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HMGA proteins in malignant peripheral nerve sheath tumor and synovial sarcoma: preferential expression of HMGA2 in malignant peripheral nerve sheath tumor

Pei Hui[1], Ning Li[1], Chaline Johnson[1], Ivo De Wever[2], Raf Sciot[3], Guidalberto Manfioletti[4] and Giovanni Tallini[5]

[1]*Department of Pathology, Yale University School of Medicine, New Haven, CT, USA;* [2]*Department of Surgery, University of Leuven School of Medicine, Leuven, Belgium;* [3]*Department of Pathology, University of Leuven School of Medicine, Leuven, Belgium;* [4]*Department of Biochemistry, University of Trieste School of Medicine, Trieste, Italy and* [5]*Department of Pathology, University of Bologna School of Medicine, Bologna, Italy*

Histological separation of synovial sarcomas from malignant peripheral nerve sheath tumors can be difficult and available immunohistochemical markers sometimes give rise to overlapping staining patterns. Additional markers are needed to better define the two entities in the routine surgical pathology practice. To this end, we explored diagnostic applications of HMGA (HMGA1 and HMGA2) protein immunohistochemistry in comparable groups of synovial sarcoma and malignant peripheral nerve sheath tumors. The histological diagnosis of these cases was confirmed by the presence or absence of synovial sarcoma specific *SYT-SSX* fusion transcript analyzed by real-time reverse transcription polymerase chain reaction. In all, 13 malignant peripheral nerve sheath tumors and 15 synovial sarcomas were included in this study. Immunohistochemically, most malignant peripheral nerve sheath tumors expressed both HMGA1 and HMGA2 protein (12/13 and 12/13 cases, respectively) with moderate to strong nuclear staining patterns. Most cases of synovial sarcomas demonstrated variable expression of HMGA1. However, significant immunoreactivity for HMGA2 was present in the glandular component of a biphasic tumor (1/1) and rarely detected in monophasic synovial sarcomas (1/14). In summary, expression of HMGA2 is a feature of MPNST but not of synovial sarcoma and immunohistochemical staining of HMGA2 may be a useful marker to separate malignant peripheral nerve sheath tumor from synovial sarcoma.
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Malignant peripheral nerve sheath tumors (MPNSTs) are defined as malignant neoplasms arising from peripheral nerves or having differentiation towards various elements of the nerve sheath. The diagnosis of MPNST has long been one of the most challenging processes for a surgical pathologist. Although generally it is acceptable that if a sarcoma arises from the peripheral nerve or a neurofibroma, a MPNST can be diagnosed, it is often difficult to establish such a diagnosis when the tumor occurs outside such settings. A major challenge resides in the differential diagnoses from other spindle cell sarcomas, notably synovial sarcoma. MPNST may bear close resemblance to synovial sarcoma and rare examples of synovial sarcoma have been described as arising in nerves.[1] In addition, biphasic synovial sarcoma can also be confused with MPNST having glandular differentiation. While immunostaining for epithelial markers can be used, rare MPNSTs can express either cytokeratin or epithelial membrane antigen.[2] Although S-100 protein immunoreactivity is a characteristic feature of MPNST, 30% synovial sarcomas can show positivity as well.[3–5] The usefulness of immunostaining for both cytokeratin seven and 19 has been proposed for the differential diagnosis since virtually all synovial sarcomas express the two markers, whereas MPNSTs rarely express both.[6] Since

Correspondence: Dr P Hui, MD, PhD, Department of Pathology, Yale University School of Medicine, BML 250, 310 Cedar Street, New Haven, CT 06520-8023, USA.
E-mail: pei.hui@yale.edu
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the elucidation of the specific chromosomal translocation and its associated fusion gene expression involved with synovial sarcoma, it has become clear that the gold standard for a definite diagnosis of synovial sarcoma requires a demonstration of the characteristic cytogenetic and/or molecular alteration, that is, chromosomal translocation t(X;18) and/or its associated *SYT-SSX* fusion transcript.[7,8]

The high mobility group proteins (HMGs) represent a subset of low molecular weight, nonhistone proteins, that function as transcription regulatory proteins that bind DNA and modify chromatin conformation.[9,10] The HMGI family members include HMGA1 and HMGA2. Disregulation of HMGA genes by chromosomal alterations has been observed in various benign mesenchymal tumors.[11] Animal experiments have suggested their roles in mesenchymal histogenesis and tumorigenesis.[12] In a pilot immunohistochemical study of HMGA1 and HMGA2 expression in a variety of malignant mesenchymal tumors, we observed that HMGA2 was consistently expressed in MPNSTs, but not in synovial sarcomas. Thus, we report herein an investigation into the expression of HMGA proteins in comparable groups of MPNST and synovial sarcoma to evaluate their diagnostic utility. Since *SYT-SSX* fusion gene is the molecular hallmark of synovial sarcoma, real-time RT-PCR detection of the fusion transcript was used to delineate the two groups of lesions and to solidify our immunohistochemical conclusions.

Materials and methods

Patients and Specimens

A total of 13 cases of MPNST and 15 cases of synovial sarcomas were selected from the files of Departments of Pathology at Yale-New Haven Hospital and at the University Hospital of Leuven. Tumor diagnosis was based on accepted criteria.[2,13] Tumors were classified as malignant peripheral nerve sheath tumors based on a combination of clinical (association with peripheral nerves, preexisting neurofibroma, setting of neurofibromatosis I) and histologic (spindle cells with wavy, comma-shaped nuclei and indistinct cytoplasm growing with alternating dense and hypodense fascicular areas) findings. According to standard diagnostic procedures, only cases with immunohistochemical positivity for S100, Leu7 or PGP9.5 and/or ultrastructural evidence of Schwann cell differentiation were included in the study.[2,13] Real-time RT-PCR was performed on all cases (see below) and the demonstration of SYT-SSX fusion transcripts was required to confirm the diagnosis of synovial sarcoma. Representative histological sections of all cases were reviewed by three pathologists before selection of the tissue for molecular analysis (PH, NL, GT).

Detection of SYT-SSX Fusion Transcripts by Real-Time Quantitative RT-PCR

RNA preparation

In all, 10–20 10 μm thick unstained sections were generated with one corresponding hematoxylin and eosin (HE) stain. Lesional tissue identified in the HE section was outlined in the unstained section, followed by scraping lesional tissue into a microcentrifuge tube. The tissue was deparaffinized twice with xylene followed by ethanol treatment twice and air-dried. The tissue was then digested with proteinase K ((100 mg/ml) (Roche, Indianapolis, IN, USA)) in the ATL tissue digestion buffer (Qiagen, Chatsworth, CA, USA) at 55°C overnight. The total RNA was then extracted by using TRIzol reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The amount of RNA was determined by measuring OD_{260}. The RNA was stored at −20°C.

Real-time quantitative RT-PCR

Real-time RT-PCR for *SYT-SSX* fusion transcript was performed according to published methodology with some modifications.[14,15] All oligonucleotide primers and probes were synthesized by the Oligo Factory (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA). Primer and probe sequences common to all three types of *SYT-SSX* were as followed: sense primer 5′-CAG CAG AGG CCT TAT GGA TAT GA-3′; antisense primer 5′-TTT GTG GGC CAG ATG CTT C-3′; *SYT-SSX* probe, 6-FAM-ATC ATG CCC AAG AAG CCA GCA GAG G-TAMRA; *GAPDH* sense primer, 5′-CCA CAT CGC TCA GAC ACC AT-3′; *GAPDH* antisense primer, 5′-CCA GGC GCC CAA TAC G-3′; *GAPDH* probe: FAM-AAG GTG AAG GTC GGA GTC AAC GGA TTT G-TAMRA. The *SYT-SSX* and control *GAPDH* mRNA quantitative assay amplified 0.2 μg of total RNA prepared from paraffin blocks of each case. Reverse transcription was performed in a total volume of 20 μl with 50 mmol/l Tris-HCl (pH 8.3), 40 mmol/l KCl, 5 mmol/l $MgCl_2$, 0.5% Tween, 0.5 mmol/l dNTP Mix, 10 mmol/l dithiothreitol, random hexamer and 4 units of M-MuLV reverse transcriptase (Roche, Indianapolis, IN, USA). Samples were incubated at 42°C for 1 h, then at 95°C for 5 min. In all, 5 μl of the reverse transcription reaction was subject to the following real-time PCR. The standard master mixes for both *SYT-SSX* and *GAPDH* were composed of reagents obtained from Perkin-Elmer Corporation (Norwalk, CT, USA) unless otherwise indicated, and included 10% TaqMan 10 × buffer, 2.5 mM $MgCl_2$, 0.2 μM of dATP, dCTP, dGTP, and dTTP, 1.5 units TaqGold DNA polymerase. All reaction mixes were brought to a 50 μl volume and placed in MicroAmp Optical Tubes and covered with MicroAmp Optical Caps (Perkin-Elmer Corporation, Norwalk, CT, USA). RT-PCR was performed in an ABI PRISM 7000 Sequence Detector (Perkin-Elmer Corporation, Norwalk, CT, USA). The reaction started with

10 min at 95°C for TaqGold activation and predenaturation, and 45 cycles with each cycle consisting of 15 s at 95°C and 1 min at 62°C. Data were normalized to the quencher dye TAMRA and analyzed using the Signal Detection software (Perkin-Elmer Applied Biosystems Division). Critical threshold (Ct) cycle numbers were obtained from amplification of both *STY-SSX* and *GAPDH*. Representative PCR results were confirmed by agarose gel electrophoresis.

Immunohistochemistry

Representative sections were cut from formalin-fixed, paraffin-embedded tissue from each of the cases and stained with antibodies according to established protocols in our laboratory, using an avidin-biotinylated peroxidase complex (ABC) technique. Anti-HMGA2 antibodies were raised in rabbit against the recombinant murine HMGA2 protein sharing a high degree of homology with the human HMGA2 protein, as previously described.[16] The HMGA2 antibodies were used at a 1:300 dilution. The antibodies against HMGA1 were developed against a HMGA1-specific synthetic peptide corresponding to the amino-terminal portion of the molecule, and were used in a 1:100 dilution in this study. Negative controls were performed by incubating the histology sections with an unrelated nuclear antibody against human papilloma virus (Dako Corp., Carrpinteria, CA, USA) and by omitting the primary antibody. Cases previously characterized for dysregulated HMGA1 and HMGA2 protein expression were used as positive controls.[15] Only tumor cells with distinct nuclear immunoreactivity comparable to that of the controls were counted as positive. The number of positive cells was evaluated in 20 high-power fields ($\times 40$) for each histology section and quantified as a percentage. The degree of staining intensity was graded as weak, moderate and strong. Only cases in which there was staining in equal to or greater than 10% of the neoplastic cell nuclei with at least moderate staining intensity were considered to have positive overall score.

Results

Clinical and Histological Features

The clinical features of these cases are summarized in Table 1. The average age of the patients diagnosed with MPNST was 42 years. Three patients presented with evidence of neurofibromatosis. All tumors except one were deep seated with sizes ranging from 1.0 to 15.0 cm (average 10.2 cm). The average age of patients with synovial sarcoma was 38 years and none was associated with clinical syndromes, especially neurofibromatosis. All synovial sarcomas were deep-seated tumors with sizes ranging from 2.0 to 15.0 cm (average 6.4 cm).

Histologically, all 13 cases of MPNSTs exhibited a spindle cell proliferation in haphazard arrays or intersecting fascicles often showing alternating dense and hypodense areas resulting in a 'tapistry' growth pattern. The neoplastic cells were spindle-shaped with variable amount of eosinophilic cytoplasm and wavy, comma-shaped hyperchromatic nuclei. Mitoses were identified in all cases. Two cases showed an involvement of the peripheral nerve. Immunohistochemical positivity for S100, Leu7 or PGP9.5 and/or ultrastructural evidence of Schwann cell differentiation was confirmed on all cases. One of the 15 cases of synovial sarcoma was a biphasic tumor with distinct spindle and glandular areas. In all, 11 of 15 cases of synovial sarcomas were of monophasic spindle cell type with the typical spindle cell proliferation arranged in lobules. Prominent fibrosis was seen and focal calcification was present in many of the tumors. Three of the 15 cases had cellular areas with a predominance of round–ovoid rather plump cells that exhibited epithelioid features and were mitotically active. These three cases shared features of the tumors referred to as monophasic epithelial synovial sarcoma in the 2002 soft tissue and bone tumors WHO fascicle[13] and, in this study they were placed under the category of monophasic synovial sarcoma with epithelioid features (Tables 1–3).

Confirming Histological Diagnosis by Real-Time RT-PCR Detection of SYT-SSX Fusion Transcript

All cases were analyzed initially by the amplification of the house keeping gene *GAPDH* to confirm the presence of amplifiable RNA. Real-Time RT-PCR detected *SYT-SSX* fusion transcript in all cases diagnosed as synovial sarcoma (Table 2). Nine cases diagnosed as MPNST showed no evidence of the fusion gene expression. One case with an initial diagnosis of spindle cell sarcoma consistent with MPNST based on the histologic features and on the finding of focal S100 immunoreactivity was found to harbor *SYT-SSX* fusion gene. Upon retrospective

Table 1 Clinicopathologic features of MPNSTs and synovial sarcomas

	MPNST	*Synovial sarcoma*			
		Spindle	*Epithelioid*	*Biphasic*	*Total*
Number of cases	13	11	3	1	15
Mean age	42				38
M:F	6:7				3:12
Site					
Upper extremity	1	0	0	0	0
Lower extremity	4	8	2	1	11
Buttock	2	0	1	0	1
Head and neck	1	0	0	0	0
Chest/breast	1	1	0	0	1
Back	1	1	0	0	1
Abdomen	3	1	0	0	1

Table 2 HMGA1 and HMGA2 immunohistochemistry and SYT-SSX real-time RT-PCR

Case #	*Diagnosis*	*HMGA2 (%)*	*HMGA2 intensity*	*HMGA2 overall score*	*HMGA1 (%)*	*HMGA1 intensity*	*HMGA1 overall score*	*MIB1*	*GRADE*	*SYT-SSX*
1	MPNST	10	2+	+	30	2+	+	20	2	NA
2	MPNST	80	3+	+	60	2+	+	40	3	NA
3	MPNST	10	2+	+	80	2+	+	15	2	NA
4	MPNST	50	2+	+	10	2+	+	60	3	Neg
5	MPNST	80	3+	+	90	3+	+	40	3	Neg
6	MPNST	0	0	–	0	0	–	10	2	NA
7	MPNST	80	3+	+	90	2+	+	30	2	Neg
8	MPNST	90	3+	+	70	3+	+	40	3	Neg
9	MPNST	90	3+	+	20	2+	+	ND	3	Neg
10	MPNST	70	2+	+	20	2+	+	40	3	Neg
11	MPNST	80	3+	+	60	2+	+	60	3	Neg
12	MPNST	80	2+	+	100	2+	+	25	3	Neg
13	MPNST	50	3+	+	20	3+	+	40	3	Neg
14	SS-MS[a]	0	0	–	90	2+	+	25	2	Pos
15	SS-MS	0	0	–	10	2+	+	20	2	Pos
16	SS-MS	0	0	–	20	2+	+	ND	2	Pos
17	SS-EP	5	2+	–	90	3+	+	15	2	Pos
18	SS-MS	0	0	–	0	0	–	30	3	Pos
19	SS-MS	20	1	–	10	1+	–	20	2	Pos
20	SS-BP	50	3+	+	90	3+	+	10	2	Pos
21	SS-EP	30	2+	+	20	3+	+	40	3	Pos
22	SS-MS	0	0	–	10	2+	+	10	2	Pos
23	SS-MS	0	0	–	10	2+	+	25	3	Pos
24	SS-MS	0	0	–	30	2+	+	40	3	Pos
25	SS-MS	0	0	–	10	2+	+	25	3	Pos
26	SS-MS	10	1+	–	40	2+	+	20	2	Pos
27	SS-MS	0	0	–	60	2+	+	15	2	Pos
28	SS-EP	10	1+	–	90	2+	+	10	2	Pos

NA—not available; neg—negative; pos—positive; SS-EP—monophasic synovial sarcoma with epithelioid features; SS-MS—monophasic spindle synovial sarcoma; SS-BP—biphasic synovial sarcoma.
[a]Initially diagnosed as sarcoma consistent with MPNST.

Table 3 Summary of HMGA1 and HMGA2 immunohistochemistry results

Diagnosis	*Total number of cases*	*HMGA1 positive*[a]	*HMGA2 positive*[a]
MPNST	13	12	12
Synovial sarcoma			
Spindle (SS-MS)	11	9	0
Epithelioid (SS-EP)	3	3	1
Biphasic (SS-BP)	1	1	1
Total	15	14	2

[a]Only cases in which there was positive staining in equal to or greater than 10% of the lesional cell nuclei with at least moderate staining intensity were considered to have positive overall score.

review of the pathology findings the case was reclassified as synovial sarcoma (Table 2, case 14). In four cases real-time RT-PCR of the *SYT-SSX* fusion transcripts was not interpretable due to insufficient mRNA template (Table 2, cases 1, 2, 3, 6).

HMGA1 and HMGA2 Immunohistochemistry

The results of immunohistochemical studies are summarized in Tables 2 and 3. Most MPNSTs expressed both HMGA1 and HMGA2 protein (12/13 and 12/13 cases, respectively). Only one case of MPNST was negative for both HMGA1 and HMGA2, possibly due to poor antigen preservation. Most of the positive cases showed strong nuclear expression with over 30% of positive neoplastic cells (Figure 1a–c). Two MPNSTs showed moderate staining intensity for HMGA2 and four showed moderate staining intensity of HMGA1 with a 10–30% range of positive neoplastic cells (Table 2). Of the 15 cases of synovial sarcomas, 14 expressed HMGA1. The three cases of synovial sarcoma with epithelioid features exhibited a high degree of HMGA1 immunoreactivity in terms of proportion of positive cells and/or nuclear staining intensity. One synovial sarcoma with epithelioid features and the biphasic synovial sarcoma showed immunoreactivity in 30 and 50% of the neoplastic cells, respectively, with at least moderate stating intensity for HMGA2 and were therefore given an overall positive HMGA2 score (Tables 2 and 3). All monophasic spindle synovial sarcomas did not show significant (overall positive score) HMGA2 expression (0/11, Figure 2a–c). In one biphasic synovial sarcoma, HMGA2 was mostly restricted to the epithelial component of the tumor (Figure 3a–c).

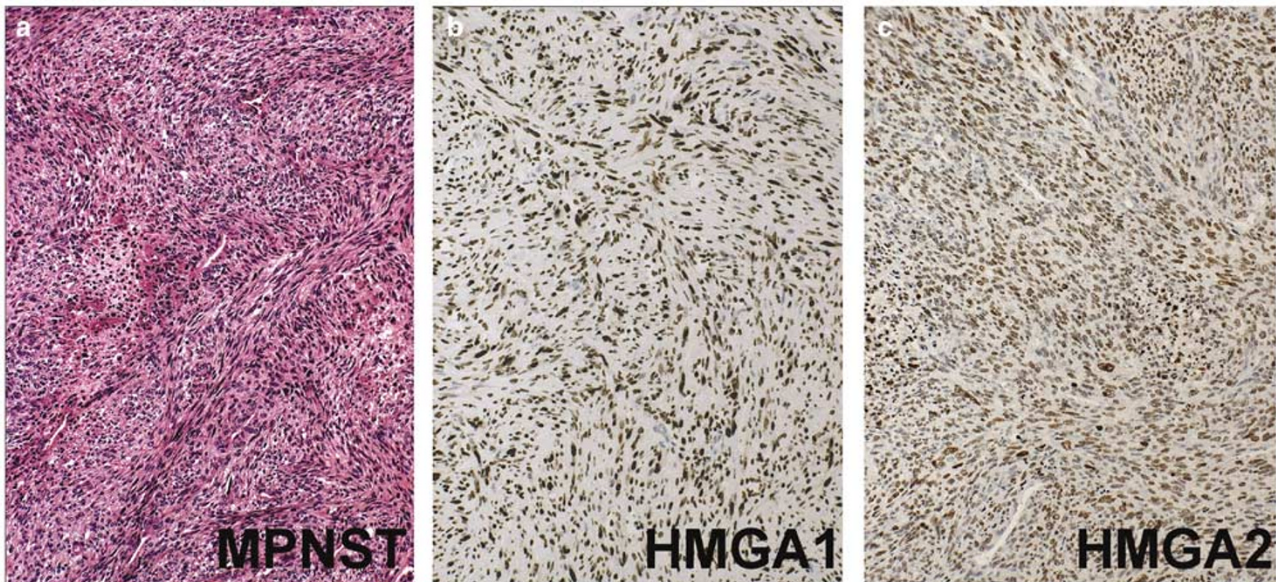

Figure 1 HMGA protein immunohistochemistry in MPNST strong expression of both HMGA1 and HMGA2 in MPNST (**a**, **b** and **c**).

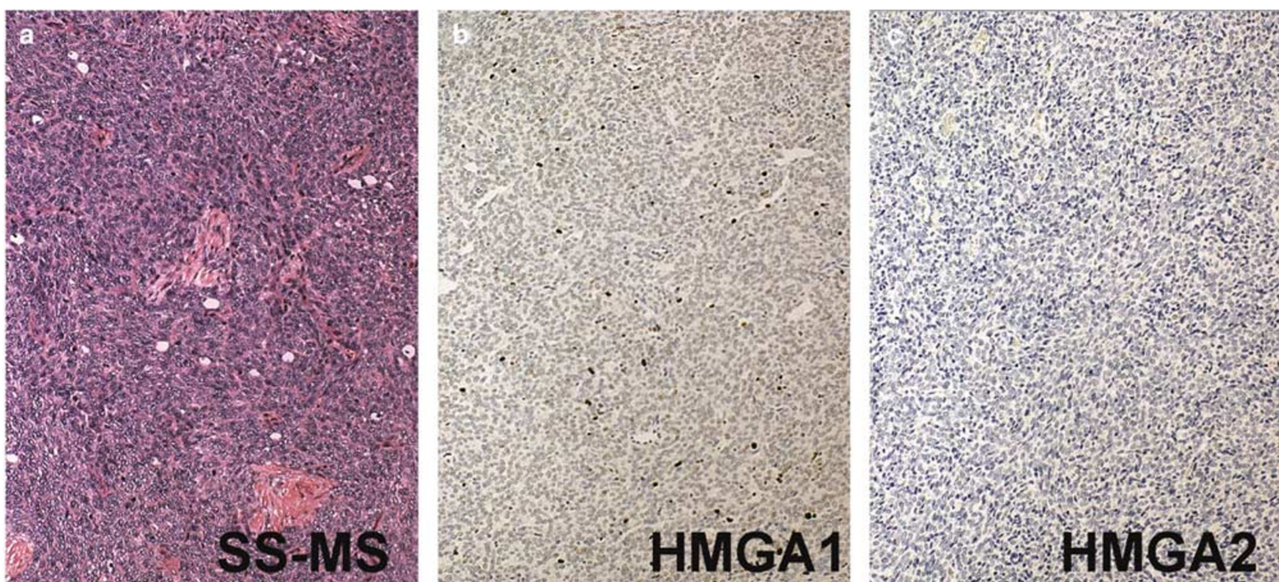

Figure 2 HMGA protein immunohistochemistry in monophasic spindle synovial sarcoma (**a**) with few scattered cells immunoreactive for HMGA1 (**b**) and no HMGA2 immunoreactivity (**c**).

Discussion

According to the new WHO classification, synovial sarcomas can be subcategorized based on histological features into biphasic, monophasic spindle, purely glandular and poorly differentiated.[13] Biphasic synovial sarcomas are soft tissue tumors with distinct histological features, generally offering an instant ‘pattern recognition’ and posing no diagnostic difficulties. On the other hand, monophasic spindle synovial sarcoma and less common subtypes of synovial sarcoma[2,13] may show many overlapping histologic features with other sarcomas. The monophasic spindle synovial sarcoma may be histologically indistinguishable from MPNST. Immunohistochemistry sometimes fails to show epithelial marker (cytokeratins and EMA) immunoreactivity in monophasic spindle synovial sarcoma and EMA can be positive in MPNST. On the other hand, S100 immunostaining is typically focal in MPNST and can be found positive in up to 30% of synovial sarcomas.[4] Overall, synovial sarcomas have a disturbing degree of immunophenotypic overlap with MPNST. The use of a panel of antibodies

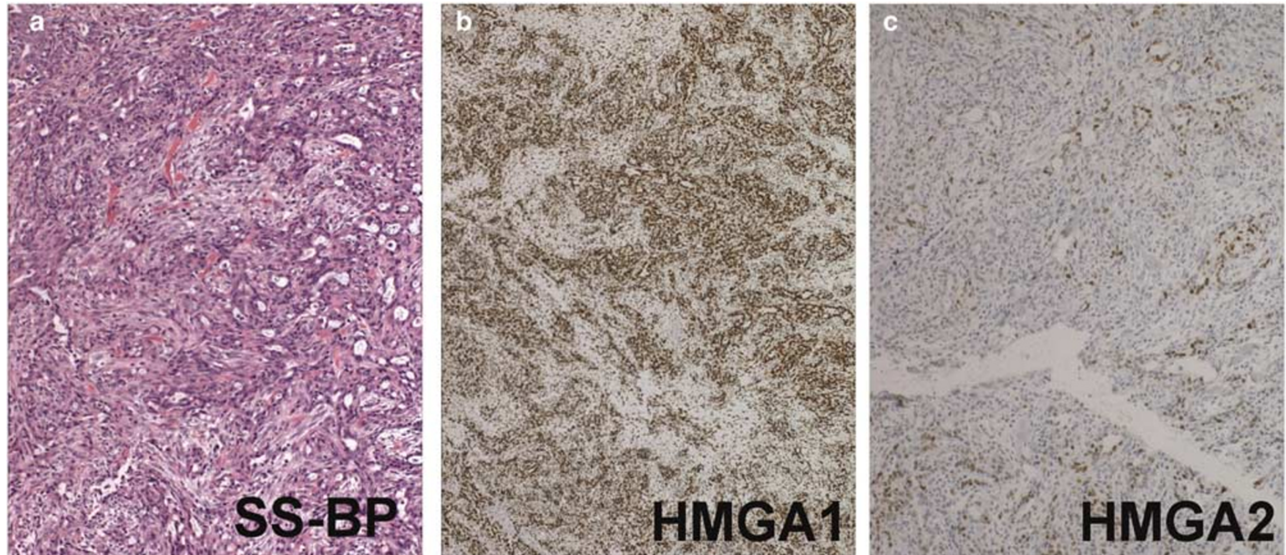

Figure 3 HMGA protein immunohistochemistry in biphasic synovial sarcoma (**a**) with HMGA1 immunoreactivity (**b**) and HMGA2-positive cells in its glandular components (**c**).

including both low and high molecular weight cytokeratin, EMA, type IV collagen, CD99, CD56, and S-100 protein has been suggested for the differential diagnosis.[5] However, identification of new immunohistochemical markers is still needed. In particular, there is the need for a marker, which positively identifies a tumor as MPNST in the differential diagnosis with monophasic spindle synovial sarcoma.

This study demonstrates that HMGA2 immunohistochemistry may be useful to separate MPNST from synovial sarcoma. Most MPNSTs (greater than 90%) were positive for both HMGA1 and HMGA2. Although 13 of 15 synovial sarcomas were positive for HMGA1, only two had an overall HMGA2-positive score. It is worth noting that significant HMGA2 immunoreactivity (cases with overall score positive) was seen only in one monophasic synovial sarcoma with epithelioid features and in the glandular component of a biphasic tumor. The absence of any significant HMGA2 protein expression in the 11 monophasic spindle synovial sarcomas predominantly composed of spindle cells indicates the potential of HMGA2 immunohistochemistry in separating synovial sarcoma from a MPNST. We do not know whether a MPNST with glandular differentiation also expresses HMGA2 in its epithelial component as all our cases were spindle cell MPNST with none exhibiting glandular differentiation. Restriction of HMGA2 immunoreactivity to the epithelial component of a biphasic synovial sarcoma should still be helpful to separate it from a MPNST with glandular differentiation, as both HMGA1 and HMGA2 are positive in the spindle cells of MPNST.

The HMGs belong to a class of low molecular weight, nonhistone, nuclear proteins.[9,10] They bind to DNA and function as transcription regulatory proteins by modulating the chromatin conformation.[17] Expression of the HMGA gene family is prominent during early stages of mammalian development, where they help regulate cell proliferation and differentiation. It has been known for some time that malignant transformation is associated with a marked increase in HMGA1 and HMGA2 expression. On the other hand, inactivation of HMGA2 gene prevents cell transformation in experimental models.[18] This is not totally unexpected considering the supposed role of HMGA proteins as transcription factors. HMGA1 and HMGA2 upregulation are best established in epithelial tumors and it has been associated with high tumor grade.[19] Interestingly, among our synovial sarcoma cases HMGA2 expression was virtually limited to the glandular elements of a biphasic tumor and to one case of monophasic synovial sarcoma with epithelioid features. HMGA1 was also positive in these two cases and in the additional two synovial sarcomas with epithelioid features. This may suggest a pattern of preferential HMGA1 and HMGA2 expression among cells with epithelial/epithelioid differentiation. We found no correlation between HMGA1 or HMGA2 expression and either cellular proliferation or tumor grade in synovial sarcoma (Table 2).

While HMGA2 proteins are required for the expression of many eukaryotic genes it has been observed that HMGA2 transactivates the leptin promoter, an adipose-specific gene and thus plays a critical role in adipocytic cell growth and differentiation. In fact, in transgenic mice models HMGA2 expression induces adiposity and lipomas[12] while HMGA2 gene inactivation induces hypoplasia of mesenchymal tissues with a specific deficit in the development of adipose tissue.[12] In humans,

HMGA1 and HMGA2 genes are mapped to chromosome 6p21 and 12q15, respectively. Chromosomal aberrations involving 12q15 and 6p21 leading to rearrangements of HMGA1 and HMGA2 genes have been observed in various benign mesenchymal tumors, such as lipoma, uterine leiomyoma, pulmonary chondroid hamartoma, salivary gland pleomorphic adenomas.[11] In addition, some locally aggressive or malignant mesenchymal tumors, including inflammatory myofibroblastic tumor, vulvar aggressive angiomyxoma, well-differentiated liposarcoma, osteosarcoma, have been recently reported to have HMGA2 gene alterations.[17]

The strong expression of HMGA proteins in MPNST suggests that they may influence the pathogenesis of MPNST. In fact, HMGA2 gene amplification has been shown in one MPNST[17] and aberrant expression of HMGA2 has been demonstrated in neuroblastoma tumors and their cell lines[20] suggesting that HMGA2 (and possibly HMGA1) may have a distinct role in the development of tumors of neuroectodermal derivation such as neuroblastoma and MPNST. The reason for the preferential expression of HMGA2 in the glandular or epithelioid components in synovial sarcoma is unclear and awaits further investigation.

Demonstration of the translocation t(X;18) by FISH or the fusion transcript, *SYT-SSX*, by RT-PCR is now considered an important diagnostic step to confirm the diagnosis of synovial sarcoma. Initial studies found that detection of the fusion gene using paraffin-embedded tissue source was highly sensitive and specific with no SYT-SSX detectable in mesenchymal tumors other than synovial sarcoma.[21,22] Although there were occasional unconventional findings,[23] recent larger series have confirmed the specificity of *STY-SSX* fusion gene for the diagnosis of synovial sarcoma.[7,8,13,14,24–26] In this report, we have used real-time RT-PCR to support and confirm the pathologic diagnosis of our cases and therefore the conclusions of our immunohistochemical study. In fact, the use of this approach has resulted in the reclassification of one MPNST as synovial sarcoma. Compared with conventional amplification methodology, real-time RT-PCR analysis offers a superior specificity by incorporating a specific internal probe and through the use of a closed laser detection system eliminates post-PCR manipulation, therefore, offering a contamination-free analysis.

In summary, we have shown that expression of HMGA2 is a feature of MPNST but not of synovial sarcoma. While the immunohistochemical diagnosis of synovial sarcoma is based on the positive identification of epithelial markers and of the aberrant SYT-SSX transcripts in the tumor, schwannian marker immunoreactivity in MPNST, when present, is usually focal. HMGA2 may be a much needed marker to positively identify a tumor as MPNST in the differential diagnosis with synovial sarcoma.

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