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SYT-SSX fusion is absent in sarcomatoid mesothelioma allowing its distinction from synovial sarcoma of the pleura

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The diagnosis of sarcomatoid mesothelioma is still a worldwide challenge and it is often difficult, both clinically and by morphological analysis, to differentiate sarcomatoid mesothelioma from synovial sarcoma, the most frequent intrathoracic sarcoma. To confirm the absence of the synovial sarcoma translocation t(X; 18) (SYT-SSX) in sarcomatoid mesothelioma, and to test its usefulness differentiating sarcomatoid mesothelioma from synovial sarcoma, 28 tumours were examined using the reverse transcriptase-polymerase chain reaction. RNA was extracted from paraffin blocks using standard methods, reverse-transcribed and PCR performed. Molecular analysis completed in two independent laboratories showed that sarcomatoid mesothelioma samples were negative for the t(X-18). This result confirms the usefulness of this analysis in differentiating sarcomatoid mesothelioma from synovial sarcoma.

The sarcomatoid variant of malignant mesothelioma accounts for about 11%[1] of all pleural mesotheliomas. The diagnosis of sarcomatoid mesothelioma is still a challenge, with recognisable pitfalls in its distinction from organising fibrous pleuritis, sarcomatoid carcinomas, primary and metastatic sarcomas. Distinguishing sarcomatoid mesothelioma from synovial sarcoma can be extremely difficult, and although synovial sarcoma is the most frequent primitive intrathoracic sarcoma, its rarity in the pleural location makes it an easily overlooked differential. The importance of making the correct diagnosis is further highlighted by the fact that synovial sarcoma is usually chemosensitive[2] while sarcomatoid mesothelioma is chemoresistant.[3,4] Classic biphasic synovial sarcoma is usually easy to recognize by light microscopy; however, most of the intrathoracic synovial sarcoma cases are monophasic histological subtypes with a predominance of monophasic fibrous cases creating the diagnostic challenge (Figure 1). As these tumours share the

same clinical presentation and as immunostains do not reliably differentiate between a sarcomatoid mesothelioma and monophasic spindle cell or poorly differentiated synovial sarcoma, additional tests are required. Both tumours typically express keratin, may express calretinin and are negative for glycoprotein markers. The advent of molecular techniques has increased both sensitivity and specificity of the diagnosis of synovial sarcoma by detecting the specific t(X; 18) translocation.[5] The demonstration of the translocation t(X; 18) in paraffin-embedded material using reverse transcription-polymerase chain reaction (RT-PCR) is now considered as a reliable and powerful tool for the diagnosis of synovial sarcoma owing to the high sensitivity and specificity of this method. The translocation results in the formation of a chimeric gene that encodes a transcription-activating protein.[6] This translocation leading to the fusion of SYT (at 18q11) with either SSX1 or SSX2 (two closely related genes at Xp11), or rarely SSX4 (also at Xp11), represents the most specific and sensitive tool for diagnosing a synovial sarcoma.[5,7,8] Bégueret et al[9] confirmed the presence of this translocation in 90% of purely sarcomatoid primary synovial sarcoma of the pleura. To verify the absence of the synovial sarcoma translocation in sarcomatoid mesothelioma,
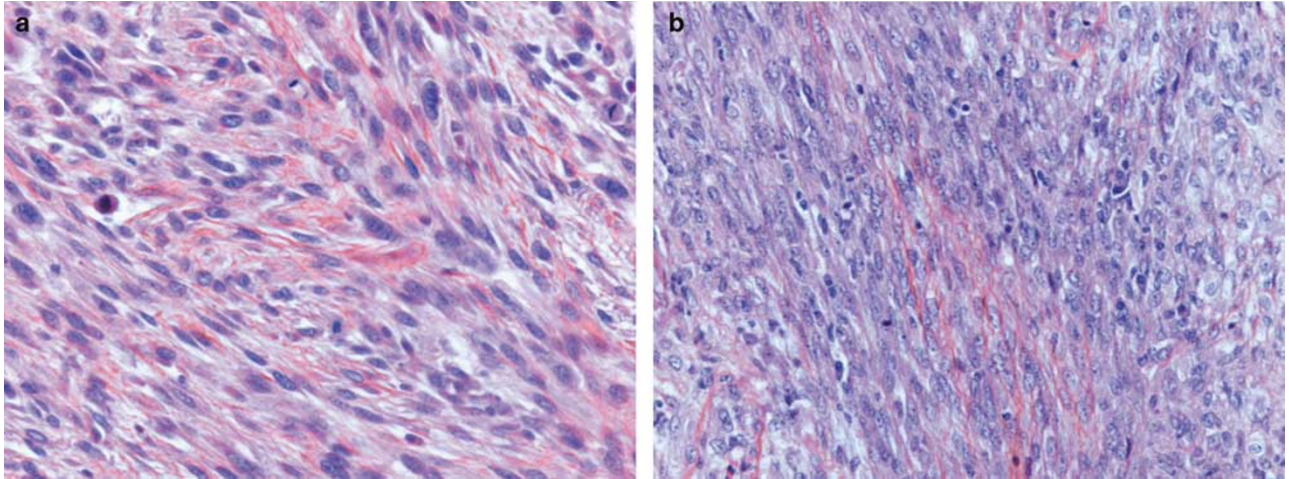
Figure 1 Pleural biopsies from patients **a** and **b**, presenting with diffuse pleural tumour, displayed very similar H&E pattern and immunohistochemical profile. Only the presence of *SYT-SSX* gene transcripts in sample tissue from patient **a** allowed to reliably identity the tumour as monophasic synovial sarcoma, whereas because of the absence from patient **b**'s tumour it was diagnosed as sarcomatoid mesothelioma.

28 tumour samples were examined, in two independent laboratories, using RT-PCR. In none of our cases of sarcomatoid mesothelioma was *SYT-SSX* fusion transcript detected.

Materials and methods

All sarcomatoid mesothelioma cases included in this study were validated according to the procedure of certification previously defined.[1] Briefly, each case included in the French National Mesothelioma Surveillance Program (PNSM) undergoes a standardized diagnostic confirmation procedure by a National group of pathologists who specialize in mesothelioma, the National Mesopath group. Three experts, blinded to asbestos exposure, classify each case as certain, uncertain, unclassifiable because of inadequate materials, or rule out in favour of another diagnosis, on the base of haematoxylin and eosin sections and immunohistochemical documents. When all three experts reach the same diagnosis, it is considered confirmed. Otherwise, the case is reviewed collectively in consensus meetings involving at least 10 experts and confirmed, ruled out, or referred for clinical expertise.

For this study 32 cases of sarcomatoid mesothelioma were identified from the files of the Mesopath group. There were two women and 30 men. All had a history of asbestos exposure and a highly suggestive clinical and CT-scan presentation for sarcomatoid mesothelioma. Three intrathoracic synovial sarcomas bearing the t(X; 18), used as positive control, were retrieved from the files of the Department of Pathology of Nancy University Hospital (France). All tissue samples had been fixed in 4% phosphate buffered-formalin. Histological typing was performed on haematoxylin and eosin sections using the WHO histological classification of lung and pleural tumours.[10]

The most frequently utilized monoclonal and polyclonal antibodies for immunohistochemical studies included: Calretinin (polyclonal, human recombinant Zymed, San Francisco, CA, USA), Cytokeratin 5/6 (clone D5/16B4, Dakocytomation, Glostrup, Denmark), cytokeratin AE1-AE3 (Dakocytomation), cytokeratin KL1 (Immunotech, Marseille, France), multicytokeratin (clone C11, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), epithelial membrane antigen (clone E29, Dakocytomation), CD34 (clone QBEND 10, Immunotech), smooth muscle actin alpha (clone alpha sm-1, Novocastra), desmin (clone D33, Dakocytomation), S100 protein (Dakocytomation), CD99 (clone M3601, Dakocytomation), bcl-2 (clone bcl-2/100/D5, Novocastra). Immunostaining was performed applying the biotin streptavidin method. Furthermore samples stained with multicytokeratin antibodies (AE1-AE3, KL1 and C11) and calretinin, additionally used a tyramin-biotin signal amplification procedure (Catalyzed Signal Amplification System, DakoCytomation), according to the manufacturer's instructions. Antigen retrieval was processed in citrate buffer (pH 6), using a pressure cooker. All steps were performed at room temperature, and diaminobenzidine was used as a chromogen. Appropriate positive and negative controls were used throughout.

RNA Extraction

Approximately 50 mg of tumour were removed from paraffin blocks. The tissue samples were deparaffinized by incubation for 5 min in 1 ml xylene at 65°C; this reaction was repeated to remove residual paraffin. After centrifugation, the tissue was washed with 1 ml of 100% ethanol, followed by 1 ml of 70% ethanol. After 5 min of vacuum drying, the pellet was suspended in 250 μl of lysis buffer (20 mmol/l Tris-HCl, pH 7.5; 20 mmol/l ethylenediaminetetra-

acetic acid; 1% sodium dodecyl sulfate; 350–500 μg proteinase K). After 16 to 48 h incubation at 55°C, 750 μl of Trizol-LS reagent (Gibco BRL, Gainthersburg, MD, USA) was added to the sample, and total RNA was extracted according to the manufacturer's instructions. The RNA pellet was dissolved in 40 μl of RNase-free water and stored at −80°C. The quantity of the isolated RNA was determined by absorbance at 260 nm and 280 nm.

RT-PCR Analysis

All samples were analysed in two independent laboratories using the same techniques (Institute of Pathology, Lausanne, Switzerland and Département de Pathologie, Nancy, France).

The RT-PCR amplification was performed with the Superscript One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μl containing 100–200 ng of total RNA, with 10 pM of each primer (2 pM of each β-actin primer). Reverse transcription was done at 50°C for 30 min. After inactivation of the reverse transcriptase at 95°C for 5 min, 40 cycles of PCR amplification were performed at 95°C for 30 s, 57°C for 45 s, and 72°C for 75 s. To check the quality of every mRNA sample, we always co-amplified a ubiquitously expressed control gene (β-actin). The primers were: *SYT*-A (5′-CAG CAGAGGCCTTATGGATATGA-3′), *SSX*-B (5′-TTTGT GGGCCAGATGCTTC-3′), β-actin-A (5′-AGGCCAAC CGCGAGAAGATGA-3′) and β-actin-B (5′-GCCGTG GTGGTGAAGCTGTAG-3′). The reaction products were subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. The size of PCR products was 97 bp for the *SYT-SSX* (*SYT-SSX1* or *SYT-SSX2*) fusion gene transcripts and 274 bp for the β-actin messenger RNA, which was used as an internal positive control. The RT-PCR procedure was performed at least twice for each sample.

For each PCR procedure, a classic t(X; 18)-positive biphasic synovial sarcoma was used as a positive control. Negative controls consisted of t(X; 18)-negative normal lung parenchyma, as well as distilled water devoid of template cDNA.

Results

Immunohistochemical Findings in the Sarcomatoid Mesothelioma Cases

All sarcomatoid mesothelioma tumour samples were variably positive for cytokeratin markers, either KL1 or AE1-AE3 (two cocktails containing antibodies to both high and low molecular-weight cytokeratins), with areas of strong staining admixed with areas of absent staining. It was also noted that, in 30% of cases, a reciprocal staining intensity pattern was observed—ie cases with strong staining for AE1-AE3 displayed weak staining for KL1 or the reverse profile. Using standard biotin streptavidin amplification procedures, 15 out of 32 samples
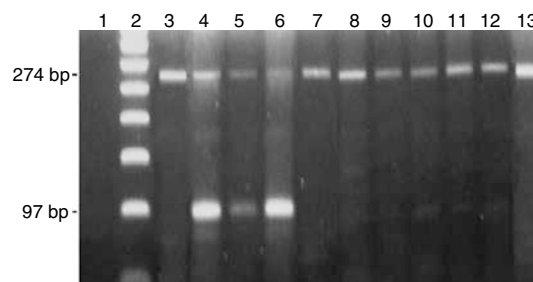
Figure 2 RT-PCR shows the absence of *SYT-SSX* transcripts in sarcomatoid mesotheliomas. Lane 1 (distilled water devoid of template cDNA) and 3 (normal lung parenchyma) are negative controls; lanes 4, 5, 6, synovial sarcoma positive controls and lanes 7 to 13 sarcomatoid mesotheliomas. PCR products are 97 bp for the *SYT-SSX* (*SYT-SSX1* or *SYT-SSX2*) fusion gene transcripts and 274 bp for the β-actin messenger RNA. Lanes 4 and 11 correspond respectively to tumours samples from patients a and b presented in Figure 1.

(54%) showed nuclear staining for calretinin, rarely diffuse (28%), mostly focal. Tyramin-biotin signal amplification showed a clear cut increase in the percentage of calretinin expressing cells, but none of the tumour samples negative for calretinin using standard biotin streptavidin amplification procedures were subsequently stained positively. Thirty-four percent of tumour samples stained positively for CK5/6, 28% for EMA, 20% for CD34 and 22% for Bcl2. All tumour samples were negative for smooth muscle actin, muscle-specific actin, desmin, CD99 and S100.

Molecular Genetic Findings

Interpretable results were obtained from 28 cases only (87.5%). All 28 sarcomatoid mesotheliomas were negative for the t(X; 18) (*SYT-SSX* translocation) in the presence of positive PCR internal controls (β-actin) (Figure 2). Four tumour samples from four patients (out of 32) showed negative results for both β-actin and *SYT-SSX* fusion gene transcripts and thus were not included in the analysis. Three classic synovial sarcoma cases used as positive controls showed the t(X; 18) translocation.

Discussion

Sarcomatoid mesotheliomas account for about 11%[1] of all pleural mesotheliomas, and typically consist of spindle cells arranged haphazardly or in fascicles. Their differential diagnosis includes benign and malignant processes such as organising fibrous pleuritis, sarcomatoid carcinomas, primary and metastatic sarcomas and more specifically synovial sarcoma.[11] Pleural metastases are a frequent event in the clinical course of soft tissue sarcomas, but primary intrathoracic sarcomas are rare. However, synovial sarcoma probably account as the most common of all primary intrathoracic sarcomas.[12] Examining a series of 40 intrathoracic synovial sarcomas, Bégueret *et al*[9] found that 27% involved

the pleura and 77.5% the pleura and lung. 97.5% of intrathoracic synovial sarcoma cases were of the monophasic histologic subtype, 60% being monophasic fibrous and 37.5% poorly differentiated with a high histologic grade, but epithelial differentiation was nevertheless detectable in 90% of them in the form of focal EMA and/or keratin reactivity. Because of its rarity and microscopic presentation in this location synovial sarcoma can be easily misdiagnosed as another type of tumour especially with sarcomatoid mesothelioma and their diagnosis most often requires molecular analysis (RT-PCR). Immunohistochemical staining is insufficient to definitively distinguish between these two malignancies. Indeed sarcomatoid mesothelioma and monophasic spindle cell or poorly differentiated synovial sarcoma show an overlap in their respective immunohistochemical spectrum with a variable staining for EMA, low and high molecular-weight keratin, calretinin, vimentin, S-100 protein and Bcl2, and with CD34 negativity. To date such immunohistochemical evaluation is not reliable in making the distinction between sarcomatoid mesothelioma and synovial sarcoma, especially when dealing with small specimens or poorly differentiated tumours.[11]

Additionally, clinical findings do not significantly help in differentiating the two entities as similar symptoms including pleural effusion, chest pain, cough, and haemothorax occur in both. Although the median age of patients presenting with synovial sarcoma is about 48 years *vs* 69 years for mesothelioma in Begueret's series,[9] 10% of patients with intrathoracic synovial sarcoma were over 70 years, thus making young age a non-reliable discriminant feature in individual cases.

At the time of diagnosis most of the cases of synovial sarcoma (80%) presented as circumscribed masses and truly localized mesotheliomas are quite uncommon;[13] however in 20% of cases both entities share a similar presentation.[9] Occupational exposure to asbestos does not definitively rule out the diagnosis of synovial sarcoma, as in France 65% of men over 65 years have a history of occupational exposure to asbestos.[14] As a consequence the true incidence of synovial sarcoma of the pleura is unknown, because only in the recent past has progress in molecular medicine provided the tools to reliably identify this tumour. However, there is growing evidence that synovial sarcoma may develop as a primary tumour in the pleura.[15–20] In this regard during the last five years (2001–2005), 3209 malignant mesotheliomas were certified by the French Mesothelioma panel (Mesopath group), 353 were sarcomatoid mesotheliomas (11%). Among sarcomatoid mesotheliomas, 54 were poorly differentiated, pleomorphic tumours (1.7% of all malignant mesotheliomas, 14% of sarcomatoid mesotheliomas). During the same period of time 58 primitive synovial sarcomas involving the pleura, all but one monophasic and all bearing the t(X; 18), considered as sarcomatoid mesotheliomas by the pathologists

responsible for the initial diagnosis, were addressed to the Mesopath group (about 2 cases of synovial sarcoma for 100 certified malignant mesotheliomas). Given the distinct strategies for clinical management of these tumour types, it is essential that pleural synovial sarcomas are not misdiagnosed as sarcomatoid mesotheliomas or sarcomatoid carcinomas. Although more aggressive than their soft tissue counterparts synovial sarcomas are less aggressive than sarcomatoid mesotheliomas with a 50 months median survival for synovial sarcoma[9] *vs* 9 months for sarcomatoid mesothelioma (Mesopath group data). As already detailed, clinical findings, histology and immunohistochemical features are insufficient to definitively distinguish between these two malignancies, detection of the t(X; 18) translocation using RT-PCR is considered as the most powerful test to make this distinction. Despite past contradictory reports,[21–23] the presence of *SYT-SSX* fusion transcripts is now considered as a diagnostic signature of synovial sarcoma.[24–27] Guillou *et al*[24] reported a prospective study of 211 mesenchymal tumours fixed in different fixatives, in which there were 135 non-synovial sarcoma tumours, 22 biphasic and 64 monophasic spindle/round cell synovial sarcomas. *SYT-SSX* gene fusion transcripts were detected in the synovial sarcoma tumour category only (100% specificity) with an overall sensitivity of 96%. This sensitivity level is in keeping with results reported in other series by Hiraga (100%)[26] and Bijwaard (96.5%).[27] Malignant mesothelioma karyotypic analysis studies performed during the past several decades failed to identify a specific karyotypic change that may prove to be of diagnostic value and showed the complexity of the karyotypes in this malignancy. Most malignant mesotheliomas display multiple clonal chromosomal abnormalities.[28–29] However a number of recurrent anomalies have been found. These include deletions at multiple sites or loss of one entire copy for chromosomes 4 and 22, polysomy for chromosomes 5, 7, 20, and losses at 1p21-2, and 3p21, 6q15-21, 9p21-22, 15q11.1-q15 and 22q12. Comparative genomic hybridisation studies confirmed the karyotype findings and identified even more chromosomal losses and gains than either conventional cytogenetics or FISH.[30] None of the significant genetic changes reported concern chromosome X or 18.

Thus because of its rarity and microscopic presentation in the thorax, synovial sarcoma can be easily misdiagnosed as another type of tumour especially with sarcomatoid mesothelioma. Taking in consideration the importance of differentiating sarcomatoid mesothelioma from synovial sarcoma in terms of treatment, survival and medico legal compensation, we looked for the t(X; 18) translocation in a series of optimally validated sarcomatoid mesotheliomas. All our samples were processed in duplicate in two independent laboratories with similar results obtained in both, contributing to demonstrate the specificity, sensibility and reprodu-

cibility of RT-PCR technnique. Therefore as none of the sarcomatoid mesotheliomas displayed the t(X; 18), in the face of appropriate positive and negative controls, we can state that absence of the translocation when dealing with this differential strongly supports the diagnosis of sarcomatoid mesothelioma.

Acknowledgement

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