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LETTER TO THE EDITOR

Upregulation of the *SOX5* by promoter swapping with the *P2RY8* gene in primary splenic follicular lymphoma

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The vast majority of follicular lymphomas (FL) originate in lymph nodes and are characterized by a specific oncogenic event, the t(14;18)(q32;q21) translocation, juxtaposing the *BCL2* gene on chromosome 18 with the immunoglobulin heavy chain locus on chromosome 14, leading to the overexpression of the antiapoptotic molecule BCL2.[1] On the other hand, there is a group of t(14;18)(q32;q21)-negative FLs, mostly found in skin or in other extranodal sites as well as in lymph nodes.[2] Follicular center cell lymphomas of the spleen rarely arise as primary splenic follicular lymphoma (PSFL), but involvement of the spleen is common in the context of a generalized lymphoma. To date, there are no data about the biological features of PSFL, due to the sporadic frequency of this pathologic entity. We report a PSFL case showing a novel fusion gene involving *P2RY8* (G-protein coupled purinergic receptor P2Y8), and *SOX5*, one of the Sry (sex-determining region Y)-box (SOX) genes. The fusion was detected by fluorescence *in situ* hybridization (FISH) and reverse transcription (RT)-PCR. The *P2RY8/SOX5* chimera resulted in the overexpression of *SOX5* and the encoded protein, evaluated by real-time quantitative PCR (RQ-PCR) and immunohistochemistry, respectively.

In January 2004, a 58-year-old male patient presented with leukopenia (WBC count 3×10^9/l) and thrombocytopenia (PLT count 65×10^9/l). Physical examination showed splenomegaly (3 cm below the costal margin). Peripheral blood, bone marrow trephine and aspirate analysis did not reveal hematological disorders. Moreover, immunophenotypic and conventional cytogenetic analysis did not show clonal diseases. Virological search and total body computed tomography (CT) scans were negative. During the follow-up, the biological and clinical features of the patient were stable with the exception of the slowly increasing spleen volume. In February 2006, the patient presented burden splenic symptoms. He was therefore treated with splenectomy (spleen maximum diameter was 26 cm, weight 1.7 kg). Spleen histological examination (Figures 1a and b) revealed a grade I FL according to the WHO classification. The neoplastic component coexpressed CD20, CD10 (Figure 1c) and BCL6 (Figure 1d) with monotypic light chain restriction; CD5, CD21, CCND1 and BCL2 were negative. The pattern of growth was almost exclusively nodular. FISH and RT-PCR analyses excluded the occurrence of both *IGH/BCL2* and *IGH/CCND1* fusion genes (data not shown). Moreover, appropriate FISH experiments showed neither *IGH/BCL3* nor *IGH/BCL6* cryptic fusion genes (data not shown). Total body CT scans, bone marrow trephine and aspirate examination were negative. In accordance with these data, the diagnosis of PSFL was made.

Chromosome analysis, performed on direct preparation of spleen cells, revealed the karyotype 46,XY,t(X;12)(p22;p12)[9]/46,XY[4] (Figure 2a). Multicolor-FISH, performed using the 24-color SpectraVysion probe (Abbott, Abbott Park IL, USA), confirmed the t(X;12) rearrangement (Figure 2a). FISH experiments, using appropriate bacterial artificial chromosome (BAC) clones, revealed that the breakpoints were encompassed by the clones RP11-261P4 (chrX:1,457,956-1,620,348, at Xp22.33; Figures 2a and b) and RP11-77H23 (chr12:24,350,453–24,538,750, at 12p12.1; Figures 2a and b). The latter BAC was found to contain the *SOX5* gene only. The FISH analysis also indicated *P2RY8* as potentially involved in the rearrangement (Figures 2a and b). RT-PCR with combinations of *P2RY8* forward and *SOX5* reverse primers successfully amplified cDNA

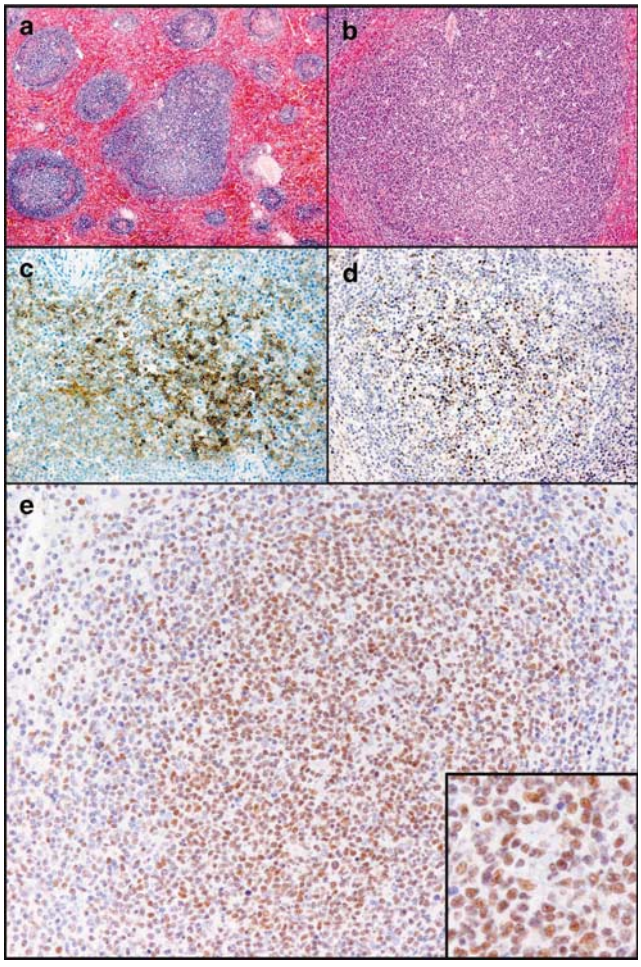
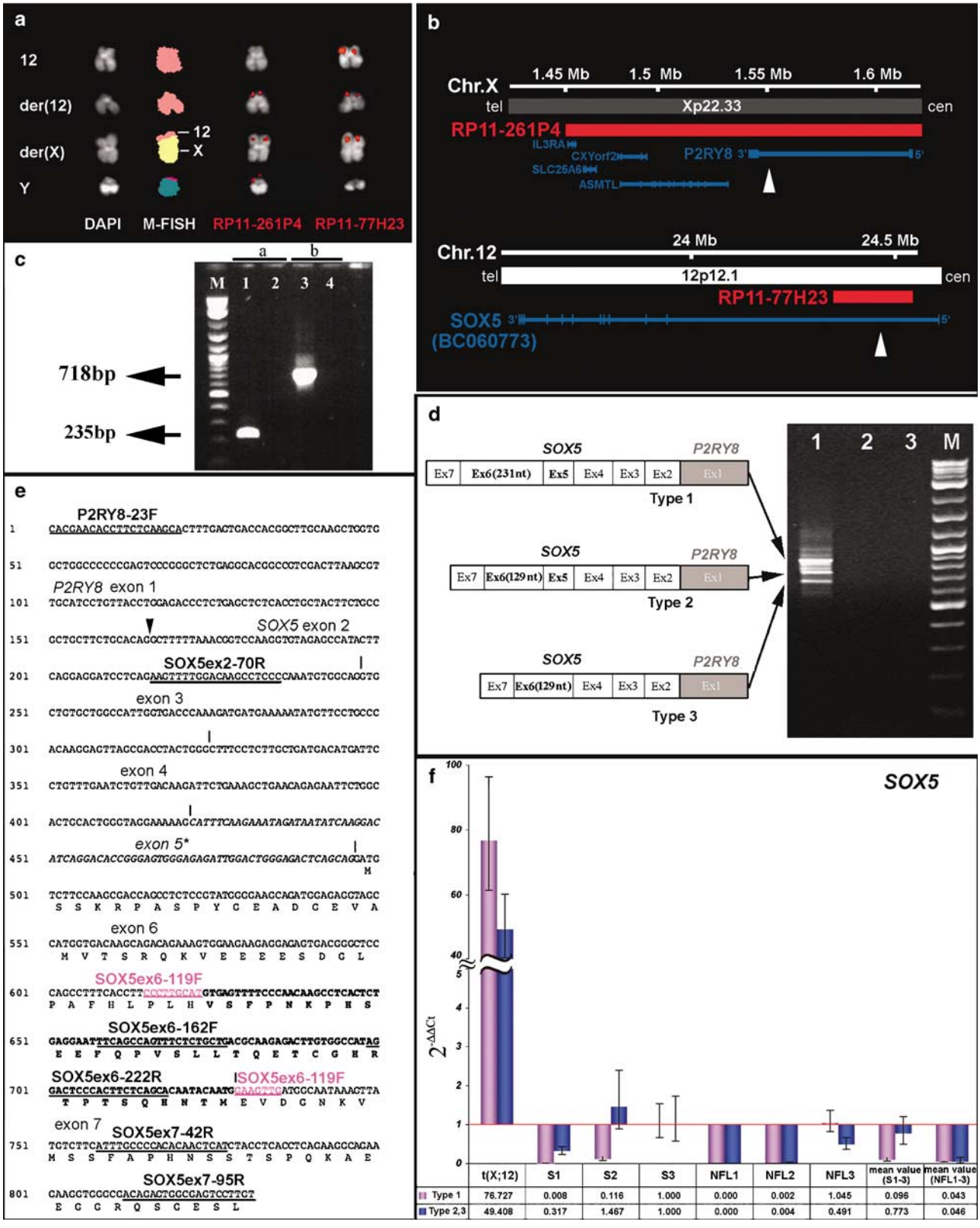


Figure 1 (**a**, **b**) Pictures, at different magnification, of spleen hystological samples from the patient (hematoxylin and eosin stain); (**a**) ×40 original magnification; (**b**) ×100 original magnification. (**c**) Immunohistochemistry analysis with anti-BCL6 monoclonal antibody (Dako, Milan, Italy). (**d**) Immunohistochemistry analysis with anti-CD10 monoclonal antibody (Novocastra, Newcastle Upon Tyne, UK). (**e**) Immunohistochemistry analysis with SOX5 guinea pig antibody, showing nuclear reactivity in the majority of FL cells (×200 original magnification).

fragments from our case. The primer combinations P2RY8ex1-23F/SOX5ex2-70R, and P2RY8ex1-23F/SOX5ex5-222R (Table 1) yielded a single band of 235 and 718 bp, respectively (Figure 2c). The sequencing of these two bands revealed the occurrence of a *P2RY8/SOX5* fusion transcript (Figure 2e). Interestingly, the primer combination P2RY8ex1-23F/SOX5ex6-95R produced three main fragments of 831, 756 and 651 bp, in addition to two fainter bands (Figure 2d). The three main bands



were sequenced for further characterization of the fusion breakpoint. In all the three transcripts, nt 187 of exon 1 of *P2RY8* (accession no. NM_178129) was fused with nt 117 of exon 2 of *SOX5* (mRNA with accession number BC060773) (Figures 2d and e). However, a precise determination of the fusion point was not possible due to the existence of an identical AG dinucleotide at the junction (position 186–187 in *P2RY8* and 117–118 in *SOX5* cDNA). In both transcripts of types 2 and 3, part of exon 6 (105 bp, position 577–681 in BC060773, downstream to the ATG starting codon[3]) was alternatively spliced out (Figures 2d and e), as well as the whole exon 5 (present as a cryptic exon in mRNA BC066773, position 375–449, but not in the RefSeq transcript NM_152989) in the type 3 transcript (Figures 2d and e).

None of the three amplified transcripts resulted in a chimeric in-frame *P2RY8/SOX5*. The fusion breakpoint, in all the three fusion transcripts, mapped before the start of the *P2RY8* coding sequence (*P2RY8* exon 2) and preserved the *SOX5* ATG initiation codon, which begins at the second nucleotide of *SOX5* exon 6 (BC060773) (Figure 2e). RT-PCR for the reciprocal *SOX5/P2RY8* fusion product, using *SOX5* exon 1 forward and *P2RY8* exon 2 reverse primers, yielded negative results (data not shown). The expression level of *SOX5* was determined by RQ-PCR using 1X PlatinumSYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA). Proper primer combinations were used to discriminate among splicing variants. In detail, the SOX5ex6-162F/SOX5ex7-42R allowed the amplification of transcript variant type I only. The other two variants (types 2 and 3) were obtained using SOX5ex6-119F/SOX5ex7-42R (Figures 2d–f). 28S rRNA was used as reference gene and one (no. 3, Figure 2f) of the three normal spleen samples was utilized as calibrator. The gene expression level variation was estimated by comparing the values of $2^{-\Delta\Delta C_t}$ (relative amount of cDNA) for the *SOX5* C_t values in the patient with t(X;12), three cases of nodal FL, and three normal spleen samples, with the relative value of the calibrator. Moreover, the C_t mean values of the three normal spleen samples and the three FL patients were also considered during the analysis. The significance was estimated by comparing the respective ranges ($2^{-\Delta\Delta C_t} \pm$s.d.) of these values (Figure 2f). This analysis revealed a clear upregulation of all the *SOX5* splicing variants in the case with the t(X;12) rearrangement (Figure 2f). Immunohistochemical analysis using two different SOX5 antibodies, based on a guinea pig antibody[4] and a rabbit anti-SOX5 antibody (Abcam ab26041), showed nuclear overexpression of the SOX5 protein in most of the neoplastic follicular cells (Figure 1e). SOX5 immunoreactivity was absent in five cases of t(14;18) positive FL (data not shown).

In the present study, we demonstrated the overexpression of *SOX5* in a case of PSFL with a t(X;12)(p22;p12) (Figure 2f). The mechanism responsible for the transcriptional deregulation

Table 1 Primers for PCR and sequencing

Designation	*Sequence (5′→3′)*	*Accession no.*	*Nucleotide position*[a]
P2RY8ex1-23F	CACGAACACCTTCTCAAGCA	NM_178129	23–42
P2RY8ex1-162F	CTACTTCTGCCGCTGCTTCT	NM_178129	162–181
P2RY8ex2-265R	AGATTTGGAAAGGCAACACG	NM_178129	433–452
P2RY8x2-901R	AAACAAACGGGTCCAGACAG	NM_178129	1069–1088
SOX5ex1-16F	CACCAAAACCCATCTCCAGT	BC060773	20–39
SOX5ex1-94F	GCATTAACGAGACCGGGTAA	BC060773	98–117
SOX5ex2-70R	GGGAGGCTTGTCCAAAACTT	BC060773	169–188
SOX5ex6-56R	TGGCTACCTCTCCATCTGCT	BC060773	486–505
SOX5ex6-222R	TGCTGAGAAGTGGGAGTCCT	BC060773	652–671
SOX5ex6-119F[b]	CCCTTGCATGAAGTTGATG	BC060773	568–576/681–687
SOX5ex6-162F[b]	TTCAGCCAGTTTCTCTGCTG	BC060773	611–630
SOX5ex7-42R[b]	ATGAGTTGTGTGGGGCAAAT	BC060773	711–730
SOX5ex7-63R	TCTGCCTTCTGAGGTGAGGT	BC060773	733–752
SOX5ex7-95R	ACAAGGACTCGCCACTCTGT	BC060773	765–784

Abbreviations: F, forward; R, reverse.
[a]Position, at nucleotide level, using the BLAST 2 sequences tool (http://genopole.toulouse.inra.fr/blast/wblast2.html).
[b]Primer used in real-time PCR analysis.

Figure 2 (**a**) Partial metaphase, from the patient's spleen, showing normal and derivative chromosomes 12 and X. Chromosome Y is also shown. From left: DAPI banding, M-FISH analysis, and FISH results using BAC clones RP11-261P4 (column 3, in red), and RP11-77H23 (column 4, in red). Whole chromosome paints of chromosomes X, Y and 12 were pseudocolored in yellow, dark green and pink, respectively (column 2). The purple signal at the tip of the short arm of chromosome Y corresponds to the pseudoautosomal region 1 (pink plus dark green). (**b**) Maps of the breakpoint regions in chromosome bands Xp22.23 (top) and 12q12.1 (bottom). White arrowheads indicate the breakpoint regions mapped by FISH experiments. (**c**) RT-PCR experiments on the cDNA of the patient (lanes 1 and 3) and of a normal control (lanes 2 and 4), using primer sets (**a**) (P2RY8ex1-23F/SOX5ex2-70R, lanes 1 and 2) and (**b**) (P2RY8-23F/SOX5ex6-222R, lanes 3 and 4). Chimeric transcripts of 235 and 718 bp are evident in lanes 1 and 3, respectively. (**d**) Analysis of the *P2RY8/SOX5* fusion transcripts. M: 2-log DNA Ladder (New England Biolabs, Milan, Italy). Lane 1: three fusion transcripts were detected using P2RY8-23F + SOX5ex6-95R in the patient's cDNA. Lane 2: no fusion transcript was detected in a normal peripheral blood sample. Lane 3: blank. (**e**) Complete nucleotide sequence of *P2RY8/SOX5* chimeric transcript 1. The sequence of all the primers used in PCR experiments are underlined (the two halves of SOX5ex6-119F are in purple). The arrowhead indicates the *P2RY8/SOX5* transcript junction, and vertical lines indicate exon boundaries. The alternatively spliced exon 5 (*) in transcript 3 is represented in italics. *SOX5* exon 6 sequence in bold is absent in type 2 and 3 chimeric transcript. (**f**) Expression analysis of *SOX5* evaluated by RQ-PCR, showing a higher *SOX5* transcriptional level in the patient with t(X;12) versus three normal spleen (S1-3) and three nodal FL control samples (NFL1-3); the mean C_t value of the spleen controls (mean value (S1-3)); and the mean C_t value of the nodal FL controls (mean value (NFL1-3)). FISH, fluorescence *in situ* hybridization.

of an oncogene through its juxtaposition to an ectopic promoter is known as 'promoter swapping'.[5] It has been reported in several tumors (pleomorphic adenoma of the salivary glands, lipoblastoma, aneurysmal bone cyst, teratoma). Since the *SOX5* (BC066773) open reading frame was preserved, its upregulation should be just the consequence of the juxtaposition to the *P2RY8* promoter sequence. Thus it can be considered a further example of a promoter swapping.

P2RY8 has not been directly implicated in tumorigenesis. Apart from the t(X;12) rearrangement described in the present study, this gene has been reported to be disrupted in a pericentric inversion of the X chromosome [inv(X)(p22.3;q13.2)] in a family with two mentally retarded males.[6] Database and a literature report[6] clearly show a high expression level of the *P2RY8* gene in lymphocytes and in lymph. Consequently, its promoter can be supposed to be potentially able to activate *SOX5*. The *SOX5* gene encodes a member of the SOX family of transcription factors, which are recognized as key players in the regulation of embryonic development and in the determination of the cell fate. *SOX5* is known to express two types of alternatively spliced transcripts, short (existing only in the testis) and long forms (*L-SOX5*), expressed in multiple tissues and showing several isoforms.[3] The L-SOX5 protein may play an important role in chondrogenesis[3] and in oligodendrocyte development.[4] There is limited knowledge about a possible role of L-SOX5 in other tissues and in human cancer. SOX5 was recently shown to be aberrantly overexpressed in glioma, and recognized as a glioma antigen using IgGs from the sera of glioma patients.[7] *SOX5* was also reported to be amplified, along with *DADR* and *ETNK1*, in testicular seminomas, although no *SOX5* overexpression was detected.[8]

In conclusion, the PSFL case we have studied presents evidence, for the first time, of the overexpression of *SOX5* consequent to a promoter swapping event juxtaposing its coding sequence to the *P2RY8* promoter. Further studies are needed to clarify the possible role of aberrant SOX5 gene expression in the pathogenesis of PSFL and of other t(14;18) negative FL.

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