Solitary fibrous tumors: loss of chimeric protein expression and genomic instability mark dedifferentiation

Gian P Dagrada¹, Rosalin D Spagnuolo¹, Valentina Mauro^{1,6}, Elena Tamborini², Luca Cesana², Alessandro Gronchi³, Silvia Stacchiotti⁴, Marco A Pierotti^{5,7}, Tiziana Negri^{1,8} and Silvana Pilotti^{1,8}

¹Laboratory of Experimental Molecular Pathology, Department of Diagnostic Pathology and Laboratory, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; ²Department of Diagnostic Pathology and Laboratory, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; ³Department of Surgery, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; ⁴Adult Mesenchymal Tumor Medical Oncology Unit, Cancer Medicine Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy and ⁵Scientific Directorate, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Solitary fibrous tumors, which are characterized by their broad morphological spectrum and unpredictable behavior, are rare mesenchymal neoplasias that are currently divided into three main variants that have the *NAB2-STAT6* gene fusion as their unifying molecular lesion: usual, malignant and dedifferentiated solitary fibrous tumors. The aims of this study were to validate molecular and immunohistochemical/biochemical approaches to diagnose the range of solitary fibrous tumors by focusing on the dedifferentiated variant, and to reveal the genetic events associated with dedifferentiation by integrating the findings of array comparative genomic hybridization. We studied 29 usual, malignant and dedifferentiated solitary fibrous tumors from 24 patients (including paired samples from five patients whose tumors progressed to the dedifferentiated form) by means of STAT6 immunohistochemistry and (when frozen material was available) reverse-transcriptase polymerase chain reaction and biochemistry. In addition, the array comparative genomic hybridization findings were used to profile 12 tumors from nine patients. The *NAB2/STAT6* fusion was detected in all of the tumors, but immunohistochemistry and western blotting indicated that chimeric protein expression was atypical or absent in 9 out of 11 dedifferentiated tumors. The comparative genomic hybridization results revealed that the usual and malignant solitary fibrous tumors had a simple profile, whereas the genome of the dedifferentiated tumors was complex and unstable, and suggested that 13q and 17p deletions and *TP53* mutations may be present in malignant lesions before the full expression of a dedifferentiated phenotype. Solitary fibrous tumor dedifferentiation is associated with the loss of chimeric oncoprotein expression, genomic instability, and cell decommitment and reprogramming. The assessment of dedifferentiated solitary fibrous tumors is based on the presence of the fusion transcripts and, in principle, negative STAT6 immunohistochemistry should not rule out a diagnosis of solitary fibrous tumor.

Modern Pathology (2015) 28, 1074–1083; doi:10.1038/modpathol.2015.70; published online 29 May 2015

Correspondence: Dr S Pilotti, MD, Laboratory of Experimental Molecular Pathology, Department of Diagnostic Pathology and Laboratory, Fondazione IRCCS Istituto Nazionale dei Tumori, Via G. Venezian 1, Milano 20133, Italy.

E-mail: silvana.pilotti@istitutotumori.mi.it

⁶Present address: Unit of Leukocyte Biology, IRCCS Ospedale San Raffaele, Fondazione del Monte Tabor, Milan, Italy.

⁷Present address: Nerviano Medical Science, Nerviano, Milan, Italy.

⁸Senior co-authors.

Received 13 January 2015; accepted 25 March 2015; published online 29 May 2015

Solitary fibrous tumors are rare mesenchymal tumors (incidence <1 per million) that were originally described on the pleurae but have subsequently been recognized at virtually all body sites.¹ The current classification includes usual, malignant and the recently described dedifferentiated variants.^{1–3} The morphobiological features distinguishing the malignant and usual variants are hypercellularity, cell pleomorphism, necrosis and a mitotic index of >4/10 high-power fields. However,

there is no strict correlation between morphobiological characteristics of a tumor and its clinical course as *bona fide* histologically benign tumors (the usual variant) can occasionally recur and metastasise after a very-long time (>10 years).⁴ The rare dedifferentiated variant is characterized by the appearance of a high-grade component mimicking a pleomorphic/spindle cell sarcoma, small-cell sarcoma and other entities that have no morphological resemblance to solitary fibrous tumors.^{2,3} This unusual component may be present at the same time as a primary malignant or usual solitary fibrous tumor, or appear often many years later during subsequent recurrences.

Until recently, there was no known, diagnostically relevant, recurrent cytogenetic/molecular alteration and immunohistochemistry was of little help insofar as solitary fibrous tumors have a relatively unspecific profile characterized by the co-expression of CD34, CD99, and BCL2. The fact that the dedifferentiated variant lacks even this immunophenotypical signature makes its diagnosis particularly challenging because it entirely relies on the presence of a usual/malignant component within the tumor or (in the case of late-recurring pure dedifferentiated tumors) the documentation of a previous solitary fibrous tumor. However, in 2013, whole exome and transcriptome sequencing-based studies^{5,6} revealed a chromosomal rearrangement leading to a *NAB2/STAT6* gene fusion that has now been recognized as the hallmark of solitary fibrous tumors on the basis of gene-specific reverse-transcription polymerase chain reaction analyses of solitary fibrous tumor series including usual and malignant variants.^{5–8}

NAB2 and *STAT6* are contiguous and partially overlapping genes with opposite transcription orientations on chromosome 12q13.3. *NAB2* is a nuclear protein that acts as a repressor of the transcription induced by some members of the early growth response (EGR) family of transactivators (particularly EGR1) and mediated by interactions with the nucleosome remodeling and deacetylase complex.⁹ Like other members of the STAT family of transcription factors, when phosphorylated by receptor-associated kinases, STAT6 translocates to the cell nucleus where it acts as transcriptional activator and has a central role in IL4-mediated biological responses. The rearrangement found in solitary fibrous tumor consists of a cytogenetically undetectable intrachromosomal inversion that causes the *NAB2* C-terminal transcriptional repression domain to be substituted by the *STAT6* transcriptional activation domain, thus deregulating the expression of a set of EGR1 targets. Cell line transformation experiments have shown that the *NAB2/STAT6* chimera stimulates proliferation in a EGR1-dependent manner.⁶ *NAB2/STAT6* mRNA chimeras are highly heterogeneous and have an unusually large number of fusion variants. The fusion types *NAB2* exon 6/*STAT6* exon 17 and *NAB2* exon 6/*STAT6* exon 18 have recently been associated with malignant

behavior, an extrapleural location and a younger age at onset,⁸ although this association has not been observed in other studies.^{6,7}

The discovery of the *NAB2/STAT6* fusion made it possible to diagnose solitary fibrous tumor molecularly using reverse-transcription polymerase chain reaction; moreover, the overexpression and phosphorylation-independent nuclear translocation of the STAT6 C-terminus can be readily identified by means of STAT6 immunohistochemistry, a sensitive, specific and more widely available means of accurate diagnosis.^{6,10,11} However, although the presence of the *NAB2/STAT6* rearrangement is pathognomonic for solitary fibrous tumors, it does not provide any known insights into the biological basis of the spectrum, prognosis or progression of the tumors. Furthermore, to the best of our knowledge, a solitary fibrous tumor diagnosis has been molecularly confirmed by reverse-transcription polymerase chain reaction in only two dedifferentiated cases.^{12,13}

The aims of this study were to validate the newly available reverse-transcription polymerase chain reaction, immunohistochemistry and biochemical analyses as methods of diagnosis in a series of tumor samples representing all of the solitary fibrous tumor variants, and to investigate the genetics of the morphopathological changes paralleling the evolution of solitary fibrous tumor, with particular reference to dedifferentiation.

Materials and methods

The case material consisted of specimens of all of the available cases of dedifferentiated solitary fibrous tumors diagnosed and treated between 2003 and 2013, together with frozen samples of two small series of usual and malignant solitary fibrous tumors surgically treated in the same period. As our institute is a referral center for patients with rare sarcomas, solitary fibrous tumors with an extrapleural location and malignant features are overrepresented. The study was approved by the Independent Ethics Committee of the Fondazione IRCCS Istituto Nazionale dei Tumori di Milano.

Patients and Primary Tumors

As summarized in Table 1, the case material consisted of 29 tumors from 24 patients: 11 males and 13 females whose median age at the time of disease onset was 51 years, range 23–76. Histology/immunohistochemistry at time of onset classified 6 patients (nos. 1–6) as having usual, 8 as having malignant (nos. 7–14), and 10 as having dedifferentiated solitary fibrous tumors (nos. 15–24).

Dedifferentiated Solitary Fibrous Tumors

Of the ten patients with dedifferentiated tumors, six (nos. 15–19 and 24) had dedifferentiated tumors at

Table 1 Overview of patients, tumor samples and results

Case number	Gender, age	Primary tumor site and histology	Samples under analysis by extent of disease (P, R, Mt), elapsed time, site and histology	PCR	IHC			STAT6 WB	aCGH G/L/B.P.	TP53 mut.
					STAT6	CD34	P53			
1	M, 53	Pelvis, U	P	ex4.ex3	pos	pos	< 50%low	pos	0/0/0	n.d.
2	M, 23	Rpn, U	P	ex6.ex18	pos	pos	neg	n.i.	0/0/0	n.d.
3	F, 55	Rpn, U	P	ex6.INT6.ex19 int	pos	pos	neg	n.i.	n.d.	n.d.
4	F, 46	Popliteus, U	P	ex6int.ex19int	pos	pos	< 50%low	n.i.	n.d.	n.d.
5	F, 63	Rpn, U	P	ex6.ex18	pos	pos	< 50%low	n.i.	n.d.	n.d.
6	F, 51	Thigh, U	P	ex6-INT6.ex4	pos	pos	< 50%low	pos	0/0/0	n.d.
7	M, 37	Meninges, M	Mt, (1 year), Lung, M	ex6.ex18	pos	pos	< 50%low	n.i.	n.d.	n.d.
8	M, 72	Abdomen, M	P	ex4.ex1b	pos	pos	< 50%low	pos	5/1/0	n.d.
9	F, 76	Psoas, M	Mt, (1 year), Rpn, M	ex4.ex3	pos	pos	< 50%low	pos	0/0/0	n.d.
10	F, 71	Thigh, M	P	ex6-INT6.ex4	pos	pos	< 50%low	pos	0/0/0	n.d.
11	M, 63	Rpn, M	R, (5 years), M	ex6.ex18	pos	pos	< 50%low	n.i.	n.d.	n.d.
12	F, 34	Pelvis, M	R, (4 years), M	ex6-ex18	pos	pos	< 50%low	n.i.	n.d.	n.d.
13	F, 53	Ileum, M	P	ex5.(GG)ex1b	pos	pos	< 50%low	pos	n.d.	n.d.
14	M, 36	Cheek, M	R, (6 years), M	ex6.ex17	pos	pos	< 50%low	n.i.	n.d.	n.d.
15	M, 59	Rpn, DD	P	ex6.ex17	P.W.	neg	> 50%pos	n.i.	n.d.	WT
16	F, 43	Meninges, DD	Mt, (9 years), orbit, DD	ex6.ex17	P.W.	P.W.	neg	n.i.	n.d.	WT
17	M, 44	Rpn, DD	R, (7 years), DD	ex6.ex18	pos	P.W.	> 50%pos	n.i.	n.d.	R273K
18 (5)	F, 55	Pleurae, DD	P ^a	n.d. FFPE only	pos	pos	n.d.	n.d.	n.d.	n.d.
19(4)	F, 47	Rpn, DD	R, (4 years), DD ^a	n.d. FFPE only	P.W.	neg	< 50%low	n.d.	n.d.	n.d.
20a (8)	F, 46	Rpn, U	P ^a	n.d. FFPE only	pos	pos	< 50%low	n.d.	n.d.	n.d.
20b (8)			Mt, (14 years), Liver, DD ^a	n.d. FFPE only	P.W.	P.W.	< 50%low	n.d.	n.d.	n.d.
21a(6)	M, 53	Pleurae, M	P ^a	n.d. FFPE only	pos	pos	> 50%pos	n.d.	n.d.	n.d.
21 b(6)			Mt, (1 year), Lung, DD	ex4.ex1b	neg	neg	> 50%pos	faint pos	n.d.	G244A
22a (1)	F, 49	Pelvis, M	P	ex6.ex18	pos	pos	> 50%pos	n.i.	0/2/4	G245S
22b (1)			R, (3 years), DD	ex6.ex18	neg	neg	> 50%pos	n.i.	3/7/13	G245S
23a(7)	M, 45	Pelvis, M	P	ex6.INT6.ex3int	pos	pos	< 50%low	pos	0/0/0	WT
23b (7)			R, (6 years), DD	ex6.INT6.ex3int	neg	neg	neg	neg	5/20/36	WT
24a	M, 46	Bladder, DD	Mt, (1 year), Lung, DD	ex6.ex17	P.W.	P.W.	neg	n.i.	9/5/21	WT
24b			Mt, (2 years), Lung,DD	ex6.ex17	P.W.	P.W.	neg	n.i.	9/4/19	WT

Abbreviations: DD, dedifferentiated; FFPE, formalin-fixed paraffin embedded; M, malignant; Mt, metastasis; n.d.: not done; n.i.: not informative (cases with undetectable protein chimeras by WB. See text); P, primary; P.W.: patchwork; R, local recurrence; Rpn, retroperitoneum; U, usual; WT, wild type TP53.

Case numbers: the original identifications of the previously published cases ³ are shown in brackets.

^aReferral cases. The time between the primary lesion and recurrences and metastases is shown in brackets. INT6: inserted sequence from *NAB2* intron 6. int: the breakpoints internal to the specified exon. The ex4-ex1b fusion has been previously described.⁵Array comparative genomic hybridization (aCGH): the number of gains, losses and breakpoints in each tested sample.

onset (Ewing-like in nos. 16, 19, and 24, pleomorphic sarcoma-like in nos. 15 and 17, embryonal rhabdomyosarcoma-like in no. 18), and four initially had usual (nos. 20 and 23) or malignant tumors (nos. 21 and 22) that evolved into Ewing-like (nos. 21 and 22), rhabdomyosarcoma-like (no. 21) and pleomorphic sarcoma-like dedifferentiated solitary fibrous tumors (no. 23).

Six of these patients have been described in a previously published morphoimmunohistochemical study,³ and Table 1 also shows their original identification numbers.

Examined Samples

The 29 analyzed samples came from primary tumors in 15 cases, local recurrences in seven, and metastases in seven (Table 1 shows the time since the primary presentation, site and histology of each recurrence/metastasis). They included formalin-fixed and paraffin-embedded and frozen samples of all of the tumors with the exception of five referred cases of which only unstained formalin-fixed and paraffin-embedded sections were available (see Table 1). Samples of dedifferentiated solitary fibrous tumors taken from five patients at two different stages of disease progression included one case of a usual solitary fibrous tumor (20a) that progressed to dedifferentiated (20b), three cases of malignant solitary fibrous tumors (21a, 22a, and 23a) that progressed to dedifferentiation (21b, 22b, and 23b), and one case with samples from two pulmonary dedifferentiated metastases (24a and 24b) separated by an interval of 1 year.

All of the tumors were assessed using a CD34, CD99, Bcl2, and Ki67 immunohistochemistry panel complemented by STAT6. The antibodies, sample processing, and immunohistochemical procedure have been previously described.³

Reverse-Transcription Polymerase Chain Reaction and Sequencing

Total RNA was extracted from all 24 frozen tumoral samples using Epicenter (Madison, WI, USA) followed by DNase treatment, and its concentration and quality were assessed by using NanoVue (VWR, Radnor, PA, USA). A total of 1 µg of RNA per sample was retrotranscribed as previously described.¹⁴

The primers used to detect NAB2/STAT6 fusion products were the previously described NAB2ex5F (CCTGTCTGGGGAGAGTCTGGATG)/STAT6ex20R (GGGGATGGAGTGAGAGTGTG)⁶ and NAB2ex21051F (GCCCAGAGCACCTACTT)/STAT6ex4762R (AGGTGATCTCCCTACTTCG);⁷ the polymerase chain reaction conditions have been described elsewhere.⁷ After treatment with ExoSap IT (Affymetrix, Santa Clara, CA, USA), the amplified products were directly sequenced using a Big Dye v1.1 cycle sequencing kit and a 3500Dx genetic analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blotting

Aliquots of nuclear extracts from all 24 frozen tumoral samples containing equal amounts of protein (as assessed using the Bio-Rad protein assay kit, Bio-Rad, Segrate, Italy) were analyzed by means of western blotting: the membranes were incubated with rabbit STAT6 antibody (Cell Signaling Technology, Danvers, MA, USA) diluted 1:1000, and rabbit anti-actin antibody (1:2500; A2066; Sigma-Aldrich, St. Louis, MO, USA) was used to normalize the results.

Immunohistochemistry

A polyclonal rabbit antibody (s20-S621, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) against the STAT6 C-terminal was used at a dilution of 1:400 and, after antigen unmasking (96 °C EDTA for 30 min), the samples were stained by using a Dako Flex+ Autostainer (Dako, Glostrup, Denmark) at room temperature per 30 min. The slides were counterstained with Mayer's hematoxylin. The antibody and immunohistochemistry procedure for the TP53 assessments have been described elsewhere.¹⁴

RB1 immunohistochemistry was performed using the R PMG 3-245 clone (Pharmingen, Beckton-Dickenson, San Jose, CA, USA) as described above.

Array Comparative Genomic Hybridization

High-resolution oligo array comparative genomic hybridization analysis was used for a subset of samples of 12 tumors from 9 patients, including three usual (nos. 1, 2, and 6), five malignant (nos. 8, 9, 10, 22a, and 23a), and four dedifferentiated tumors (nos. 22b, 23b, 24a, and 24b). The samples taken from three patients (nos. 22, 23, and 24) were from the primary (no. 22a) or the earliest available lesion (Nos. 23a and 24a) and the subsequent dedifferentiated recurrence (nos. 22b, 23b, and 24b). Genomic DNA was extracted from the frozen samples by using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) including RNase treatment, and its quality was assessed by using NanoVue (VWR) and agarose gel electrophoresis. The molecular karyotype of the samples was assessed by using an Agilent 144K array and an Agilent Genomic DNA ULS labeling kit in accordance with the manufacturer's protocol, and the raw data were processed using 'Feature extraction' software (Agilent Technologies, Santa Clara, CA, USA).

The data were analyzed using Agilent's Genomic Workbench Standard Edition 6.5.0.58. Copy-number variations, low-level copy-number abnormalities (0.3 < log2 ratio > -0.3), and copy-number abnormalities involving chromosomes X and Y were not considered.

TP53 Mutational Analysis

TP53 mutational status was assessed in ten solitary fibrous tumors (nos. 15, 16, 17, 21b, 22a and b, 23a

and b, and 24a and b) with suitable genomic DNA; exons 5–8 were amplified and sequenced as previously described.¹⁴

Statistical Analysis

The data were analyzed using the two-tailed non-parametric Mann–Whitney test and Star View Software; a *P*-value of ≤ 0.05 was considered significant.

Results

Diagnostic Tools Assessing NAB2/STAT6 Fusion: Strength and Pitfalls

Reverse-transcription polymerase chain reaction. The classification of *NAB2/STAT6* fusion types is challenging because of their variety and complexity. Furthermore, the absence of an acknowledged numbering system for *STAT6* exons causes confusion and makes it cumbersome to compare results from different groups.

In terms of size, *NAB2/STAT6* fusion variants can be tentatively grouped into two categories: those with distal *STAT6* breakpoints (exons 17–19) whose chimeric proteins have a similar size to that of *STAT6* (small), and those with *STAT6* proximal breakpoints (exons 1b–4), which produce outsized chimeric proteins (large).⁶ *NAB2* breakpoints are also

heterogeneous and fall in proximal (exons 3–5) or distal exons (exons 6–7). Furthermore uncommon variants with intronic and/or intraexonic breakpoints have been described.^{5–7}

NAB2/STAT6 fusion transcripts were detected in all of the tested tumor samples, and corresponded to nine different fusion types: four fusions have been previously described (see Table 1); the remaining five involved intronic sequences and/or exonic fusion/breakpoints while retaining the reading frame (Table 1 and Supplementary Figure S1). There was no correlation between the fusion type and tumor malignancy/dedifferentiation or site, but there was a significant association between a younger age at the time of onset and fusions with *STAT6* (Mann–Whitney, two-tailed, $n_1 = 13$, $n_2 = 8$, $P < 0.05$) or *NAB2* distal breakpoints (Mann–Whitney, two-tailed, $n_1 = 16$, $n_2 = 5$, $P < 0.05$). This finding is in line with previous observations.⁸

STAT6 immunohistochemistry. All of the 7 usual and 11 malignant tumors showed strong and diffuse STAT6 immunostaining restricted to the nucleus, a hallmark of solitary fibrous tumors^{6,10,11} (Figure 1a), whereas immunohistochemistry profile was more heterogeneous in the dedifferentiated tumors: only two (nos. 17 and 18) showed typical STAT6 immunostaining, six samples from five patients (nos. 15, 16, 19, 20b, 24a, and 24b) showed patchwork STAT6 immunodecoration characterized by focally

Figure 1 STAT6 immunolabeling modulation in dedifferentiated solitary fibrous tumors. (a) Case No. 13. Standard strong and diffuse nuclear immunoreactivity for STAT6 in malignant solitary fibrous tumor. (b) Case No. 16. Example of dedifferentiated solitary fibrous tumor with patchwork immunoreactivity: areas of null STAT6 nuclear immunostaining abutting immunolabeled tumoral clusters. (c) Higher magnification of panel b showing paired multinucleated positive and negative tumor cells. (d) Case number 22b. An Ewing-like dedifferentiated solitary fibrous tumor in which STAT6 immunolabeling is completely lost. (e) Case number 23b. Example of spindle cell dedifferentiated solitary fibrous tumor showing the complete loss of immunoreactivity: the tumoral nuclei appear STAT6 negative throughout the sample. (f) Higher magnification of panel e showing faint cytoplasmic immunoreactivity.

Figure 2 STAT6 immunophenotypal and biochemical profiles of two malignant solitary fibrous tumors progressing to dedifferentiation. Both cases (23: a, b, and c and 21: d, e, and f) show the loss of STAT6 protein expression upon immunohistochemical (b and e) and western blotting analysis (c: 23b and f: 21b). (f) An unmatched positive control (case no. 13) has been used alongside case 21b because there was no cryopreserved material of the primary tumor.

nuclear positive areas flanked by immunonegative areas (Figures 1b and c); and the remaining three (nos. 21b, 22b, 23b) showed negative STAT6 nuclear immunoreactivity (Figures 1d–f). The STAT6 negative and patchwork cases occasionally showed faint STAT6 cytoplasmic immunoreactivity (Figures 1e and f). It is particularly worth noting that the four patients for whom material was available from an initial usual/malignant tumor and a subsequent dedifferentiated recurrence/metastasis (nos. 20–23), the earlier lesions showed canonical STAT6 immunostaining (Figures 2a and d), but not the later lesions (Figures 2b and e). These four cases have been included in previous studies.^{3,15}

Western blotting. Immunoblotting of all of the 24 tested samples revealed the presence of a 100-kDa band corresponding to STAT6 protein (data not shown). The expected additional bands of higher molecular weight corresponding to ‘large’ NAB2-STAT6 chimeras⁶ were detected in all of the usual and malignant cases (nos 1, 6, 8, 9, 10, 13, and 23a) with NAB2/STAT6 fusions involving proximal STAT6 exons (from exons 1b to 5); examples of such ‘large’ chimeric proteins are shown in Figure 2c and f (lanes 23a and 13). Unlike in a previous study⁶, we did not detect any additional bands corresponding to ‘small’ chimeric products in the tumors with distal STAT6 breakpoints (exons 17 and 19). It is worth noting that, although both the initial malignant tumor and subsequent dedifferentiated recurrence

of no. 23 were reverse-transcription polymerase chain reaction positive, only the protein extracts of the former showed the presence of the expected ‘large’ chimeric protein (Figure 2c, lane 23a). The findings were similar in case no. 21: although both lesions were reverse-transcription polymerase chain reaction positive, the primary lesion was STAT6 immunohistochemistry positive, whereas the dedifferentiated metastasis was immunohistochemistry negative and western blotting revealed only a very-faint chimeric product (Figure 2f, lane 21b).

Taken together, these findings suggest that reverse-transcription polymerase chain reaction is the diagnostic gold standard for solitary fibrous tumors as it can detect NAB2/STAT6 fusions throughout the spectrum, including dedifferentiated variants that have lost their immunohistochemical signature.

Genomic Characterization of Usual, Malignant and Dedifferentiated Solitary Fibrous Tumors

Despite their generally ‘simple’ genomic and cytogenetic profiles,^{16–18} it has been reported that solitary fibrous tumors undergo genetic alterations during their progression to malignancy^{19,20} and dedifferentiation.² Given the availability of suitable representative samples of all types of solitary fibrous tumors (including two cases progressing to dedifferentiated), we used array comparative genomic hybridization analysis in order to define the

Figure 3 Rb and TP53 status in a progressive solitary fibrous tumor (case no. 22). (a and b) The Rb nuclear immunolabeling of the primary malignant tumor (a) is lost in the Ewing-like dedifferentiated recurrence (b) (upper left corner: internal positive control). (c) Corresponding array comparative genomic hybridization profiles showing hemizygous and homozygous losses of the RB1 locus (the lighter and darker areas, respectively). TP53 immunohistochemistry (d), molecular (e) and genomic profile (f) of the recurrence showing positive nuclear immunolabeling (d), a disabling GGC to AGC mutation (e) corresponding to the G245S substitution and chromosome 17 array comparative genomic hybridization profile of both the primary tumor (22a) and the dedifferentiated recurrence (22b) (f).

genetic events associated with their morphophenotypical evolution.

Array comparative genomic hybridization. All of the usual tumors had a normal profile, and only two of the malignant solitary fibrous tumors showed abnormalities: no. 8 had copy-number abnormalities involving entire chromosomes (gains for chromosomes 5, 8, 18, 19, and 21 and the loss of chromosome 13), and no. 22a had two (13q and 17p) interstitial deletions (Figures 3c and f; Supplementary Fig. S2). On the contrary, all of the dedifferentiated solitary fibrous tumors had a complex genomic profile that was mainly characterized by intrachromosomal gains and more prevalent losses (Table 1 and Supplementary Fig. 2) and, interestingly, were all genomically unstable as shown by the acquisition of new copy-number abnormalities in successive samples (Table 1 and Supplementary Fig. S2). All of the dedifferentiated solitary fibrous tumors showed losses in chromosome 13 (which always included the *RB1* gene) and 16q losses with a minimal common deleted region within the 16q 23.1 chromosomal band. The deletion of 17p

(including the *TP53* gene) was observed in two cases (nos. 22b and 23b).

***RB1* and *TP53*: the oncosuppressors most frequently involved in dedifferentiated solitary fibrous tumors copy-number abnormalities.** The immunohistochemical profile of RB1 of case no. 22 showed nuclear immunoreactivity in the primary malignant tumor (Figure 3a) and complete loss in the dedifferentiated recurrence (Figure 3b) with a homozygous *RB1* deletion (Figure 3c).

None of the usual solitary fibrous tumors was TP53 immunopositive, and only two of the 11 malignant samples (nos. 21a and 22a) had more than 50% of nuclear-positive cells suggesting *TP53* disabling mutations, while 4 out of the 10 tested dedifferentiated samples (nos. 15, 17, 21b, and 22b) from 11 patients were immunopositive (Figure 3d). *TP53* disabling mutations were confirmed in one malignant (no. 22a) and three dedifferentiated solitary fibrous tumors (nos. 17, 21b, and 22b), and both TP53-positive malignant cases (one TP53 immunohistochemistry positive and one TP53 immunohistochemistry positive/*TP53* mutated) later evolved into dedifferentiated solitary fibrous tumors (see Table 1).

Discussion

The recent identification of *NAB2/STAT6* fusion as a hallmark/initiating event of solitary fibrous tumors has allowed the use of dedicated molecular (reverse-transcription polymerase chain reaction), immunohistochemical (STAT6 immunohistochemistry) and biochemical (western blotting) techniques as means of supporting their diagnosis and studying their progression. We used these techniques together with genome-wide (array comparative genomic hybridization) analysis, TP53 immunohistochemistry and mutational analysis to investigate a series of 29 tumors taken from 24 patients. We also used the molecularly confirmed diagnosis to focus on the recently described and relatively unexplored dedifferentiated solitary fibrous tumor variant in order to document the changes that take place during dedifferentiation, ie, the loss of chimeric protein expression and increased genomic instability, which can be interpreted as evidence of tumor reprogramming.

The results confirm that reverse-transcription polymerase chain reaction is the diagnostic gold standard albeit with some limitations due to the broad splicing landscape and the frequent occurrence of 'noncanonical' *NAB2/STAT6* fusions. Its overall sensitivity was 100% as it detected *NAB2/STAT6* fusion in all of the tumors, including the seven dedifferentiated solitary fibrous tumors for which frozen material was available. Regarding the prognostic value of fusion types, only one study to date⁸ reports an association between specific fusions (namely the 'small' variants ex6-ex17, ex6-ex18) and clinicopathological characteristics such as younger age at onset, extrapleural site and malignancy. However, none of such associations were reported in two other studies.^{6,7} In our series we did find significant association between 'small' type fusions and younger age but not between fusion type and the pathological characteristics of the tumors such as malignancy or site (pleuropulmonary vs others). The lack of association between pleuropulmonary site and fusion type in our series is not surprising since only two tumors arising at this site are included. It is worth noting that both papers reporting no association^{6,7} had similarly unbalanced series with an overwhelming majority of extrapleural tumors. More studies on large series with balanced representation of sites of origin are needed in order to assess the reproducibility and true extent of these observations.

A reliable and widely applicable alternative means of diagnosing solitary fibrous tumors is STAT6 immunostaining, which is characterized by strong and diffuse nuclear immunopositivity. However, although this pattern was found in all of the usual and malignant variants, the immunohistochemical profile of the dedifferentiated tumors was less typical: only 2 out of the 11 tumors (from 10 patients) were typically positive, 3 were negative, and the remaining 6 had an ambiguous pattern characterized by patchwork nuclear positivity and a focal loss of nuclear

immunostaining. The STAT6-negative and patchwork cases occasionally showed faint cytoplasmic STAT6 immunoreactivity, and the tumors showing the loss or modulation of STAT6 immunoreactivity often also showed the loss of CD34 immunolabeling (Table 1). This makes the immunohistochemistry-based diagnosis of dedifferentiated solitary fibrous tumors particularly challenging and totally reliant on reverse-transcription polymerase chain reaction. It is important to point out that the loss of STAT6 immunoreactivity was further supported by western blotting readouts showing the absence or greatly reduced levels of chimeric protein in two STAT6 immunohistochemistry-negative cases. Post-transcriptional mechanisms of STAT6 downregulation are known in hematological cell lineages and include CBL-B mediated ubiquitination followed by proteasome degradation in T-cells,²¹ and the proteolytic generation of a shorter STAT6 isoform (65 kDa) lacking the COOH terminal transactivation domain in mast cells.²² Similar mechanisms may be responsible for *NAB2/STAT6* chimera downregulation and loss in dedifferentiated solitary fibrous tumors, in which the light cytoplasmic immunostaining in association with the decrease or loss of STAT6 nuclear labeling favor the ubiquitination-mediated mechanism.

Taken together, our findings suggest that *NAB2/STAT6* downregulation is frequent in dedifferentiated solitary fibrous tumors and may reflect a loss of oncogene addiction,^{23,24} thus supporting the hypothesis that dedifferentiation participates in tumor reprogramming. An association between tumor dedifferentiation and the loss of oncogene expression has been found in the case of GISTs (gastrointestinal stromal tumors)²⁵ and chordomas,²⁶ and it is interesting to note that, as in the case of solitary fibrous tumors, the oncogene in both cases is thought to be the initiating/defining lesion (mutated *KIT* in the case of GISTs, and brachyury overexpression in the case of chordomas). At the same time as the loss of *NAB2/STAT6* expression, we also observed the acquisition of a complex array comparative genomic hybridization profile, which seems to be the hallmark of solitary fibrous tumor dedifferentiation.

It is known that genome instability plays a role in sarcoma dedifferentiation by favoring the acquisition of critical new genetic changes such as oncogene independence, the reinstatement/acquisition of stem-cell potential (reprogramming), and an ability to display divergent phenotypes.²⁷⁻²⁹ Nonrandom genomic alterations associated with dedifferentiation have also been reported in dedifferentiated chondrosarcomas³⁰ and liposarcomas³¹ and, in the latter, specific copy-number abnormalities are associated with a distinct morphology and poor prognosis.

All of the dedifferentiated solitary fibrous tumors investigated by means of array comparative genomic hybridization were characterized by a complex genomic profile including a number of interstitial gains and losses which, in two cases, were acquired during progression from a STAT6-positive malignant tumor

(nos. 22a and 23a) to a STAT6-negative dedifferentiated solitary fibrous tumor (nos. 22b and 23b). On the contrary, only two of our nondedifferentiated tumors showed the presence of copy-number abnormalities. To the best of our knowledge, the only other dedifferentiated-solitary fibrous tumor previously profiled by means of comparative genomic hybridization² was a tumor characterized by a divergent evolution³ whose high-grade component showed a complex profile with multiple gains and losses without any abnormalities in the low-grade component. In line with previously published findings, the most frequent copy-number abnormalities in our series were the loss of 13q,^{16–18} which always involved *RB1* and included the only homozygous deletion; deletion 16q with its minimal common region in 16q23.1; and deletion 17p always involving *TP53*. It is worth noting that the losses of 17p and 13q were the only copy-number abnormalities in one malignant tumor that later evolved into a dedifferentiated solitary fibrous tumor and acquired further copy-number abnormalities.

Unlike Mohajery *et al.*,⁷ who found frequent rearrangements and copy-number abnormalities on 12q13, we observed only one case: a 4 Mb gain involving the *NAB2/STAT6* loci. This difference is probably because of the different resolution of the array platforms (144K vs the 1.2M array of Mohajery *et al.*). It is worth pointing out that the recurrent 12q rearrangements, which have also been found by means of classical cytogenetic^{7,32} and fluorescent *in situ* hybridization analyses,⁷ are probably related to the primary *NAB2/STAT6* genomic rearrangement rather than being acquired during progression to malignancy or dedifferentiation.

An immunohistochemistry profile suggesting a *TP53* mutation was found in four dedifferentiated solitary fibrous tumors and confirmed by sequencing in three (two of which harbored the same alteration in their earlier malignant presentations). *TP53* mutations/overexpression have been reported in high-grade/dedifferentiated solitary fibrous tumors,^{12,13,33–35} and both deletion 13 and *TP53* mutations have been observed to appear during disease progression in longitudinal studies of solitary fibrous tumors.^{19,20}

Cumulatively, the main features of dedifferentiated solitary fibrous tumors are the complete or partial loss of *NAB2/STAT6* chimeric protein expression (despite the retention of chimeric RNA expression), and the acquisition of genome instability. It can therefore be hypothesized that a dysregulated post-translational mechanism (responsible for the chimeric protein downregulation) is involved in the acquisition of the dedifferentiated phenotype. Such changes can be interpreted as being part of tumor cell reprogramming, and may explain the previously described different responses to targeted therapies for malignant and dedifferentiated solitary fibrous tumors.^{15,36,37}

On the other hand, genomic changes such as del17p/*TP53* mutations and del13q (which seem to be prevalent in dedifferentiated solitary fibrous tumors)

can also be occasionally found in tumors whose histology and immunoprofiles are fully consistent with a diagnosis of a malignant solitary fibrous tumor. Two of our malignant *TP53* immunopositive cases (nos. 21 and 22, the latter with a 13q deletion) were characterized by dedifferentiated recurrences within 3 years, a much shorter latency than the more than 10 years observed in other patients with recurrent dedifferentiated solitary fibrous tumors.⁴ This suggests that these abnormalities may at least sometimes precede the expression of a fully dedifferentiated phenotype and that they may have prognostic/predictive value.

The size (24 cases) and the composition (only two pleural /24 and 10 dedifferentiated /24 cases) of our series are not suitable to draw conclusions of broad reliability and statistical validity with regard to issues such as the correlation between fusion type and clinicopathological features. However, our aim was to define the applicability of the newly available molecular and immunohistochemical tools to the diagnosis of dedifferentiated solitary fibrous tumors and to describe the changes occurring during the process of evolution toward a dedifferentiated phenotype. The results uncover some biological peculiarities of dedifferentiation in solitary fibrous tumor underlining, at the same time, the pitfalls and strengths of the newly available diagnostic tests in the setting of dedifferentiated solitary fibrous tumor.

Acknowledgments

AIRC grant IG 14102 to SP; AIRC grant MFAG 11817 to TN.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

- 1 Fletcher CDM, Bridge JA, Lee JC. Extrapleural solitary fibrous tumor. In: WHO Classification of Tumors of Soft Tissue and Bone. Fletcher CDM, Bridge JA, Hogendoorn PCW, Mertens F. International Agency of Research on Cancer (IARC), 2013, pp 80–82.
- 2 Mosquera JM, Fletcher CD. Expanding the spectrum of malignant progression in solitary fibrous tumors: a study of 8 cases with a discrete anaplastic component – is this dedifferentiated SFT? *Am J Surg Pathol* 2009;33: 1314–1321.
- 3 Collini P, Negri T, Barisella M *et al.* High-grade sarcomatous overgrowth in solitary fibrous tumors: a clinicopathologic study of 10 cases. *Am J Surg Pathol*. 2012;36:1202–1215.
- 4 Baldi GG, Stacchiotti S, Mauro V *et al.* Solitary fibrous tumor of all sites: outcome of late recurrences in 14 patients. *Clin Sarcoma Res* 2013;3:4.
- 5 Chmielecki J, Crago AM, Rosenberg M *et al.* Whole-exome sequencing identifies a recurrent *NAB2-STAT6* fusion in solitary fibrous tumors. *Nat Genet*. 2013;45: 131–132.

- 6 Robinson DR, Wu YM, Kalyana-Sundaram S *et al*. Identification of recurrent NAB2-STAT6 gene fusions in solitary fibrous tumor by integrative sequencing. *Nat Genet*. 2013;45:180–185.
- 7 Mohajer A, Tayebwa J, Collin A *et al*. Comprehensive genetic analysis identifies a pathognomonic NAB2/STAT6 fusion gene, nonrandom secondary genomic imbalances, and a characteristic gene expression profile in solitary fibrous tumor. *Genes Chromosomes Cancer* 2013;52:873–886.
- 8 Barthelmeß S, Geddert H, Boltze C *et al*. Solitary fibrous tumors/hemangiopericytomas with different variants of the NAB2-STAT6 gene fusion are characterized by specific histomorphology and distinct clinicopathological features. *Am J Pathol*. 2014;184:1209–1218.
- 9 Srinivasan R, Mager GM, Ward RM *et al*. NAB2 represses transcription by interacting with the CHD4 subunit of the nucleosome remodeling and deacetylase (NuRD) complex. *J Biol Chem*. 2006;281:15129–15137.
- 10 Doyle LA, Vivero M, Fletcher CD *et al*. Nuclear expression of STAT6 distinguishes solitary fibrous tumor from histologic mimics. *Mod Pathol*. 2014;27:390–395.
- 11 Yoshida A, Tsuta K, Ohno M *et al*. STAT6 immunohistochemistry is helpful in the diagnosis of solitary fibrous tumors. *Am J Surg Pathol* 2014;38:552–559.
- 12 Masuda Y, Kurisaki-Arakawa A, Hara K *et al*. A case of dedifferentiated solitary fibrous tumor of the thoracic cavity. *Int J Clin Exp Pathol* 2013;7:386–393.
- 13 Kurisaki-Arakawa A, Akaike K, Hara K *et al*. A case of dedifferentiated solitary fibrous tumor in the pelvis with TP53 mutation. *Virchows Arch* 2014;465:615–621.
- 14 Licitra L, Perrone F, Bossi P *et al*. High-risk human papillomavirus affects prognosis in patients with surgically treated oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2006;24:5630–5636.
- 15 Stacchiotti S, Tortoreto M, Bozzi F *et al*. Dacarbazine in solitary fibrous tumor: a case series analysis and preclinical evidence vis-a-vis temozolomide and anti-angiogenics. *Clin Cancer Res*. 2013;19:5192–5201.
- 16 Bertucci F, Bouvier-Labit C, Finetti P *et al*. Comprehensive genome characterization of solitary fibrous tumors using high-resolution array-based comparative genomic hybridization. *Genes Chromosomes Cancer* 2013;52:156–164.
- 17 Martin AJ, Summersgill BM, Fisher C *et al*. Chromosomal imbalances in meningeal solitary fibrous tumors. *Cancer Genet Cytogenet*. 2002;135:160–164.
- 18 Krismann M, Adams H, Jaworska M *et al*. Patterns of chromosomal imbalances in benign solitary fibrous tumors of the pleura. *Virchows Arch*. 2000;437:248–255.
- 19 Moritani S, Ichihara S, Hasegawa M *et al*. Dedifferentiation and progression of an intracranial solitary fibrous tumor: autopsy case of a Japanese woman with a history of radiation therapy of the head during infancy. *Pathol Int*. 2011;61:143–149.
- 20 Ness GO, Lybaek H, Arnes J, Rødahl E. Chromosomal imbalances in a recurrent solitary fibrous tumor of the orbit. *Cancer Genet Cytogenet*. 2005;162:38–44.
- 21 Qiao G, Ying H, Zhao Y *et al*. E3 ubiquitin ligase Cbl-b suppresses proallergic T cell development and allergic airway inflammation. *Cell Rep* 2014;6:709–723.
- 22 Suzuki K, Nakajima H, Kagami S *et al*. Proteolytic processing of Stat6 signaling in mast cells as a negative regulatory mechanism. *J Exp Med*. 2002;196:27–38.
- 23 Jonkers J, Berns A. Oncogene addiction: sometimes a temporary slavery. *Cancer Cell*. 2004;6:535–538.
- 24 Perl A, Carroll M. BCR-ABL kinase is dead; long live the CML stem cell. *J Clin Invest*. 2011;121:22–25.
- 25 Antonescu CR, Romeo S, Zhang L *et al*. Dedifferentiation in gastrointestinal stromal tumor to an anaplastic KIT-negative phenotype: a diagnostic pitfall: morphologic and molecular characterization of 8 cases occurring either *de novo* or after imatinib therapy. *Am J Surg Pathol*. 2013;37:385–392.
- 26 Jambhekar NA, Rekhi B, Thorat K *et al*. Revisiting chordoma with brachyury, a 'new age' marker: analysis of a validation study on 51 cases. *Arch Pathol Lab Med* 2010;134:1181–1187.
- 27 Lagasse E. Cancer stem cells with genetic instability: the best vehicle with the best engine for cancer. *Gene Ther* 2008;15:136–142.
- 28 Liang Y, Zhong Z, Huang Y *et al*. Stem-like cancer cells are inducible by increasing genomic instability in cancer cells. *J Biol Chem* 2010;285:4931–4940.
- 29 Vicente-Dueñas C, Romero-Camarero I, Cobaleda C *et al*. Function of oncogenes in cancer development: a changing paradigm. *EMBO J*. 2013;32:1502–1513.
- 30 Sakamoto A. The molecular pathogenesis of dedifferentiated chondrosarcoma. *Indian J Orthop* 2014;48:262–265.
- 31 Crago AM, Socci ND, DeCarolis P *et al*. Copy number losses define subgroups of dedifferentiated liposarcoma with poor prognosis and genomic instability. *Clin Cancer Res*. 2012;18:1334–1340.
- 32 Mandahl N, Orndal C, Heim S *et al*. Aberrations of chromosome segment 12q13-15 characterize a subgroup of hemangiopericytomas. *Cancer* 1993;71:3009–3013.
- 33 Subramaniam MM, Lim XY, Venkateswaran K *et al*. Dedifferentiated solitary fibrous tumor of the nasal cavity: the first case reported with molecular characterization of a TP53 mutation. *Histopathology* 2011;59:1269–1274.
- 34 Yokoi T, Tsuzuki T, Yatabe Y *et al*. Solitary fibrous tumor: significance of TP53 and CD34 immunoreactivity in its malignant transformation. *Histopathology* 1998;32:423–432.
- 35 Schirosi L, Lantuejoul S, Cavazza A *et al*. Pleuropulmonary solitary fibrous tumors: a clinicopathological, immunohistochemical, and molecular study of 88 cases confirming the prognostic value of de Perrot staging system and TP53 expression, and evaluating the role of c-kit, BRAF, PDGFRs (alpha/beta), c-met, and EGFR. *Am J Surg Pathol*. 2008;32:1627–1642.
- 36 Stacchiotti S, Libertini M, Negri T *et al*. Response to chemotherapy of solitary fibrous tumor: a retrospective study. *Eur J Cancer*. 2013;49:2376–2383.
- 37 Stacchiotti S, Negri T, Libertini M *et al*. Sunitinib malate in solitary fibrous tumor (SFT). *Ann Oncol* 2012;23:3171–3179.

Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)