

## Expression of Emmprin and Matrix Metalloproteinases (MMPs) in Peripheral Nerve Sheath Tumors: Emmprin and Membrane-type (MT)1-MMP Expressions are Associated with Malignant Potential

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**Abstract.** *Background:* Matrix metalloproteinases (MMPs), including membrane-type (MT)-MMPs, correlate with biological aggressiveness in many carcinomas. However, their roles in peripheral nerve sheath tumors (PNSTs) have rarely been investigated. *Materials and Methods:* In this study, the immunohistochemical expression of 6 MMPs, their 3 inhibitors and emmprin, an MMP inducer, was examined in 14 schwannomas, 14 neurofibromas and 12 malignant peripheral nerve sheath tumors (MPNSTs) in relation to malignant potentials. *Results:* Higher expression levels (>3+) of emmprin and MT1-MMP were noted in 83.3% and 16.7% of MPNSTs, respectively, versus none in schwannomas and neurofibromas ( $p < 0.0001$ ). The overall expression rate (1-4+) of MT1-MMP was 58.3% in MPNSTs versus 7.1% in both schwannomas and neurofibromas ( $p = 0.0093$ ). Gelatinase A (MMP-2) showed higher expression levels (>3+) in all the tumors without significant differences. Moreover, the expression patterns of MMP-1 and gelatinase B (MMP-9) could divide PNSTs into two groups: schwannoma versus neurofibroma/MPNST. Higher expression levels (>3+) of MMP-9 were observed in 50% of schwannomas versus none in neurofibromas and MPNSTs, while those of MMP-1 were found in 35.7% of neurofibromas and 66.7% of MPNSTs versus none in schwannomas. RECK was the main inhibitor expressed in these 3 tumors, with no significant differences. *Conclusion:* These results suggest that emmprin and MT1-MMP may be malignant potential-related

proteins in PNSTs, and that MMP-1 and 9 may help differentiation between schwannoma and neurofibroma, especially in their plexiform types.

Cancer metastasis continues to be the greatest obstacle for cancer cure. In metastasis, degradation and the consequent rearrangement of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) are involved in multiple steps, such as invasion through the stroma at both primary and metastatic sites, intravasation and extravasation (1). Among the MMPs, those most frequently involved in human tumor invasion and metastasis are gelatinase A (MMP-2) and B (MMP-9) (2, 3). Since the ratio of activated MMP-2 to proMMP-2 has been shown to correlate with metastasis in breast and thyroid carcinomas (4), the membrane type (MT)1-MMP that activates proMMP-2 also appears to be important (5). The MMPs mainly involved in metastasis are different in some tumors: e.g. matrilysin (MMP-7) works predominantly in endometrial carcinomas (6) and MMP-9 in malignant lymphomas (7, 8). These lines of evidence suggest that it is necessary to know which types of MMP are predominantly expressed in certain types of tumor and effectively block them. In this regard, little is known about MMPs involved in tumorigenesis of peripheral nerve sheath tumors, including schwannoma, neurofibroma and malignant peripheral nerve sheath tumor (MPNST).

Emmprin is a transmembrane glycoprotein, which is frequently expressed on carcinoma cells and stimulates nearby fibroblasts to produce interstitial collagenase (MMP-1), MMP-2 and stromelysin (MMP-3) (9). Moreover, emmprin cDNA-transfected breast cancer cells were considerably more tumorigenic and invasive than plasmid-transfected cancer cells when implanted into mice, indicating a role of emmprin in cancer progression (10). *In vivo*, the expression levels of emmprin are up-regulated in

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carcinomas, such as urinary bladder, breast, lung and oral carcinomas (11-13). Additionally, emmprin up-regulation in non-epithelial tumors, such as high-grade gliomas and malignant lymphomas, has been recently reported (14, 15). However, emmprin expression in sarcomas has rarely been explored.

In this study, the expressions of MMP-1, -2, -3 and -9 and MT1- and MT2-MMP, their inhibitors including tissue inhibitors of matrix metalloproteinases (TIMP)-1 and -2 and RECK, and emmprin in schwannomas, neurofibromas and MPNST were investigated. Emmprin and MT1-MMP expressions were closely associated with the malignant potential of tumors in these peripheral nerve sheath tumors. MMP-9 was frequently expressed in schwannomas, whereas the expression of MMP-1 was associated with neurofibromas and MPNST.

## Materials and Methods

**Tumor samples.** This study included 14 cases of schwannoma (12 conventional, 2 plexiform; 6 males, 8 females; age range, 25 – 90 [mean = 52.0] years), 14 cases of neurofibroma (10 cutaneous, 4 plexiform; 5 males, 9 females; age range, 6 – 94 [mean = 52.1] years) and 12 cases of MPNST (7 males, 5 females; age range, 31-83 [mean = 57.3] years), diagnosed at the Department of Pathology, Fukuoka University, Japan. Each specimen obtained at biopsy or surgery was fixed in 20% formalin and embedded in paraffin.

Histological diagnosis of MPNST was made according to the widely accepted criteria that requires the tumor to conform to one of the following: i) arises within a peripheral nerve; ii) arises in transition from a benign or other malignant peripheral nerve tumor; iii) develops in a patient with NF1 (von Recklinghausen's disease) and exhibits the same histological features as do a majority of MPNSTs arising from nerves; or iv) develops in a patient without NF1, but exhibits the same histological features as most MPNSTs and shows either or both immunohistochemical and ultrastructural features of Schwann or perineural cell differentiation (16).

**Immunohistochemistry.** The antibodies used in this study included: monoclonal antibodies (mAb) to human MT1-MMP (Daiichi Fine Chemical Co., Takaoka, Japan; clone 114-6G6), MT2-MMP (Daiichi Fine Chemical; clone 162-22G5), MMP-1 (Daiichi Fine Chemical; clone 41-1E5), MMP-2 (Daiichi Fine Chemical; clone 75-7F7), MMP-3 (Daiichi Fine Chemical; clone 55-2A4), MMP-9 (Daiichi Fine Chemical; clone 56-2A4), TIMP-1 (Daiichi Fine Chemical; clone 147-6D11), and TIMP-2 (Daiichi Fine Chemical; clone 67-4H11); and goat polyclonal antibodies to human emmprin (R & D Systems, Minneapolis, MN, USA) and RECK (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunostaining of formalin-fixed, paraffin-embedded tissue sections was performed using a biotin-streptavidin method, as described before with some modifications (17). Briefly, sections were deparaffinized, rehydrated in descending alcohol dilutions, and washed in Tris-buffered saline, pH7.6 (TBS). After non-specific sites were blocked with 3% bovine serum albumin and 1% non-fat dry milk in TBS for 30 min 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 anti-mouse (Vector Laboratories, Burlingame, CA, USA) or swine anti-rabbit IgG (DAKO, Carpinteria, CA, USA) for 30 min at room temperature, followed by streptavidin conjugated to alkaline phosphatase (DAKO) for another 30 min. The reaction was revealed with naphthol AS-BI phosphate (Sigma Chemical Co., St. Louis, MO, USA) in 100 ml of 0.2 M TBS (pH 8.2) containing 4% hydrochloric acid and 4% nitric acid and counterstained with Mayer's hematoxylin or methylgreen.

The immunohistochemical specificity of the antibody was confirmed by 2 types of negative controls: substituting mouse (for mAb) or rabbit (for polyclonal antibody) non-immune IgG for the primary antibody and omitting the primary antibody in the staining protocol.

The staining results were evaluated semi-quantitatively by 2 independent observers. Immunostaining was considered negative if less than 10% of the tumor cells failed to stain. In specimens considered positive, staining of the tumor was quantitated on a scale from 1 to 4 based on the percentage of positive tumor cells. The scale was structured as follows: 1+ = 10% to 25%; 2+ = 25% to 50%; 3+ = 50% to 75%; and 4+ = >75% (18).

Fisher's exact test was used to evaluate the statistical significance of the immunostaining results.

## Results

MPNST often arise in normal peripheral nerves. A typical tumor that arose in the ishiadic nerve of a 31-year-old Japanese female is illustrated in Figure 1. A transition from neurofibroma-like low cellular portions within the nerve is demonstrated in Figure 1a, b. Spindle tumor cells have tapered nuclei and exhibit mitotic figures (Figure 1c) and occasional S-100 protein immunoreactivity (Figure 1d). A case of diffuse cutaneous neurofibroma consisting of tumor cells with ovoid to thin, elongated nuclei is illustrated in Figure 1e, f, and a case of schwannoma in which tumor cells are arranged in Antoni A and B patterns is exhibited in Figure 1g, h.

MPNSTs frequently showed strong and diffuse emmprin expression. The positivity was demonstrated as cytoplasmic and membrane staining with anti-emmprin antibody (Figure 2a-c), which was not present in negative controls with non-immune IgG (Figure 2f). Epithelioid-type tumor cells showed especially strong positivity with accentuation along the cell membrane (Figure 2a). At the invasion front, emmprin-positive tumor cells were well demonstrated, which helped to discriminate tumor cells from the surrounding reactive fibroblastic cells (Figure 2c). On the contrary, most of the schwannoma and neurofibroma cells stained negative (Figure 2d, e). The results of emmprin immunostaining in peripheral nerve sheath tumors are summarized in Figure 3. About 65% of neurofibromas and schwannomas were negative, and their expression levels were always <2+, whereas MPNST showed expression levels of >3+ in 83.3% of cases ( $p < 0.0001$ ). Plexiform and non-plexiform neurofibromas showed no difference in emmprin expression levels (data not shown).



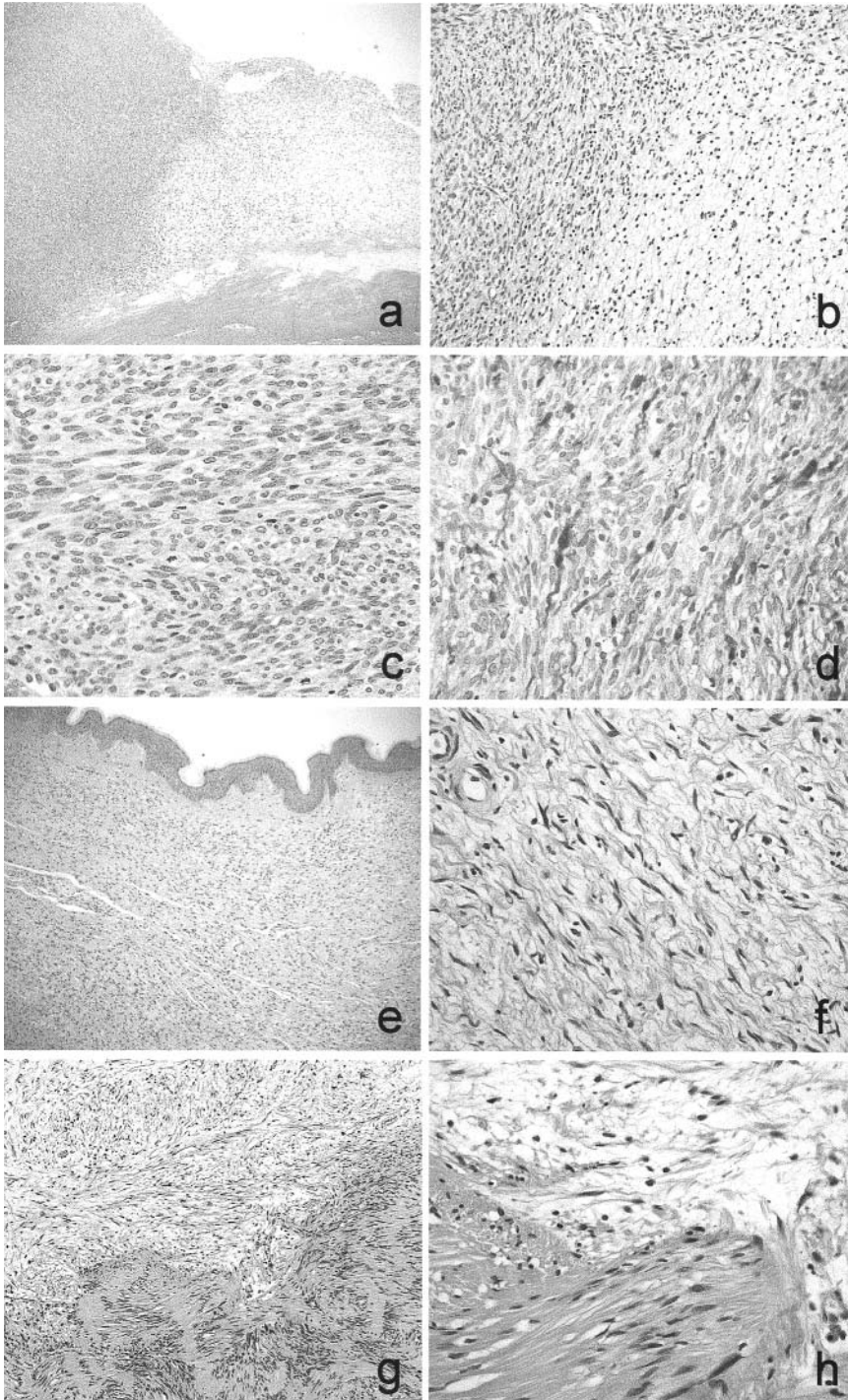


Figure 1. Histology of MPNST, neurofibroma and schwannoma. a-d, MPNST arising from the Nervus Ischiadicus. e, f, Diffuse cutaneous neurofibroma. g, h, Schwannoma with Antoni A and B patterns. a-c and e-h, H.E.; d, S-100 protein immunostaining. Original magnifications, a, 40x; b, 200x; e, g, 100x; c, d, f, h, 400x.

MMP-2 was expressed diffusely as cytoplasmic staining in all of MPNSTs, neurofibromas and schwannomas (Figure 4a-c), and was occasionally associated with staining of the surrounding ECM. Cytoplasmic positive reactivity to MT1-MMP, an activator of proMMP-2, was found in

MPNST tumor cells, but was rarely seen in neurofibroma and schwannoma cells (Figure 4d-f). MMP-9 positivity was demonstrated more frequently in schwannoma cells than in MPNST and neurofibroma cells (Figure 4g-i). In contrast, MMP-1 positivity was shown more frequently in

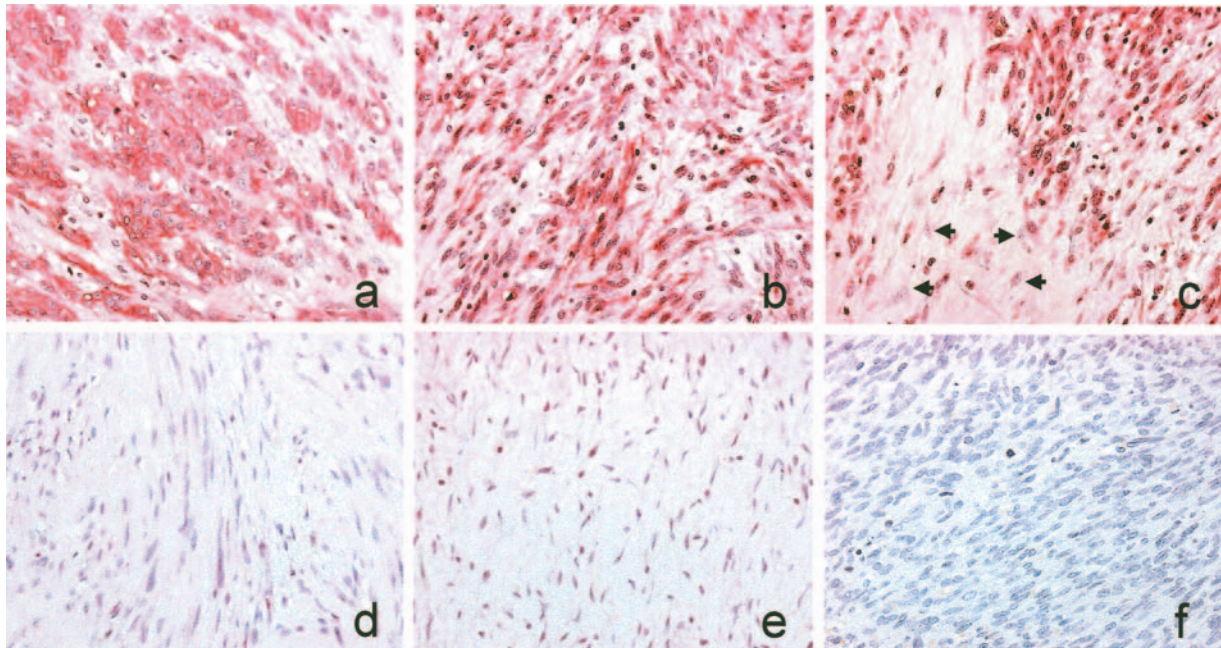


Figure 2. *Emmprin* expression in MPNST (a-c), schwannoma (d) and neurofibroma (e). MPNST tumor cells (epithelioid cells in (a) and spindle cells in (b)) frequently show diffuse reactivity in the cytoplasm or along the cell membrane, while most schwannoma and neurofibroma cells stain negative. At the invasion front of MPNST (c), *emmprin*-positive tumor cells face *emmprin*-negative fibroblastic cells (arrows). f, Negative control (MPNST) with non-immune IgG. Original magnifications, 400x.

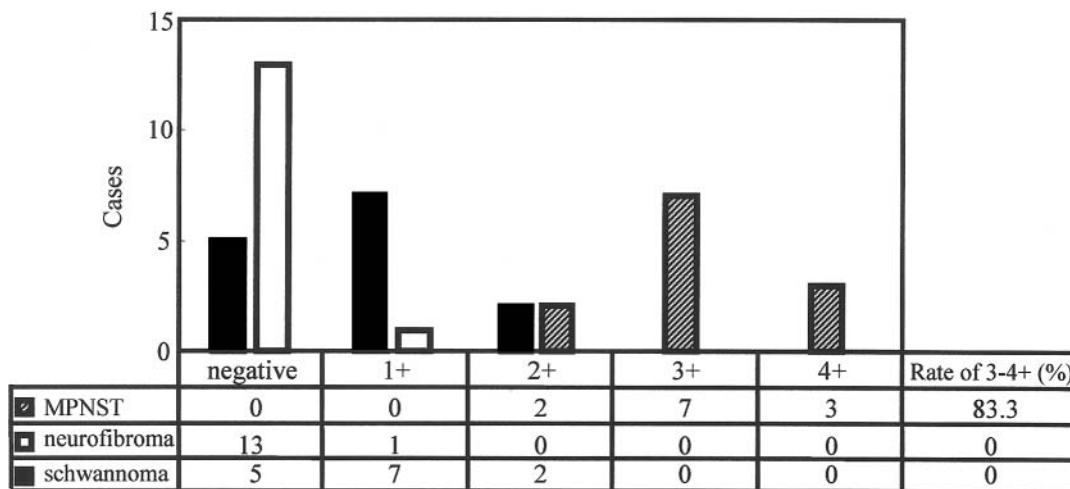


Figure 3. Immunostaining levels of *emmprin* in MPNST, neurofibroma and schwannoma. The number of cases of each expression level is shown in the figure and the table underneath. The rate of 3 and 4+ cases is also shown in the table. Black solid column, schwannoma; white open column, neurofibroma; hatched column, MPNST.

MPNST and neurofibroma cells than schwannoma cells (Figure 4j-l). The results of MMP immunostaining are summarized in Figure 5. MT1-MMP expression was demonstrated in 58.3% of MPNSTs versus 7.1% each in

neurofibromas and schwannomas ( $p=0.0093$ ) (Figure 5a), whereas more than 90% of MPNSTs, neurofibromas and schwannomas showed high levels (>3+) of MMP-2 expression (Figure 5b). With regard to the expression



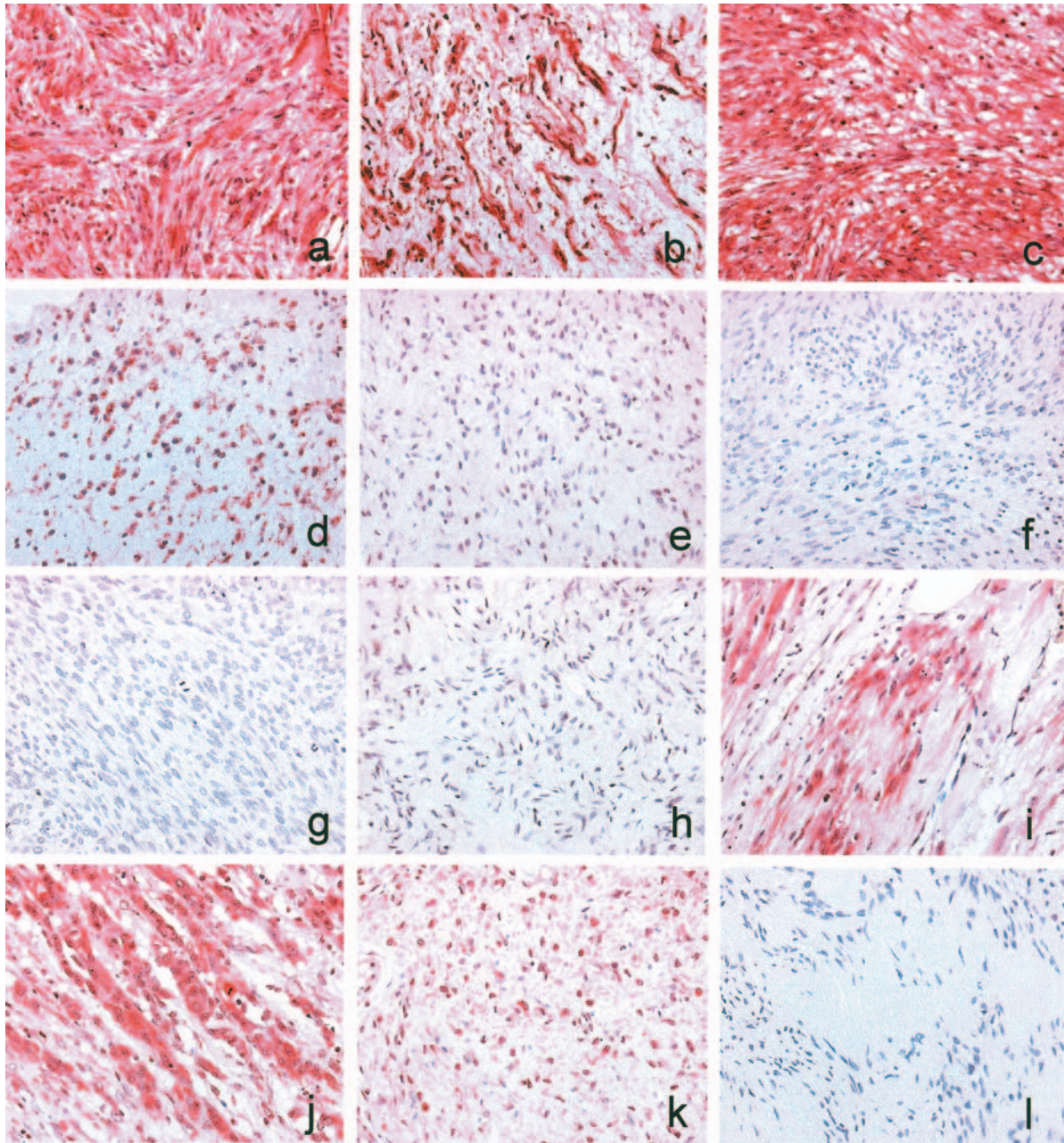


Figure 4. MMP expression in MPNST (a, d, g, j), neurofibroma (b, e, h, k) and schwannoma (c, f, i, l). a-c, MMP-2 immunostaining; d-f, MT1-MMP immunostaining; g-i, MMP-9 immunostaining; j-l, MMP-1 immunostaining. MMP-2 expression is seen diffusely in all the tumors. MT1-MMP cytoplasmic positivity is seen in MPNST, but rarely in neurofibroma and schwannoma. MMP-9 positivity is seen in schwannoma but not in MPNST and neurofibroma, whereas MMP-1 positivity is observed in MPNST and neurofibroma but not in schwannoma. Original magnifications, 400x.

levels of MMP-1 and -9, MPNSTs and neurofibromas tended to show similar expression patterns. Fifty percent of schwannomas *versus* none (0%) of MPNSTs and neurofibromas exhibited high expression levels of MMP-9 (Figure 5c), while high expression levels of MMP-1 were demonstrated in 66.7 and 35.7% of MPNSTs and

neurofibromas, respectively, *versus* none (0%) of schwannomas (Figure 5d).

MMP inhibitors did not show any significant differences among MPNSTs, neurofibromas and schwannomas. Low expression levels of TIMP-1 were found only in a small number of schwannomas and MPNSTs (Table Ia). TIMP-2

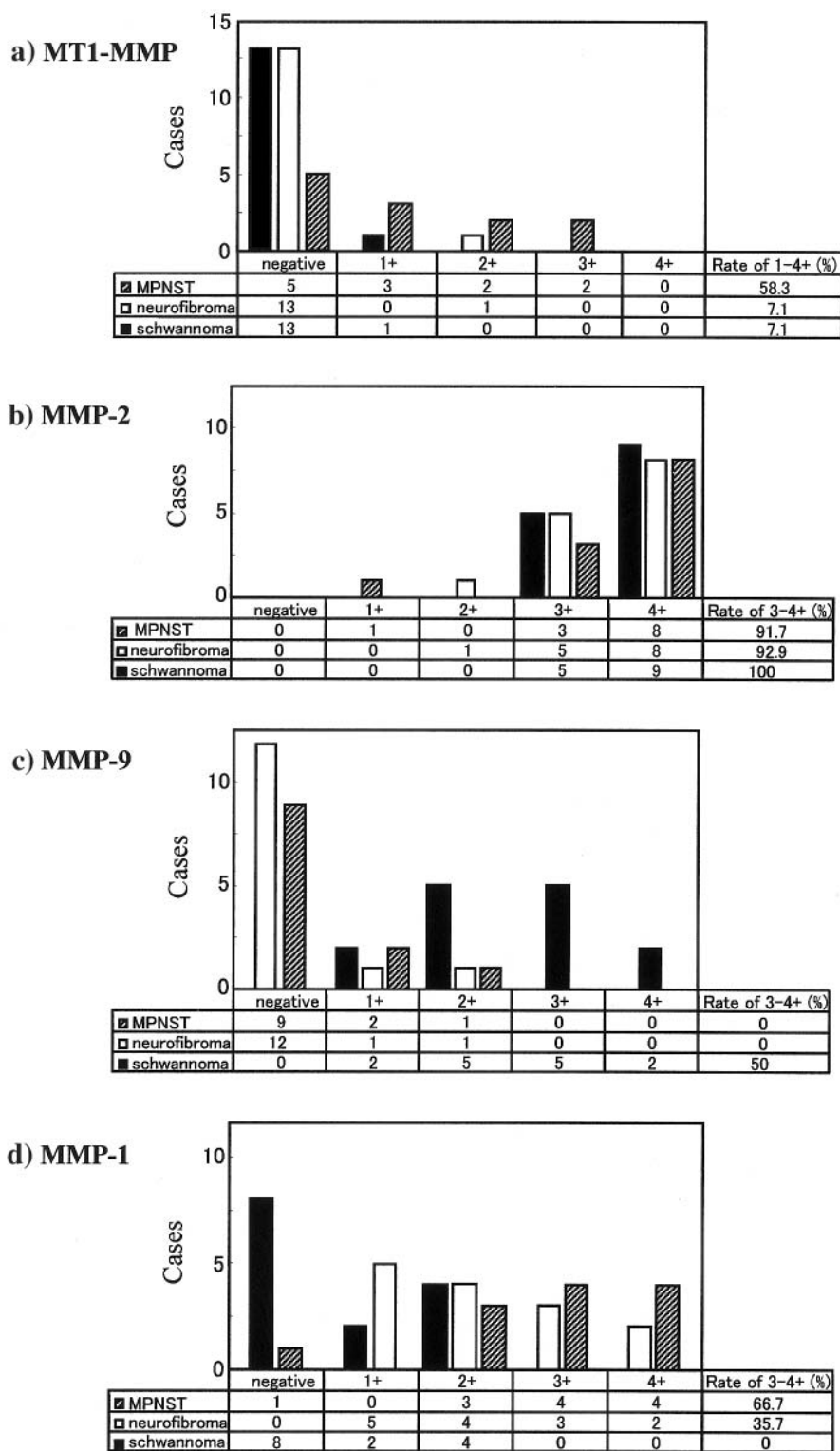


Figure 5. Immunostaining levels of MT1-MMP (a), MMP-2 (b), MMP-9 (c) and MMP-1 (d). The number of cases of each expression level is shown in the figure and the table underneath. The rate of 3 and 4+ cases is also shown in the table. Black solid column, schwannoma; white open column, neurofibroma; hatched column, MPNST.



Table Ia. *TIMP-1* expression.

		negative	positive				rate of 3-4+ (%)
case #			1+	2+	3+	4+	
schwannoma	14	12	2	0	0	0	0
neurofibroma	14	14	0	0	0	0	0
MPNST	12	8	3	0	1	0	8.3

Table Ib. *RECK* expression.

		negative	positive				rate of 3-4+ (%)
case #			1+	2+	3+	4+	
schwannoma	14	7	5	0	2	0	14.3
neurofibroma	14	5	3	1	5	0	35.7
non-plexiform	10	4	2	1	3	0	30.0
plexiform	4	1	1	0	2	0	50.0
MPNST	12	4	2	4	2	0	16.7

expression was not demonstrated at all (data not shown). Positivity (1-3+) for RECK was shown in 50%, 64% and 67% of schwannomas, neurofibromas and MPNSTs, respectively (Table Ib).

## Discussion

Our results indicate an association of higher expression levels of emmprin and MT1-MMP with the malignant potential in peripheral nerve sheath tumors. Moreover, the expression patterns of MMP-1 and -9 showed a similarity in neurofibroma and MPNST *versus* schwannoma.

Emmprin expression has never been investigated in sarcomas or in soft tissue tumors, except for alveolar soft part sarcoma (ASPS) and giant cell tumor (GCT) of the bone (19, 20). In ASPS, emmprin (CD147) is accumulated in the specific cytoplasmic granules together with monocarboxylate transporter 1 (MCT1) (19). CD147 binds to MCT1 and facilitates its appropriate targeting to the cell membrane (21). It has been suggested that MCT1-CD147 complexes accumulate intracellularly in ASPS because of overproduction or impaired trafficking to their normal destination at the cell membrane. However, cell surface localization and function as an inducer of MMPs were not demonstrated in ASPS (19). In GCT, expression of emmprin was shown at both mRNA and protein levels, and the expression levels were significantly higher in stage III GCTs when compared with stage I/II GCTs (20). Furthermore, the receptor activator of NF-kappaB ligand

(RANKL) up-regulated emmprin expression during osteoclastogenesis. Our study demonstrated emmprin expression in peripheral nerve sheath tumors. Its higher expression levels were associated with tumor progression: significantly higher levels of emmprin in MPNST compared with those in schwannomas and neurofibromas. Additionally, immunohistochemical demonstration of emmprin helped to pick up infiltrating MPNST cells at the invasion front, suggesting it can be an effective adjunct for the histological estimation of surgical resection margins. Emmprin expressed on tumor cells can stimulate both neighboring tumor cells themselves and host fibroblasts (22, 23). MMPs that can be up-regulated by emmprin *in vitro* include MMP-1, -2, -3 and MT1-MMP (23-25). However, up-regulated MMPs vary depending on the cell type that is stimulated by emmprin (22, 24-26). Our study suggested that MT1-MMP was a possible responder in MPNST.

The present study showed the ubiquitous expression of MMP-2 in almost all peripheral nerve sheath tumors. The occasional extracellular staining could conceivably indicate binding to the matrix and may be a result of locally increased synthesis/release of MMP-2, since it does not seem to be an artifact of the immunohistochemical technique, as reported previously (27). The peripheral nerves are shown to up-regulate MMP-2 in response to nerve injuries (28). Axonal regeneration occurs within the Schwann cell basal laminae of degenerated nerves, depending on the neurite-promoting activity of endoneurial laminin. This activity of laminin is blocked by its association with chondroitin sulfate proteoglycans (CSPG). MMP-2 up-regulated in the injured nerves degrades CSPG, thus disinhibiting laminin and allowing extension to proceed (29). Based on the above findings, this MMP-2-producing ability of cells within the peripheral nerve may be preserved even during neoplastic transformation. MMP-2 expression in MPNST was also reported before, and no significant association between MMP-2 immunoreactivity and metastasis or histological grades was demonstrated (30). In that report, the activation status of MMP-2 was not discussed. However, activation is essential for MMP-2 to exert its enzymatic activity, and MT1-MMP is the major MMP-2-activating protease (5). Once activated, overexpressed MMP-2 in immortalized Schwann cells or the schwannoma cell line D6P2T causes rapid invasion of a reconstituted basement membrane *in vitro* (31). Therefore, up-regulation of MT1-MMP in MPNST, shown for the first time, to our knowledge, in our study, may be responsible for the invasive and aggressive biological features of MPNST. Similarly, the expression levels of MT1-MMP demonstrated a significant correlation with the tumor grade in human cartilaginous tumors (32).

In our study, neurofibroma and MPNST showed a similar pattern of expression of MMP-1 and -9 (more MMP-1 and

less MMP-9), while schwannoma exhibited a different pattern (more MMP-9 and less MMP-1). The expression of MMP-9 in schwannomas has also been reported *in vitro* (33) and *in vivo* (34, 35). Moreover, MMP-9 mRNA was localized to schwann cells as well as infiltrating macrophages in injured peripheral nerves (36). Although one report showed MMP-9 immunoreactivity in more than 25% of tumor cells in 4 out of 15 MPNSTs (27%), blotting analysis using protein extracts from the tumors revealed only weak or no reactivity for MMP-9 (30). Cutaneous neurofibroma cells were shown to secrete MMP-1 *in vitro* (31). Plexiform neurofibromas and neurofibromas of major nerves are considered to be precursor lesions to the majority of MPNSTs (37), whereas schwannomas are slowly growing benign tumors that only rarely undergo malignant change (38). Additionally, neurofibromas have more infiltrating margins than conventional schwannomas. These lines of evidence may relate to common patterns of expression of MMP-1 and -9 in neurofibromas and MPNSTs. Moreover, these features may help to differentiate plexiform neurofibromas from plexiform schwannomas. Plexiform neurofibromas occasionally contain microscopic nodules composed entirely of schwann cells, some featuring Verocay bodies (plexiform neurofibroma with multifocal schwannoma-like nodules) (39). When sizable, these may resemble small schwannomas. It is pointed out that such tumors pose a problem in differential diagnosis between neurofibroma and schwannoma (39). The expression patterns of MMP-1 and -9 may help the differential diagnosis.

The balance between the activity of MMP inhibitors and that of MMP determines the proteolytic activity and may, in part, determine the overall invasiveness and potential for metastasis (40). In our study, the most frequently expressed inhibitor in peripheral nerve sheath tumors was RECK, which showed no significant difference in expression levels in the tumors. Thus, we speculate that an imbalance towards excessive proteolytic activity may be caused by up-regulation of emmprin and MT1-MMP rather than down-regulation of inhibitors in MPNSTs. In this light, the future anti-invasion therapy for MPNST may include inhibition of emmprin-mediated up-regulation of MMPs, including MT1-MMP, for example by using emmprin activity-blocking synthetic peptides (14).

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