Malignant Peripheral Nerve Sheath Tumors are t(X;18)-Negative Sarcomas. Molecular Analysis of 25 Cases Occurring in Neurofibromatosis Type 1 Patients, Using Two Different RT-PCR-Based Methods of Detection

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To verify the absence of the synovial sarcoma translocation t(X;18) (SYT-SSX) in malignant peripheral nerve sheath tumors, 34 tumor samples from 25 neurofibromatosis type 1 patients were examined in two independent laboratories (Bordeaux, France, and Lausanne, Switzerland) using reverse transcriptase polymerase chain reaction (RT-PCR)-based techniques. RNA was extracted from paraffin blocks using standard methods, reverse transcribed, and conventional (in one laboratory) *versus* real-time (in the other laboratory) PCR performed. Twenty-seven tumor samples from 19 patients were negative for the t(X;18) in both laboratories; six additional tumors that were t(X;18)-negative in one laboratory gave noninterpretable results in the other, due to lack of internal positive controls; one case was noninterpretable in both places. In conclusion, malignant peripheral nerve sheath tumors in neurofibromatosis type 1 patients do not bear the synovial sarcoma t(X;18) (SYT-SSX). Laboratories that use PCR-based techniques for diagnostic purposes would benefit from quality assurance programs.

KEY WORDS: Malignant peripheral nerve sheath tumor, Neurofibromatosis type 1, RT-PCR, t(X;18).
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Most synovial sarcomas bear the translocation t(X;18), which may be detected in formalin-fixed, paraffin-embedded material by reverse transcriptase-polymerase chain reaction (RT-PCR). This technique is particularly useful for consultation cases for which paraffin-embedded material only is available.

Several large series have shown that this method is highly sensitive and specific, with virtually no t(X;18) (SYT-SSX) detected in mesenchymal tumors other than synovial sarcomas (1–4). This specificity was recently challenged by O'Sullivan *et al.* (5). These authors detected SYT-SSX fusion gene transcripts not only in synovial sarcomas but also in 15 of 20 malignant peripheral nerve sheath tumors (MPNSTs) as well as in several other types of benign and malignant mesenchymal tumors, thus raising major concerns and controversies regarding t(X;18) specificity. In a letter to the Editor that strongly disagreed with O'Sullivan *et al.*, Ladanyi *et al.* (6) regretted that the authors did not attempt to confirm their results using alternative techniques such as Southern blotting, conventional cytogenetics, and/or *in situ* hybridization. In addition, they reported 145 cases of MPNST that always proved to be t(X;18)-negative in several different laboratories using different techniques, a finding that would strongly support their view.

In this article, we report our experience with 34 cases of MPNST that occurred in 25 neurofibromatosis type 1 (NF1) patients. In none of our cases was the t(X;18) (SYT-SSX) detected using two different PCR-based techniques performed in two different laboratories.

MATERIALS AND METHODS

Cases of MPNST occurring exclusively in NF1 patients were retrieved from the files of the Laboratory of Pathology, Institut Bergonié, Bordeaux, France, and the Institute of Pathology, Lausanne, Switzerland. Thirty-four samples from 25 patients were selected. One paraffin block of tumor, at least, was available for molecular testing. Twenty-six

samples were fixed in phosphate-buffered formalin and eight in the Holland Bouin fluid (a mixture consisting of water, 9% formaldehyde, 1.35% acetic acid, 3.6g/100 mL picric acid, and 2.25g/100 mL copper acetate [Verbièse, Wasquehal, France]).

In one laboratory (Bordeaux, France), 20 10-μm-thick sections were obtained by cutting paraffin blocks with a microtome. To eliminate any contamination of one case by material from another, the microtome knife was carefully cleaned with absolute alcohol before cutting. In the other laboratory (Lausanne, Switzerland), approximately 50 mg of tumor were removed from paraffin blocks by scraping with a sterile and disposable scalpel. Specific attention was paid to eliminate potential contamination.

RNA extraction protocol was the same in both laboratories (4, 7). Briefly, tissue samples were deparaffinized twice in xylene. After centrifugation, the tissue was washed in ethanol, and the pellet was resuspended with 500 μL of ATL buffer (Qiagen) added with proteinase K and incubated 16 to 48 hours at 60°C. RNA was extracted according to the method of Chomzynski and Sacchi (8) using 1.5 mL of Trizol-LS reagent (Gibco BKL) for 500 μl of cellular lysate. The RNA pellet was resuspended in 50 μl of RNase-free water and stored at −80°C.

Protocol for reverse transcription was the same in Bordeaux and Lausanne (4, 7). Reverse transcription of 5 μg RNA was performed in a total volume of 20 μL with 50 mM Tris-HCl, pH 8,3, 40 mM KCl, 5 mM MgCl2, 0,5% Tween, 0,5 mM dNTP Mix, 10 mM dithiothreitol, specific reverse primer (65 ng SSX-B or 50 ng GAPDH in Bordeaux and 15 ng of β-actin-B in Lausanne), 12 U RNAse inhibitor (Promega), 10U Expand Reverse Transcriptase (Roche Diagnostics). Samples were incubated at 42°C for 1 hour, then at 95°C for 5 minutes.

A real time PCR technique was used in one laboratory (Bordeaux, France) (7), and a "conventional" technique was used in the other laboratory (Lausanne, Switzerland) (4). The real-time PCR was carried out using the ABI/PRISM 5700 (Applied Biosystems). PCR reaction was performed in triplicate in a total volume of 50 μL with PCR Core Reagent kit (Applied Biosystems) consisting of 4 mM MgCl2, 0.4 mM of dATP, dCTP, dGTP and 0,8 mM of dUTP, 1,5 U *Taq* Gold polymerase, 0,5 U Amperase UNG, 4 μL of the cDNA reaction, 65 ng forward primer, 65 ng reverse primer and 5 pmole probe. Thermal cycling conditions were 2 minutes at 50°C for amperase activation, 10 minutes at 95°C for *Taq* polymerase activation then 50 cycles of 3 PCR steps consisting of 30 seconds at 95°C, 45 seconds at 63°C and 75 seconds at 72°C. GAPDH transcript expression has been studied as a housekeeping gene to control the RNA ability to be reverse transcribed and PCR amplified for all the tumors.

For the conventional PCR method, the amplification reactions (20 μL) were performed, in presence of 2 μL of the cDNA reaction, with an initial incubation step at 95°C for 5 minutes. The amplification profile of the PCR consisted of 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 75 seconds. For each sample, two PCR amplifications were performed in parallel: one contained only the primer set necessary for the amplification of the SYT-SSX fusion gene transcripts, whereas in the second a set of primers at a lower concentration (1 pmole in place of 10 pmoles for the SYT-SSX fusion gene in each 20 μL PCR reaction) for the ubiquitously expressed β-actin gene transcripts were also added. The reaction products were subjected to electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. The RT-PCR procedure was performed at least twice for each sample. For both methods, PCR amplifications were performed with the following primers: STY-A (5'-CAGCAGAGGCCTTATGGATATGA-3'), SSX-B (5'-TTTGTGGGCCAGATG). For PCR amplification of GAPDH, the following primers were used: GAPDH-A (5' CCA CAT CGC TCA GAC ACC AT 3'), GAPDH-B (5' CCA GGC GCC CAA TAC G 3') and for β-actin; β-actin-A (5'-AGGCCAACCGCGAGAAGATGA-3') and β-actin-B (5' GCCGTGGTGGTGAAGCTGTAG-3'). For the real-time PCR method, the following probes were used (9): Syno S1 probe (5' *FAM*-ATC ATG CCC AAG AAG CCA GCA GAG G 3') and GAPDH probe (5' *FAM*-AAG GTG AAG GTC GGA GTC AAC GGA TTT G 3').

RESULTS (see Table 1)

Clinicopathologic Data

There were 17 males and eight females, whose ages ranged from 14 to 79 years (mean: 42 years). All patients had clinical features of neurofibromatosis type 1, and for some of them, a familial history was also on records. Tumors were located in lower extremity ($n = 4$), upper extremity ($n = 3$), neck ($n = 3$), trunk wall ($n = 6$), inner trunk ($n = 7$), and head ($n = 2$). From these patients, 34 tumor samples were analyzed, including 20 primary tumors, 12 local recurrences, and two distant metastases.

Histologically, four samples were malignant neurofibromas with an immunopositivity for S100 protein, 22 samples were conventional MPNST with S100 protein reactivity for 17 of them, and eight samples were undifferentiated spindle and/or pleomorphic cell tumors, of which four were S100-positive.

Molecular Findings

Molecular analysis performed in one laboratory (Bordeaux) showed that 30 samples from 21 pa-

TABLE 1. Clinicopathologic Data and SYT-SSX Fusion Transcript Status in 25 Neurofibromatosis Type 1 Patients with MPNST in NF1

Case	Sex/Age	Site	Event (*)	Histology (**)	S100 Protein	Fixative (***)	PCR Bordeaux (****)	PCR Lausanne (****)
1	F/68	Forearm	P	Malignant NF	+Diffuse	Formalin	—	—
			R	Malignant NF	+Focal	Formalin	—	—
			R	Malignant NF	+Diffuse	Formalin	—	—
			R	Conventional	+Focal	Formalin	—	—
2	F/79	Parotid	R	Conventional	+Diffuse	Formalin	—	—
			R	Conventional	+Diffuse	Formalin	NI	—
3	F/41	Paravertebral	P	Conventional	—	Formalin	—	—
			R	Undifferentiated	—	Formalin	—	—
4	M/57	Mediastinum	R	Conventional	—	Formalin	—	—
			R	Conventional	—	Formalin	—	—
5	M/51	Thoracic wall	R	Conventional	+Focal	Formalin	—	—
			M	Conventional	+Diffuse	Formalin	—	—
			M	Conventional	+Diffuse	Formalin	—	—
6	M/33	Iliac fossa	R	Undifferentiated	—	Formalin	—	—
7	F/16	Axillary area	P	Conventional	+Focal	Formalin	—	—
			R	Conventional	—	Formalin	—	—
8	F/47	L4 nerve root	P	Conventional	+Focal	Formalin	—	—
9	M/72	Thoracic wall	R	Conventional	+Diffuse	Formalin	—	—
10	M/63	Thoracic wall	P	Conventional	+Focal	Formalin	—	—
11	M/44	Psoas	P	Conventional	+Focal	Formalin	—	—
12	M/29	Paravertebral	P	Conventional	+Focal	Formalin	NI	—
13	M/26	Buttock	P	Conventional	+Focal	Formalin	—	—
14	F/18	Peritoneum	P	Undifferentiated	+Focal	HB	NI	NI
15	F/69	Leg	P	Undifferentiated	—	HB	—	—
16	M/41	Knee	P	Undifferentiated	+Focal	HB	—	—
17	M/20	Leg	P	Undifferentiated	+Focal	Formalin	NI	—
18	M/21	Thoracic wall	P	Undifferentiated	+Focal	HB	—	NI
19	M/20	Neck	P	Undifferentiated	—	Formalin	—	—
20	M/59	Elbow	P	Conventional	+Diffuse	Formalin	—	—
21	M/37	Trunk wall	P	Conventional	+Focal	HB	—	—
22	M/24	Neck	P	Conventional	—	HB	—	NI
23	F/48	Forearm	P	Malignant NF	+Diffuse	HB	—	—
24	M/56	Scalp	P	Conventional	+Focal	HB	—	—
25	M/14	Neck	P	Conventional	+Focal	HB	—	NI

(*) P, primary; R, local recurrence; M, metastasis.

(**) NF, neurofibroma; Conventional, conventional MPNST; Undifferentiated, undifferentiated spindle cell/pleomorphic sarcoma.

(***) HB, Holland Bouin fluid.

(****) PCR was considered negative (−) when SYT-SSX fusion gene transcripts were not detected in the presence of positive PCR internal controls (GAPDH or β-actin). Results were considered noninterpretable (NI) when neither SYT-SSX fusion gene transcripts nor PCR internal controls were detectable.

tients were negative for the t(X;18) (SYT-SSX) in the presence of positive PCR (GAPDH) internal controls (Ct value ranged from 21 to 37, mean: 27). Four tumor samples from four patients (Cases 2, 12, 14, and 17) showed negative results for both SYT-SSX fusion gene transcripts and GAPDH and, thus, were considered noninterpretable. For one of these patients (Case 2) a sample taken from another tumoral event was also negative for the t(X;18) with positive GAPDH PCR internal controls. In the other laboratory (Lausanne, Switzerland), 30 tumor samples from 21 patients were negative for the t(X;18) and positive for β-actin (internal control), whereas four samples from four patients (Cases 14, 18, 22, and 25) were noninterpretable (*i.e.*, lack of amplification of both β-actin and SYT-SSX sequences). Combining the results from the two different laboratories regarding the same tumor population, it appeared that 27 tumor samples from 19 patients were t(X;18)-negative with positive GAPDH/β-actin internal controls, and one sample (Case 14) was noninterpretable due to negative internal controls in both laboratories. Six additional samples from six different patients (Cases 2, 12, 17, 18, 22, and 25) were considered noninterpretable due to negative PCR internal controls obtained in one of the two laboratories (three cases in Bordeaux and three cases in Lausanne).

DISCUSSION

Diagnosing synovial sarcomas may be difficult, and this tumor may be easily confused with a large number of other benign and malignant spindle cell tumors. The demonstration of the translocation t(X;18) (SYT-SSX) in paraffin-embedded material using RT-PCR is now considered a useful and powerful adjunct for the diagnosis of synovial sarcoma due to the high sensitivity and specificity of this method. In a recent study (4) on 250 paraffin-embedded soft-tissue lesions, we obtained PCR products for

221 tumors. t(X;18) (SYT-SSX) fusion gene transcripts were observed in 83 of 86 synovial sarcomas (96% sensitivity) and never detected in any of the 135 tumors of other type that had been examined (100% specificity). Using the same technique on the same kind of material, O'Sullivan *et al.* (5) found (SYT-SSX) fusion gene transcripts in 29 of 34 (85%) synovial sarcomas, but also in 15 of 20 (75%) MPN-STs, 1 of 4 adult fibrosarcomas, 1 of 10 malignant fibrous histiocytomas, 1 of 7 congenital-infantile fibrosarcomas, and 2 of 3 neurofibromas. Based on these results, the authors concluded that the translocation t(X;18) is not specific for synovial sarcoma and, thus, should be used with great caution in the differential diagnosis of spindle cell tumors.

In a subsequent article on "gold standard" methods for diagnosing soft-tissue tumors (10), the same authors assumed that "molecular genetic testing is an ancillary technique comparable to electron microscopy and immunohistochemistry." Unfortunately, O'Sullivan *et al.* (5) did not try to confirm their unexpected and provocative results with techniques other than RT-PCR such as *in situ* hybridization, which could have been applied on paraffin-embedded material as well. They did not ask another independent laboratory to verify their results on the same samples either. In a letter to the Editor, Ladanyi *et al.* (6) raised the possibility of contamination during the block cutting procedure or PCR technique based on the fact that none of the 145 cases of MPNST molecularly examined heretofore in different laboratories using different techniques (*i.e.*, RT-PCR, conventional cytogenetics, and FISH) proved to be t(X;18) (SYT-SSX)-positive.

In the current report, we tested 34 samples from 25 cases of MPNST in NF1 patients in two independent laboratories using conventional and real-time PCRs. None of MPNSTs were t(X;18) (SYT-SSX)-positive, regardless of the laboratory involved and the PCR techniques used. PCR is a very sensitive technique. As a consequence, a false-positive result can easily be obtained by means of tissue contamination, even if minimal (*i.e.*, microscopic). The way the tissue is collected from paraffin blocks before being submitted to PCR is crucial. In our opinion, disposable material should be used for each case

and PCR-based techniques, especially when they are used for diagnostic purposes, should benefit from quality assurance programs. Along the same line, it should ideally always be possible to verify results from one laboratory by another independent laboratory, using the same material but not necessarily the same technique, as shown in the current study. Real-time PCR, which appeals to strict automation techniques (9), also could improve the quality of the results by decreasing the risk of contamination.

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