivin in six of eight cases of MPNST (fig 3A). Tenascin C staining was positive in tumorous stroma (fig 3B) and at the invasive front (fig 3C; eight of eight cases). MPNST tumour cells showed focal positivity in some cases (fig 3D; four of nine). Tenascin C was negative in the tumour cells of BPNST (table 3). Tenascin C showed weak and focal positivity in the tumorous stroma of BPNST. Survivin could not be analysed immunohistochemically in cases 6 and 16 because all the frozen tissue was used for RNA isolation. Tenascin C expression at the invasive front and in the tumorous stroma could not be assessed in case 9 because those parts of the tumour were not included.

Next, we compared survivin and tenascin C expression and clinical behaviour in MPNST (table 4). Tumour size was not significantly different between patients who were positive

and negative for survivin or tenascin C. Clinical follow up was possible in seven patients (cases 1–4 and 7–9). All patients were alive without disease (24–132 months); however, all except one (case 7) experienced recurrence after first surgery. Two patients (cases 3 and 8) were treated with chemotherapy after repeat surgery. Survivin negative and tenascin C positive patients had a relatively lower recurrence rate, but this was not significant because of the low numbers.

DISCUSSION

Little is known about gene expression profiles and the malignant potential of MPNST except for topoisomerase IIa, the expression of which was identified via chromosome 17 specific cDNA microarrays.⁵ We compared the expression of a wide range of genes in MPNST and BPNST using cDNA microarrays. Supervised clustering revealed that seven genes were differentially expressed in MPNST compared with BPNST. Keratin 18, survivin, tenascin C, adenosine deaminase, collagen type VI α 3, and collagen type VII α 1 were upregulated, whereas IGFBP6 was downregulated. Upregulation of survivin and tenascin C was confirmed at the protein level.

Survivin has been implicated in both cell cycle control and apoptosis resistance.¹³ The survivin promoter exhibits typical M phase inducible transactivation,¹⁴ and interference with the expression of survivin causes caspase dependent cell

Table 4 Survivin and tenascin C expression and clinical behaviour in malignant peripheral nerve sheath tumours

	Mean tumour size (cm)	Recurrence*
Survivin		
Positive (n=6)	4.4†	5/5 (100%)
Negative (n=2)	6.6†	1/2 (50%)
Tenascin C		
Positive (n=4)	5.4‡	2/3 (67%)
Negative (n=5)	7.3‡	4/4 (100%)

Immunohistochemical staining graded + to 3+ was considered positive.

*Seven patients were available for follow up analysis; †p=0.67 (Student's *t* test); ‡p=0.48 (Student's *t* test).

Figure 3 Immunohistochemical demonstration of survivin and tenascin C in malignant peripheral nerve sheath tumours. (A) Case 2: positive survivin staining in neoplastic cells; frozen section; original magnification, $\times 100$. (B) Case 3: positive tenascin C staining in the fibrous stroma (thick arrow), although the neoplastic cells (thin arrow) are negative; paraffin wax embedded section; original magnification, $\times 40$. (C) Case 2: positive tenascin C staining at the invasive front (thick arrow) of the tumour, which invades adipose and muscular tissue (thin arrow). Note that the interior tumour cells are negative for tenascin C (arrowhead); paraffin wax embedded section; original magnification, $\times 20$. (D) Case 7: in some cases, neoplastic cells are focally positive for tenascin C; paraffin wax embedded section; original magnification, $\times 200$.

death in the G2–M phase of the cell cycle.¹⁵ Survivin is not expressed in most terminally differentiated normal tissues, but is highly expressed in malignancies and embryonic tissues.¹⁶ Survivin expression is upregulated in all phases of the cell cycle, and the cancer specific activity of the survivin promoter has been detected both in vivo and in vitro.¹⁷ This overexpression in malignant tumours has been associated with aggressive behaviour and poor prognosis in patients with various malignancies—for example, colorectal cancer and hepatocellular carcinoma.^{18–21}

“Because malignant peripheral nerve sheath tumour is an aggressive cancer for which no consensus treatment exists, survivin and tenascin C could potentially be used as targets for novel treatments”

Tenascin C is an isoform of the tenascin family—large hexameric glycoproteins found in the extracellular matrix, which play an important role in cell adhesion and tissue repair.²² The expression of tenascin C is normally low or undetectable in healthy adult tissues,²³ but increases in malignant diseases.²⁴ In our present study, the tenascin C protein was expressed in all MPNST cases, predominantly in the tumorous stroma and at the invasion border between neoplastic and non-neoplastic tissues. The tenascin C protein was occasionally detected in the cytoplasm of MPNST cells, but it was not found in the tumour cells of BPNST. This expression pattern is consistent with that seen in other malignant neoplasms.^{11–25} At the invasive front tenascin may play an important role in enhancing the invasiveness of sarcoma cells and facilitating the metastatic potential of MPNST, and may be associated with poor prognosis, as seen in malignant mesothelioma and cholangiocarcinoma.^{25–26} Recently, antitenascin monoclonal antibody has been used successfully to treat malignant glioma.^{27–28} It is possible that this antibody may provide a useful treatment for MPNST in the future.

We investigated the association between the expression of these two molecules and clinical behaviour in MPNST (table 4), but there was not effect on survival or tumour size. Patients who were negative for survivin and positive for tenascin C had a relatively lower recurrence rate, but this was not significant because of the low numbers. Clinical analyses with larger numbers of patients should be performed in the future.

Other genes upregulated in MPNST may also be important although their expression at the protein level has not been

confirmed. Keratin 18 is an epithelial cell associated marker, and is expressed in various carcinomas, including those of the breast, prostate, lung, colon, and ovary.²⁹ MPNST sometimes shows epithelial structures.³⁰ In our study, no definite epithelial arrangement of cells was seen, but the upregulation of keratin 18 may indicate the epithelial differentiation of MPNST at the mRNA level. Two types of collagen were upregulated in MPNST. This may merely reflect an increase in the amount of collagen in the tumour, but an association between upregulated collagen and extracellular matrix remodelling and progression of malignant neoplasms has been reported.³¹

IGFBP6 was the only gene that was downregulated in MPNST compared with BPNST. Insulin-like growth factors (IGFs), particularly IGF-II, are potent proliferation stimuli for many types of tumour cells, and often function as autocrine/paracrine growth factors.^{32–33} IGFBPs modulate these IGF functions.^{34–36} IGFBP6, a 30 kDa O-glycosylated protein, shows 100-fold higher affinity for IGF-II than for IGF-I.³⁷ Its expression induces cell differentiation and/or the arrest of proliferation.³⁸ Although the exact function of IGF-II has not been clarified in MPNST, downregulation of IGFBP6 in MPNST compared with BPNST may be associated with its malignant phenotype.

In summary, the gene products differentially upregulated in MPNST, such as survivin and tenascin C, may be associated with the malignant potential of MPNST. Because MPNST is an aggressive cancer for which no consensus treatment exists, these molecules could potentially be used as targets for novel treatments.

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Take home messages

- The keratin 18, survivin, tenascin C, adenosine deaminase, collagen type VIa3, and collagen type VIIa1 genes were significantly upregulated in malignant peripheral nerve sheath tumour (MPNST), and insulin-like growth factor binding protein 6 was downregulated
- Upregulation of survivin in MPNST at the protein level was confirmed in six of eight cases compared with benign tumours
- Tenascin C was also expressed at the invasive front and tumorous stroma in all MPNST cases, whereas MPNST cells expressed tenascin C in four of nine cases
- Survivin and tenascin C may be associated with the malignant potential of MPNST and could be considered as potential therapeutic targets

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