Microtubule-Associated Protein-2 and Class III β-Tubulin Are Expressed in Extraskeletal Myxoid Chondrosarcoma

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Extraskeletal myxoid chondrosarcoma is a rare soft tissue sarcoma of uncertain histogenetic origin. Because recent reports have indicated neural–neuroendocrine differentiation in some extraskeletal myxoid chondrosarcomas, we investigated 25 tumors for expressions of microtubule-associated protein-2 and Class III β-tubulin, which are major components of microtubules and specifically localized in neurons and their derivatives. Immunohistochemical expression of microtubule-associated protein-2 and Class III β-tubulin was studied in extraskeletal myxoid chondrosarcomas using formalin-fixed, paraffin-embedded tissues. Cytoplasmic expressions of microtubule-associated protein-2 and Class III β-tubulin were detected in 21 (84%) and 13 (52%) of the 25 extraskeletal myxoid chondrosarcomas, respectively, although the number of positively stained tumor cells varied. Expression of the Class III β-tubulin gene was also assessed in two immunohistochemically positive cases by *in situ* hybridization using an oligonucleotide probe specific for its transcript, and both cases showed expression of Class III β-tubulin transcript. Another case was examined with immunoelectron microscopy, and immunogold particles for Class III β-tubulin were localized to microtubular aggregates. Our data indicate that microtubules in extraskeletal myxoid chondrosarcoma are similar to those found in neurons, further supporting the concept that neural–neuroendocrine differentiation occurs in a significant number of extraskeletal myxoid chondrosarcoma.

KEY WORDS: Extraskeletal myxoid chondrosarcoma, Microtubule, Microtubule-associated protein, Tubulin.

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Extraskeletal myxoid chondrosarcoma is a rare soft tissue sarcoma with distinct clinicopathologic and cytogenetical features (1–8). It is typically myxoid and arises in the deep soft tissue of the proximal extremities and limb girdles of middle-aged adults (1–4). Characteristic chromosomal translocations including t(9;22)(q22;q12), resulting in a fusion of the *EWS* gene at 22q12 and the *CHN/TEC* gene at 9q22, have been reported in extraskeletal myxoid chondrosarcoma, as well as two other less common rearrangements, including fusion of the EWS-related gene *TAF2N* to *CHN/TEC* and fusion of the NH2-terminal domain of the basic helix-loop-helix protein *TCF12* to *CHN/TEC* (5–8). Extraskeletal myxoid chondrosarcoma has generally been regarded as a chondroid tumor because of morphologic similarities between extraskeletal myxoid chondrosarcoma and embryonic chondrogenesis, the presence of chondroitin-4 and 6-sulfate in its myxoid matrix, frequent S-100 protein positivity, and Type II collagen synthesis by tumor cells (1,

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9–13). However, its chondroid nature has been questioned because it is an extraskeletal tumor lacking discernible hyaline cartilage. In addition, recent ultrastructural identification of dense core granules as well as immunohistochemical detection of synaptophysin, chromogranin A, and PGP9.5 indicate that some extraskeletal myxoid chondrosarcomas exhibit neuroendocrine-neural differentiation (3, 13–15).

Microtubular aggregates within rough endoplasmic reticulum are a characteristic ultrastructural finding of extraskeletal myxoid chondrosarcoma, with at least one third of the tumors reported to show these structures (5, 6, 16). Microtubules have crucial biological functions, including mediating mitosis, intracellular transport, and cell motility.

Tubulin is a major constituent protein of microtubules and consists of two 50-kDa subunits designated as α and β. Expression of Class III β-tubulin, one of the six isotypes of the β subunit expressed in mammals (17), is restricted almost entirely to neurons with the known exception of Sertoli cells in the testis (18–20).

Microtubule-associated proteins (MAP) are major components of a family of cytoskeletal proteins associated with microtubule assembly and are implicated in key roles in morphogenesis, function, and maintenance of the nervous system (21). MAP-2, including high-molecular-weight MAP-2 (MAP-2a, MAP-2b) and low-molecular-weight MAP-2 (MAP-2c, MAP-2d), is the most abundant MAP isoform in the brain and is primarily localized to neuronal dendrites (22, 23). However, it recently has been reported that MAP-2 is expressed in the companion layer of the anagen hair follicle (24).

Class III β-tubulin and MAP-2 have been used as sensitive and specific markers for neural differentiation, and a variety of neural or neuroendocrine tumors such as neuroblastoma, medulloblastoma, central neurocytoma, ganglioglioma, dysembryoplastic neuroepithelial tumor, pulmonary carcinoid, and small cell carcinoma have been shown to express Class III β-tubulin and/or MAP-2 (23, 25, 26). However, the expression of these molecules has been demonstrated also in variable fractions of non-neural or non-neuroendocrine tumors such as astrocytoma, pulmonary squamous cell carcinoma, and adenocarcinoma (25–27).

The aim of our study was to assess neural–neuroendocrine differentiation in extraskeletal myxoid chondrosarcoma using microtubule-related proteins, Class III β-tubulin, and MAP-2 as specific markers.

MATERIALS AND METHODS

Twenty-five cases of extraskeletal myxoid chondrosarcoma were retrieved from the institutional pathology files of all of the authors. Detailed clinicopathologic and molecular features of some of these cases have been described elsewhere (2, 7, 8).

Four-μm-thick sections of representative formalin-fixed, paraffin-embedded tissues of extraskeletal myxoid chondrosarcoma were prepared and used for immunohistochemical analysis and *in situ* hybridization. Normal cerebral tissue was included as a positive control in both analyses. Sixteen other myxoid or chondroid tumors (3 myxoid liposarcomas, 3 myxoid malignant fibrous histiocytomas, 3 soft tissue myxomas, 2 enchondromas, 2 chondrosarcomas, and 3 chordomas) were also analyzed immunohistochemically for expressions of MAP-2 and Class III β-tubulin.

Standard antigen retrieval with a microwave oven was performed on deparaffinized sections. After treatments with 3% hydrogen peroxide for 10 minutes and normal goat serum for 5 minutes, the sections were incubated with commercialized monoclonal antibodies raised against MAP-2 (AP-20; Sigma, Saint Louis, MO, dilution 1:200) and Class III β-tubulin (TUJ1; BabCO, Richmond, CA, dilution 1:400) for 16 hours at 4° C. Although the expression of Class III β-tubulin appears to be limited to neuronal tissues, this molecule has been detected in some non-neuronal tumors such as pulmonary squamous cell carcinoma with the antibody TUJ1 (25). However, this immunoreactivity might have been antibody clone dependent, because another clone (TU-20) of the antibody raised against Class III β-tubulin has failed to support this finding (23). The labeled streptavidin-biotin-peroxidase complex method (LSAB kit; DAKO Japan, Kyoto, Japan) was used for immunostaining followed by visualization with 3,3'-diaminobenzidine. Immunostaining results of tumor cells were tabulated as follows: focal, 5% to less than one third positively staining cells; moderate, one third to less than two thirds positively staining cells; and diffuse, two thirds or more positively staining tumor cells.

In two extraskeletal myxoid chondrosarcomas that were immunohistochemically positive for MAP-2 and Class III β-tubulin, the gene expression of Class III β-tubulin was investigated by *in situ* hybridization using digoxigenin-end-labeled oligonucleotide probes flanking parts (45 bases) of exons 3 and 4 of human Class III β-tubulin mRNA (GenBank accession no. AF427491): antisense probe 5'-ACTCTGACCAAAGATGAAATTGTCAGGCCTGAAGAGATGTCCAAA, sense probe 5'-TTTGGACATCTCTTCAGGCCTGACAATTTCATCTTTGGTCAGAGT. After treatment with 50 μg/mL proteinase K (Sigma) for 15 minutes at 37° C, the sections were postfixed in 4% paraformaldehyde, neutralized with 2 mg/mL glycine in phosphate buffered saline, and immersed in 40% formamide. Hybridization was carried out in 40% formamide, 10 mmol/L Tris-HCl (pH 8.0), 0.6 mol/L NaCl, 1 mmol/L EDTA, 1× Denhardt's solution (Sig-

TABLE 1. Immunohistochemical Results of MAP-2 and Class III β-Tubulin in Myxoid and Chondroid Tumors

Tumor*	Number of Cases Examined	MAP-2			Class III β-Tubulin		
		Focal	Moderate	Diffuse	Focal	Moderate	Diffuse
EMCS	25	11	8	2	8	5	0
Soft tissue myxoma	3	1	0	0	0	0	0
Myxoid liposarcoma	3	0	0	0	0	0	0
Myxoid MFH	3	0	2	0	1	0	0
Enchondroma	2	0	2	0	0	0	0
Chondrosarcoma	2	0	2	0	1	0	0
Chordoma	3	0	0	3	0	2	0

* EMCS = extraskeletal myxoid chondrosarcoma; MFH = malignant fibrous histiocytoma.
Focal, positive tumor cells < 1/3; moderate, 1/3< positive tumor cells <2/3; diffuse, 2/3< positive tumor cells.

ma), 250 μg/mL yeast tRNA, 125 μg/mL salmon sperm DNA, and 10% dextran sulfate, together with 1 μg of the labeled probe for 16 hours at 42° C. After hybridization, the sections were rinsed briefly with 50% formamide in 2× SSC and washed 5 times with the same washing solution for 1 hour at 37° C, followed by 2× SSC for 15 minutes. at room temperature. Signal detection was performed using a monoclonal anti-digoxigenin antibody (21H8; Abcam, Cambridge, UK) and a polymeric conjugate made up of a large number of peroxidase and secondary antibody molecules (EnVision, DAKO Japan) according to the manufacturer's instructions. Positive signals were visualized with 3,3'-diaminobenzidine. Specificity of the antisense probe was attested by a positive hybridization signal to cDNA of human brain tissue blotted on a nylon filter membrane (Amersham Pharmacia Biotech, Tokyo, Japan) and negativity in genomic human DNA and products (247 bp) of polymerase chain reaction for transcripts of human phosphoglycerate kinase gene on the same membrane.

One of the cases immunohistochemically positive for Class III β-tubulin was further investigated using immunoelectron microscopy. Small pieces of the specimen were cut from the formalin-fixed tumor tissue and thoroughly washed with phosphate buffered saline at 4° C. The specimen was dehydrated with a graded series of dimethylformamide at −20° C and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany), which was polymerized by ultraviolet irradiation at −20° C. Ultrathin sections were mounted on nickel grids coated with 0.5% formvar (Nisshin EM, Tokyo, Japan) and incubated with the primary antibody (TUJ1, 1:50) at 4° C overnight. Fifteen-nanometer gold particles conjugated with anti-mouse IgG antibody (BBInternational, Cardiff, UK) were applied for 30 minutes at room temperature, followed by brief double staining with uranyl acetate and lead citrate. The primary antibody was omitted from the procedure in a negative control.

RESULTS

Immunohistochemical staining results of all tumors are summarized in Table 1, and detailed results as well as molecular findings of the fusion genes in each case of extraskeletal myxoid chondrosarcoma are shown in Table 2.

Cytoplasmic expression of MAP-2 and Class III β-tubulin was detected immunohistochemically in 21 cases (84%) and 13 cases (52%) of 25 cases, respectively. Diffuse (more than two thirds of tumor cells) and mostly intense staining for MAP-2 was seen in two cases, whereas eight cases showed moderate positive staining (one third to two thirds of tumor cells) (Fig. 1). Moderate immunohistochemical staining for Class III β-tubulin was seen in five cases, and focal positive staining, in 8 cases. Co-expression of MAP-2 and Class III β-tubulin was noted in 10 of 25 cases (40%).

Focal MAP-2 expression was seen in one of three soft tissue myxomas, and moderate MAP-2 expression, in two of three myxoid malignant fibrous histiocytomas; one of these malignant fibrous histiocytomas also focally expressed Class III β−tubulin.

TABLE 2. Immunohistochemical Results and Molecular Features of 25 Extraskeletal Myxoid Chondrosarcomas

Number of Cases	Immunohistochemistry		Fusion Gene
	MAP-2	Class III β-Tubulin	
1	Focal	Moderate	TAF2N-CHN
2	Focal	Negative	EWS-CHN type 1
3	Diffuse	Moderate	EWS-CHN type 1
4	Focal	Negative	EWS-CHN type 1
5	Moderate	Focal	EWS-CHN type 1
6	Negative	Focal	EWS-CHN type 1
7	Focal	Focal	EWS-CHN type 1
8	Diffuse	Focal	EWS-CHN type 1
9	Negative	Focal	EWS-CHN type 1
10	Negative	Negative	Not detected
11	Focal	Moderate	Not detected
12	Negative	Focal	EWS-CHN type 2
13	Focal	Negative	EWS-CHN type 1
14	Focal	Negative	TAF2N-CHN
15	Focal	Negative	EWS-CHN type 1
16	Moderate	Focal	EWS-CHN type 1
17	Focal	Negative	Not detected
18	Focal	Focal	Not examined
19	Focal	Negative	Not examined
20	Moderate	Negative	Not examined
21	Moderate	Moderate	Not examined
22	Moderate	Moderate	Not examined
23	Moderate	Negative	Not examined
24	Moderate	Negative	Not examined
25	Moderate	Negative	Not detected

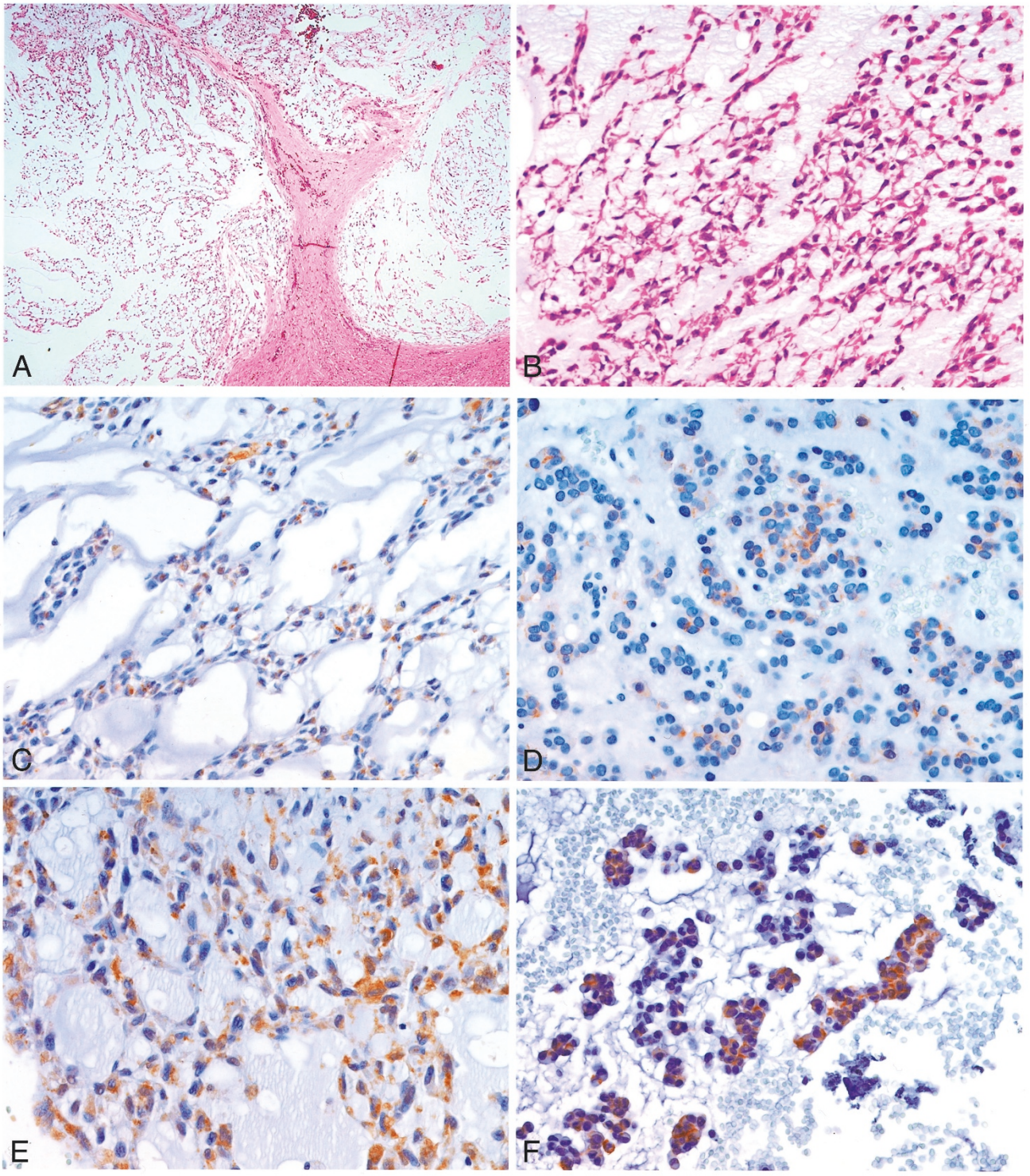

FIGURE 1. Morphology of EMCS (**A, B**) and immunohistochemical expression of Class III β-tubulin (**C, D**) and MAP-2 (**E, F**).

In contrast, all 7 chondroid tumors showed moderate to diffuse expression of MAP-2 (Fig. 2). One of two chondrosarcomas and two of three chordomas also expressed Class III β-tubulin focally to moderately (Fig. 2).

In situ hybridization performed in two extraskeletal myxoid chondrosarcomas using the antisense probe demonstrated positive signals for Class III β-tubulin in tumor cells of both cases; focal or moderate immunohistochemical expression of MAP-2 and Class III β-tubulin was identified in these cases (Fig. 3).

The extraskeletal myxoid chondrosarcoma analyzed with immunoelectron microscopy showed prominent microtubular aggregates within variably dilated cisternae. Immunogold particles for Class III

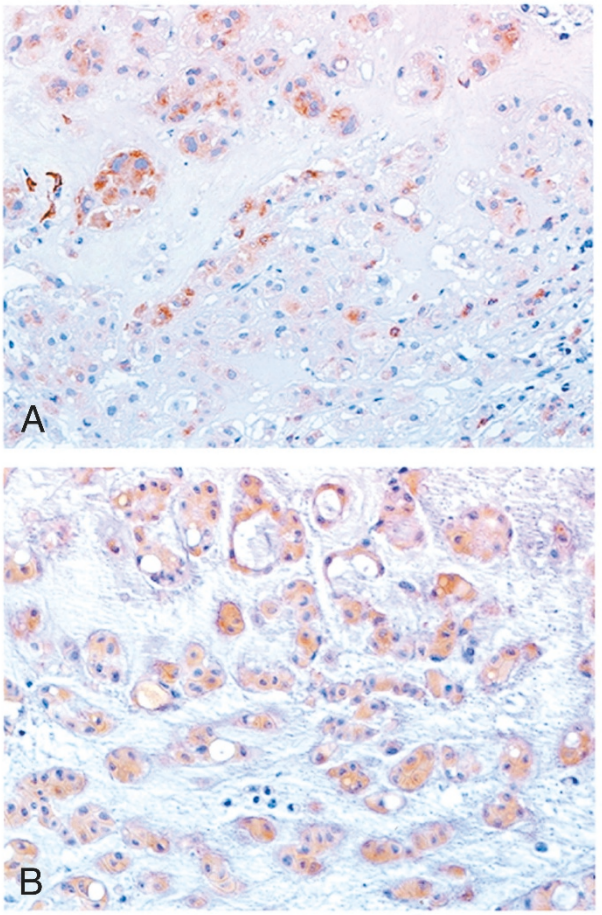

FIGURE 2. Chordoma with immunohistochemical expression of class III β-tubulin (**A**) and MAP-2 (**B**).

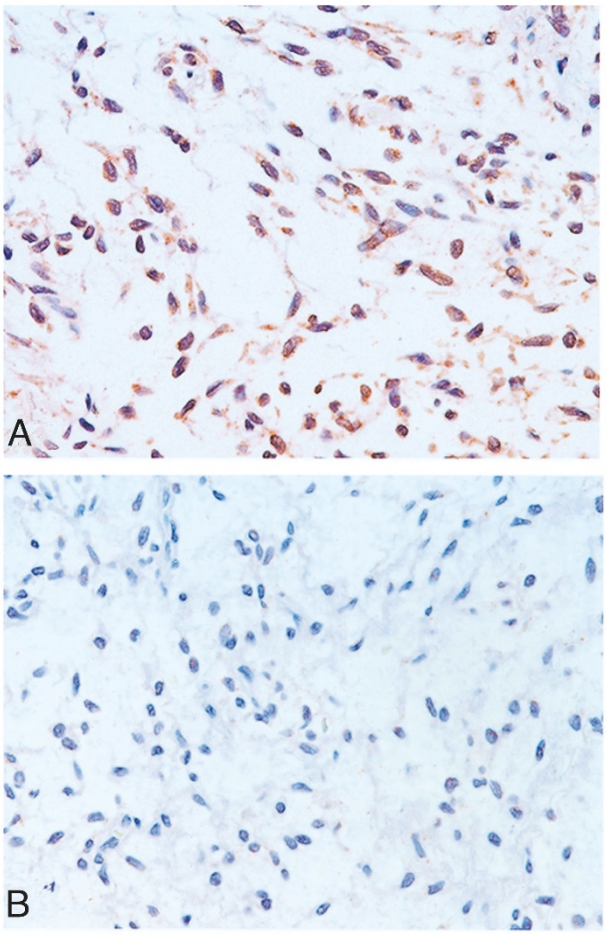

FIGURE 3. Expression of Class III β-tubulin in EMCS by *in situ* hybridization using an antisense oligonucleotide probe (**A**). The tumor is negatively stained with a sense probe (**B**).

β-tubulin were localized primarily to these microtubular aggregates (Fig. 4).

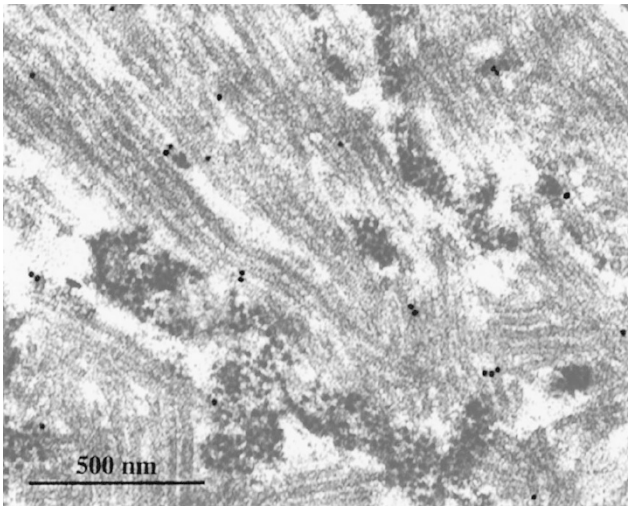

FIGURE 4. Immunolabeling of Class III β-tubulin in EMCS. Immunogold particles are localized on microtubules aggregated within cisternae.

DISCUSSION

The recent detection of dense core granules ultrastructurally as well as synaptophysin, chromogranin A, neuron-specific enolase, PGP9.5, and CD57 (Leu 7) immunohistochemically in some extraskeletal myxoid chondrosarcomas has raised the possibility of neural–neuroendocrine differentiation in extraskeletal myxoid chondrosarcoma and has posed further questions regarding its chondroid nature (3, 4, 13–15). In addition, the recent immunohistochemical detection of peripherin in extraskeletal myxoid chondrosarcoma, a type III-intermediate filament involved in the growth and development of the peripheral nervous system, as well as tau, an axonal protein promoting tubulin polymerization, has provided additional support for the notion of limited neural differentiation in extraskeletal myxoid chondrosarcoma (28, 29). Our immunohistochemical and immunoelectron microscopy findings of the expression of neuron-specific microtubule-related proteins, MAP-2, and Class III β-tubulin, as well as the detection of Class III β-tubulin gene expression by *in situ* hybridiza-

tion, lend further support to the notion that a subset of extraskeletal myxoid chondrosarcoma shows neural–neuroendocrine differentiation. Our findings indicate that the abundant microtubules typically occurring in extraskeletal myxoid chondrosarcoma as aggregates in dilated endoplasmic reticulum or cisternae are similar to the microtubules of neurons and their derivatives.

Microtubules are ubiquitous organellae involved in various cellular functions such as mortility, mitosis, and intracellular transport. The β-tubulin isoforms have been found to differ in their cellular distributions within human tissues (30). Tubulin expression was recently reported in two extraskeletal myxoid chondrosarcomas and six chordomas: however, the tubulin isoforms were not determined (29). In this study, we selected a monoclonal antibody to the Class III β-tubulin isoform, TUJ1, which recognizes an epitope specifically expressed in neurons (17, 18). The Class III β-tubulin isoform as well as MAP-2 have recently been used as markers of neuroendocrine differentiation in pulmonary carcinoid and small cell carcinoma (25, 26).

The significance of Class III β-tubulin expression in extraskeletal myxoid chondrosarcoma is not entirely clear. Microtubule formation characteristically occurs in early cartilage development (31) and is of particular interest with regard to extraskeletal myxoid chondrosarcoma's histologic and histochemical resemblance to embryonal cartilage. Moreover, up-regulation of β-tubulin is believed to regulate differentiation and hypertrophy in growth-plate chondrocytes (32). Which β-tubulin isoforms are expressed in developing cartilage and growth plate chondrocytes, however, is unknown.

MAP-2, high– and low–molecular weight components of the MAP family, is expressed mainly in neuronal cells and has been used as a sensitive and specific marker for cells with neural differentiation. The monoclonal antibody AP20 that was used in our study specifically detects 280-kDa-high molecular forms (MAP2a, MAP2b) of MAP-2. The expression of MAP-2 in 21/25 extraskeletal myxoid chondrosarcomas and 3/3 chordomas in our study is in keeping with the consistent expression of tau, another microtubule-associated protein family that is required for axonal outgrowth and associated with neurodegenerative diseases such as Alzheimer's disease (29).

The expression of MAP-2 and Class III β-tubulin in myxoid or chondroid tumors other than extraskeletal myxoid chondrosarcoma is not easily explained. Cartilage and neural tissue may have overlapping microtubule-related protein molecules, because pluripotent precursor cells of the neural crest can give rise to many derivatives, including neurons and cranial skeletal components such as cartilage, bone, and dermis (33). Besides, developing cartilage as well as notochordal remnants or the nucleus pulposus in the vertebral column of two fetuses (9 and 23 weeks of gestation, respectively) that we examined expressed MAP-2 but not Class III β-tubulin (data not shown). This finding may be related to the frequent expression of MAP-2 in chondroid tumors. Alternatively, the expressions of neuron-specific microtubule-related proteins could represent unscheduled or divergent differentiation in subsets of tumor cells, a phenomenon encountered in a variety of neoplastic conditions. To further address these issues, a large panel of tumors and tissues could be assessed for both markers.

The morphologic spectrum of extraskeletal myxoid chondrosarcoma is quite wide, and histologic recognition of its unusual variants may be very difficult (2). The ultrastructural demonstration of microtubular aggregates in extraskeletal myxoid chondrosarcoma is of limited diagnostic value because it occurs in only one third of cases and because similar structures may be seen in metastatic melanoma, osteosarcoma, embryonal rhabdomyosarcoma, and chordoma (34–38). The finding of MAP-2 and Class III β-tubulin in extraskeletal myxoid chondrosarcoma *per se* is probably of limited diagnostic value because these proteins are also expressed in myxoid and chondroid tumors simulating extraskeletal myxoid chondrosarcoma. However, this finding may help explain part of the reason behind neural–neuroendocrine differentiation seen in some extraskeletal myxoid chondrosarcomas.

In summary, our study demonstrates the presence of the neuron-specific microtubule-related proteins MAP-2 and class III β-tubulin in extraskeletal myxoid chondrosarcoma and further underscores the unique combination of potential chondroid and neural–neuroendocrine differentiation in this tumor.

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