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The *SYT-SSX1* fusion type of synovial sarcoma is associated with increased expression of cyclin A and D1. A link between t(X;18)(p11.2; q11.2) and the cell cycle machinery

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A recent large multi-centre study convincingly confirmed previous observations that the *SYT-SSX1* fusion type, compared to *SYT-SSX2*, of synovial sarcoma is associated with a worse clinical outcome. Apart from the clinical impact, this fact also suggests (1) that the *SYT-SSX* fusion gene may influence molecular mechanisms involved in tumour growth and progression; and (2) that the *SYT-SSX1* fusion type has a stronger influence on these mechanisms than *SYT-SSX2*. The nature of the underlying mechanisms is, however, still unknown. In this study we made use of the *SYT-SSX1* vs *SYT-SSX2* concept to investigate whether major, tumour relevant, and growth regulatory proteins (e.g. cyclins and cyclin-dependent kinases) may be involved. Using Western blotting analysis on 74 fresh, fusion variant-typed, tumour samples from localized synovial sarcoma, we found a significant correlation between *SYT-SSX1* and high expression of cyclin A ($P = 0.003$) and D1 ($P = 0.025$). Our data suggest that *SYT-SSX* may influence the cell cycle machinery, and that the more aggressive phenotype of the *SYT-SSX1* variant is due to an accelerated tumour cell proliferation.
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Synovial sarcoma constitutes a highly malignant soft tissue sarcoma and has the propensity to occur in the extremities of young and middle-aged adults (dos Santos *et al*., 2001; Ladanyi, 2001). Cytogenetically, synovial sarcoma is characterized by the translocation t(X;18)(p11.2;q11.2) (Turc-Carel *et al*., 1987) resulting in a fusion between the *SYT* gene on chromosome 18 and *SSX1, SSX2* or, rarely, *SSX4* on the X chromosome and the formation of new chimeric genes, *SYT-SSX1, SYT-SSX2* or *SYT-SSX4* (Clark *et al*., 1994; de Leeuw *et al*., 1995; Skytting *et al*., 1999).The function of these fusion genes is, however, still unclear. However, increasing evidence has implicated that *SYT-SSX* could play an important role in synovial sarcoma development and progression (Ladanyi, 2001).

Several independent studies showed that *SYT-SSX1*, compared to *SYT-SSX2*, has a significantly unfavourable prognosis (Kawai *et al*., 1998; Nilsson *et al*., 1999; Inagaki *et al*., 2000). These results were recently confirmed by a multi-institutional retrospective study on 243 cases of synovial sarcoma, in which the *SYT-SSX1* fusion type was found to be the only independent significant factor for overall survival in patients with localized disease at diagnosis (Ladanyi *et al*., 2002). Apart from the clinical impact of this study, it also suggests that *SYT-SSX* interferes with mechanisms involved in tumour growth and progression, as well as that *SYT-SSX1* has a stronger positive influence on these mechanisms compared to *SYT-SSX2*.

Two previous independent studies demonstrated an association between *SYT-SSX1* and an increased proliferation activity, as assessed by Ki67 (Nilsson *et al*., 1999; Inagaki *et al*., 2000). Even though these results are based on limited numbers of cases, the concept is intriguing and consistent with the general assumption that deregulated cellular growth is the most fundamental property for tumour development and progression (Evan and Vousden, 2001). In a recent study we approached this issue by blocking the expression of *SYT-SSX* in cultured synovial sarcoma cells using antisense oligonucleotides. Our results showed that *SYT-SSX* was crucial for expression of cyclin D1. In fact, antisense oligonucleotides to *SYT-SSX* specifically destabilized cyclin D1 and subsequently inhibited the tumour cells growth (Xie *et al*., 2002). These observations imply that the *SYT-SSX*

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fusion gene may play an important role in the cell cycle machinery, and based on them we have now undertaken a study on 74 fresh frozen samples of localized synovial sarcoma to investigate whether *SYT-SSX1* and *SYT-SSX2* fusion types may affect major, cancer relevant, cell cycle regulators differentially.

First we analysed different amounts of proteins (10–90 μg) to determine the optimal amount of loaded protein. For this purpose cyclin D1 was measured. Cyclin D1 signals increased with the amount of loaded proteins (Figure 1a, left panel), and the correlation was found to be linear (Figure 1a, right panel). In the following analyses, 50 μg proteins from each sample were used for Western blotting.

Expression levels of cyclins (A, D1 and E), cyclin-dependent kinases (CDKs), p27 and the proto-oncogene protein Bcl-2 were quantitatively assessed in fresh-frozen tissues from all 74 cases by Western blotting (Figure 1b). β-actin was used as a loading control. The intensities of the signals were quantified by scanning densitometry. All obtained data are presented in Table 1. As shown in Figure 1b and Table 1, the signal intensities of cyclin A, E and D1 and p27 showed the largest variations among the cases.

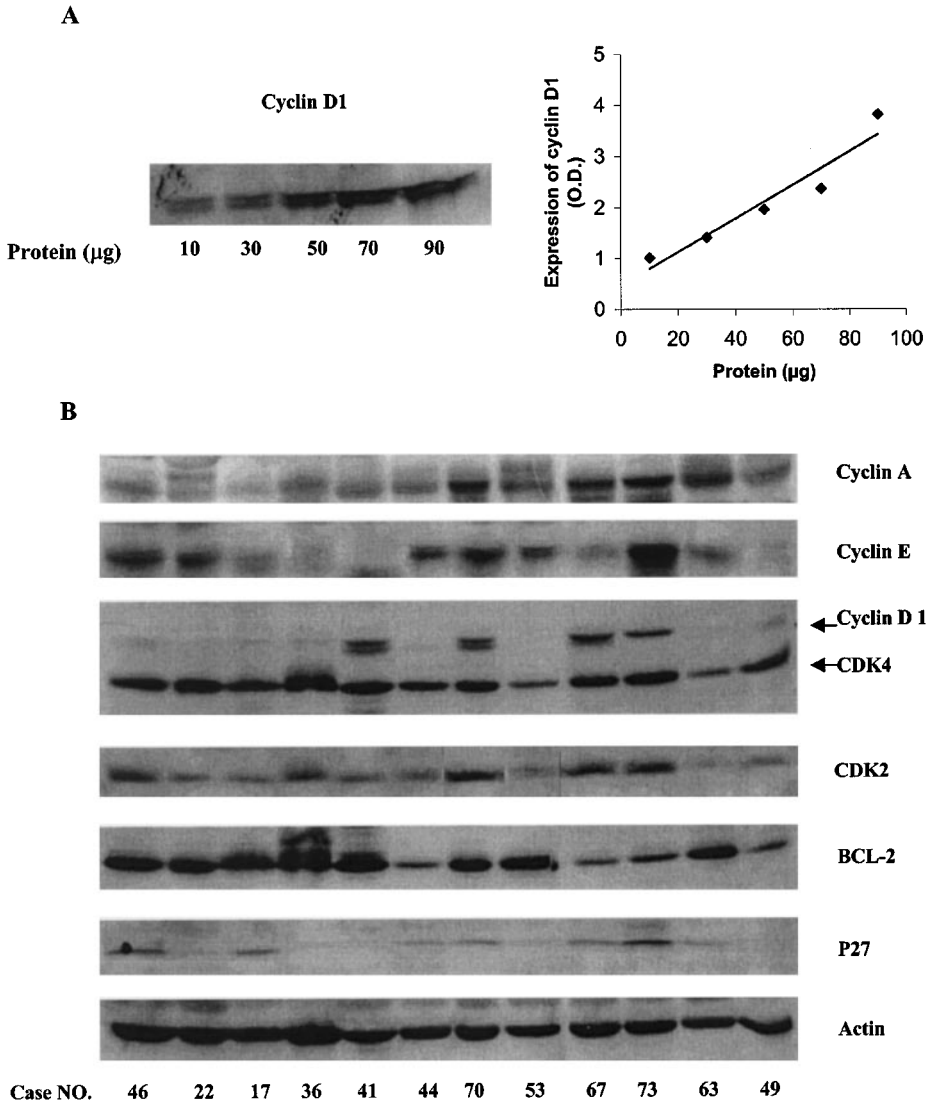

Figure 1 (**a**) Different amounts (10–90 μg) of total proteins were subjected to Western blotting for analysis of signals for cyclin D1 (left panel). The right panel shows densitometric data. (**b**) Cyclin A, D1 and E and their catalytic partners CDK 4 and CDK2, as well as p27 and BCL-2 were detected by Western blotting in 74 fresh-frozen synovial sarcoma samples. All samples were proven to be highly representative for synovial sarcoma tissue. The total protein of the tumour samples was extracted with a lysis buffer (50 mM TRIS-HCl pH 7.5, 150 mM NaCl, 1% NP-40 supplemented with protease inhibitor tablet) for 1 h on ice. After centrifugation at 1600 *g* for 5 min at 4°C, the supernatants, containing the total protein, were assessed by dye-binding Bio-Rad protein assay for protein concentration. For all samples, the same amount of total protein (50 μg) was subjected to SDS–PAGE. The membranes were probed with: anti-cyclin D1 (M-20) (1 : 500), anti-cyclin E (HE-12) (1 : 500), anti-cyclin A (BF-683) (1 : 500), anti-CDK4 (H-22) (1 : 500), anti-CDK2 (M-2) (1 : 500), anti-p27 (1 : 500) and anti-BCL-2 (100) (1 : 500). All these antibodies were from Santa Cruz (CA). β-actin (1 : 500) was used as a loading control. The density of the bands was assessed using Multianalyst software

Table 1 *SYT-SSX* fusion types and expression of cell cycle regulators in 74 samples of localized synovial sarcoma[a]

Case no.	*Fusion type*	*Cyclin A*	*Cyclin D1*	*Cyclin E*	*CDK4*	*CDK2*	*BCL-2*	*P27*
1	2	0	0	2.09	3.72	2.02	5.52	0.12
2	2	0	0.02	0	0.39	0.12	0.15	0.28
3	1	0	0.08	0.91	1.02	0.7	1.97	0.04
4	2	0.04	0	0.36	0.72	1.03	2.08	0.18
5	2	0.05	0	2.18	1.08	0.53	1.6	0.59
6	1	0.07	0.13	0.18	1.04	0.91	2.75	0.01
7	1	0.1	0	0.24	1.2	0.76	2.95	0.28
8	1	0.12	0.19	0.93	1.91	0.83	5.78	0.53
9	1	0.13	0	0	0.23	0.29	1.29	0.02
10	1	0.13	0.08	1.2	2.65	2.14	6.62	0.37
11	2	0.16	0.07	0.68	2.87	1.25	1.2	0.48
12	2	0.18	0.02	1.1	1.36	1.01	2.87	0.02
13	2	0.2	0.37	0.96	5.09	1.17	4.01	0
14	2	0.26	0	0.74	2.01	0.72	5.77	0.13
15	1	0.26	0.01	0.07	1.32	0.47	3.89	0
16	1	0.29	2.74	0.83	8.24	2.11	4.82	0.06
17	2	0.33	0.21	0.54	3.23	0.93	4.23	0.34
18	2	0.35	0	0.91	1	0.77	1.76	0.06
19	2	0.43	0.17	0	3.11	1.01	6.33	0.1
20	2	0.43	0.24	0.51	2.23	0.95	4.86	0.17
21	1	0.44	0.08	0.34	3.33	0.72	3.16	0.34
22	2	0.53	0.03	1.58	4.7	1.08	4.65	0.05
23	2	0.56	0	1.58	4.78	1.42	5.46	0.62
24	1	0.56	0.14	0.06	2.64	0.91	2.1	0
25	2	0.57	0	0.16	1	0.35	0.23	0.04
26	2	0.58	1.13	1.24	4.39	2.34	4.43	0.39
27	2	0.61	0.04	0.74	2.37	0.96	2.04	0.04
28	2	0.63	0.1	3.24	5.36	1.05	6.98	0.28
29	1	0.64	0.18	0.36	3.15	1.19	2.52	0.06
30	1	0.65	0.09	0.72	1.18	0.92	1.87	0
31	2	0.66	0.02	1.23	3.1	1.37	6.61	0.19
32	2	0.67	0	0.61	0.59	0.34	0.08	0.05
33	1	0.69	0.84	1.32	1.61	0.66	4.08	0.11
34	1	0.72	0	0	1.89	1.67	2.47	0.09
35	1	0.72	1.27	0.73	2.95	1.75	5.11	0.07
36	1	0.74	0.24	0.25	4.98	2.11	3.78	0.04
37	2	0.77	0.15	0.75	2.78	1.54	4.99	0.49
38	1	0.78	1.51	0.53	2.58	1.56	2.66	0.21
39	1	0.78	0.3	1.09	4.45	1.34	3.58	0.16
40	2	0.85	0.25	0.78	3.41	2.3	5.6	0
41	2	0.92	2.31	0	4.68	1.18	5.48	0.03
42	2	0.94	0.09	1.26	4.44	1.68	3.37	0.38
43	1	0.95	0.63	0	3.35	0.94	2.54	0.2
44	2	0.97	0.04	1.55	3.33	1.29	1.43	0.18
45	2	1	0.98	1.38	5.01	1.09	3.72	0.03
46	2	1.04	0.38	2.85	4.77	2.82	6.15	0.87
47	2	1.08	0.11	0.42	6.17	0.88	3.56	0.33
48	1	1.08	0.62	0.68	2.49	1.93	4.2	0.03
49	2	1.14	0.38	0.03	3.6	1.04	1.23	0.03
50	1	1.2	2.21	2.64	3.24	1.66	0.28	0.06
51	1	1.22	0.57	1.89	4.27	2.56	2.95	0.09
52	1	1.25	0.17	0.32	1.67	0.89	4.88	0.51
53	2	1.27	0	1.42	1.48	0.91	6.65	0.1
54	1	1.48	0.54	0.35	3.42	1.14	3.93	0.37
55	1	1.58	0.81	0.41	5.18	2.32	6.16	0.4
56	2	1.68	0	2.88	2.88	1.16	1.24	0.03
57	1	1.73	0.26	1.16	4.99	1.54	1.35	0.32
58	2	1.82	0.04	0.62	4.82	2.1	5.7	0.31
59	1	1.87	2.27	0.43	5.58	0.95	4.66	0.56
60	1	1.91	0.09	0.99	2.96	2.09	4.37	0.44
61	1	1.98	0.25	1.49	1.04	1.45	3.87	0.42
62	1	2.01	0.21	0.97	2.01	1.3	2.06	0.08
63	1	2.05	0	0.64	0.97	0.5	5.32	0.18
64	1	2.06	0.27	2.41	3.42	0.36	4.42	0.33
65	1	2.25	0.16	1.97	1.73	1.33	1.8	0.63
66	1	2.27	0.72	0.68	4.52	1.48	4.29	0.57
67	1	2.27	2.35	0.68	4.29	2.63	1.25	0.32
68	2	2.28	2.27	0.58	4.92	2.33	4.16	0.3
69	1	2.29	0.24	0.02	7.23	1.09	5.56	0.2

Continued

Table 1 (*Continued*)

Case no.	*Fusion type*	*Cyclin A*	*Cyclin D1*	*Cyclin E*	*CDK4*	*CDK2*	*BCL-2*	*P27*
70	2	2.31	1.97	2.23	4.2	3.22	4.82	0.33
71	1	2.51	0.32	1.68	4.38	0.72	3.09	0.5
72	1	2.64	0.36	0.2	2.71	1.1	0.66	0.25
73	1	2.98	1.6	4.07	5.18	2.71	2.26	0.81
74	1	3.13	1.19	2.47	2.02	1.49	3.35	0.88

[a]Types of *SYT-SSX* fusion were determined by RT–PCR and sequence analysis as previously described (Nilsson *et al*., 1999). Expression levels of the proteins were assessed by Western blotting and densitometry

To determine suitable 'cut offs' between low and high expression for the investigated proteins, we made distribution histograms regarding intensities for each of them. The distribution histograms for cyclins A and D1 are shown in Figure 2. As can be seen, cyclin A exhibits a bimodal distribution and the cut off was set between the two peaks at baseline resolution level (Figure 2a). This means that the cut off is set at a comparably insensitive level, and that limited adjustments of it would not affect the outcome of statistical analysis. From this it follows that 53 and 21 cases having a low and high cyclin A expression, respectively. The variation of cyclin D1 expression was also large but the distribution pattern differed considerably from that of cyclin A. There was a large and relative narrow peak with no or very weak expression (OD 0–0.4) representing the majority of cases (54 cases), followed by a very broad 'peak' with higher expression (OD 0.5–2.8) representing the remaining 20 cases (Figure 2b). The cut off was therefore set between these peaks at OD 0.4–0.5, which is also a quite insensitive level. In a corresponding manner, using the distribution curves, we could identify suitable cut off levels with base line resolutions for the other proteins investigated. These were for cyclin E, p27, Bcl-2, CDK4 and CDK2; 1.0 (range 0–4.1), 0.4 (range 0–0.9), 3.7 (range 0.1–7.0), 3.5 (range 0.2–8.3) and 1.4 (range 0.1–3.2), respectively.

Using RT–PCR and sequence analysis it was found that 40 and 34 cases expressed *SYT-SSX1* and *SYT-SSX2*, respectively (Table 1). No atypical transcripts were encountered in these cases. Applying the above cut off values, we analysed the correlation between high expression of each cell cycle regulatory protein and *SYT-SSX* fusion type. Statistical analysis showed that a high level of either cyclin A or D1 was significantly associated with the *SYT-SSX1* fusion type. In the *SYT-SSX1* group, 17 cases expressed a high level of cyclin A, compared to only four cases of high cyclin A in the *SYT-SSX2* group ($P=0.003$) (Figure 3a). Even if we used the mean value of cyclin A expression (1.02) as cut off, the correlation was still significant ($P=0.007$) (see Table 1). For cyclin D1, 15 cases with high level of cyclin D1 was located in the *SYT-SSX1* group, compared to only five cases in the *SYT-SSX2* group ($P=0.025$) (Figure 3b). Even if we used the median value as cut off, there was still a significant correlation between *SYT-SSX1* and high cyclin D1.

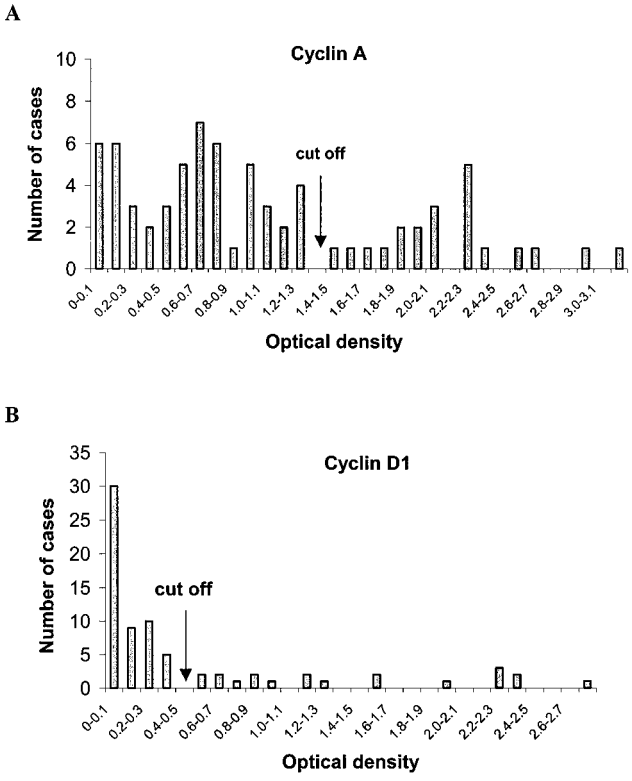

Figure 2 Distribution of cyclin A (**a**) and cyclin D1 (**b**) expression in 74 synovial sarcoma samples. The signals of cyclin A and D obtained by Western blotting were quantified by densitometry

In contrast to cyclin A and cyclin D1, no significant correlations were found between the expressions of Bcl-2, cyclin E, CDK2, CDK4, p27 and *SYT-SSX* fusion type. Even when we changed the cut off levels, none of them exhibited any significant association to the fusion variants (data not shown).

We also calculated the mean values ± standard error of means (mean ± s.e.m.) of cyclin A and D1 expression, using the O.D. values (Table 1), for all 40 *SYT-SSX1* and 34 *SYT-SSX2* cases (see Table 1). Herewith the mean ± s.e.m. of cyclin A expression was 1.26 ± 0.14 and 0.74 ± 0.10 in the *SYT-SSX1* and *SYT-SSX2* cases ($P=0.0005$), respectively. The corresponding values for cyclin D1 were 0.59 ± 0.11 and 0.34 ± 0.11 ($P=0.01$). These data provide further support for a link between *SYT-SSX1* and cyclin A

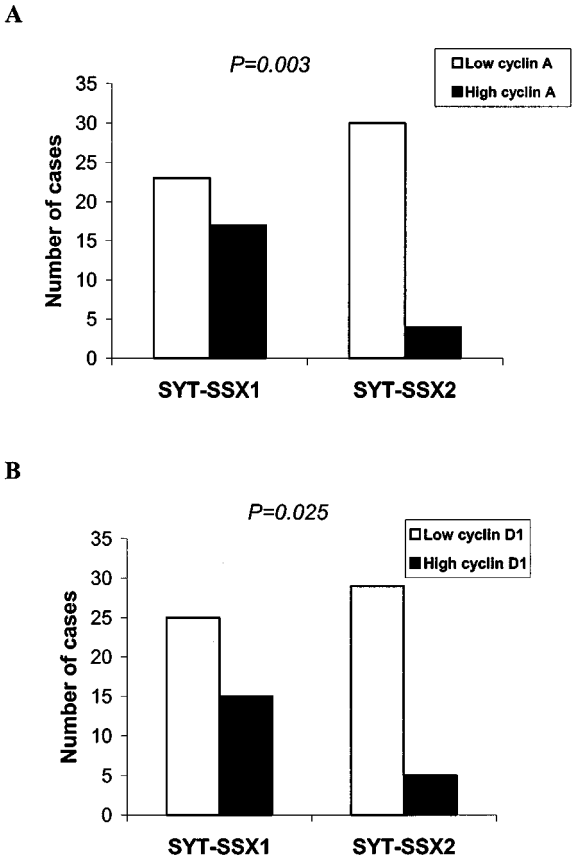

Figure 3 The association between high or low expression of cyclin A (**a**) or cyclin D1 (**b**) and *SYT-SSX1* or *SYT-SSX2*. Statistical analysis was performed using the χ^2-test, *P* values less than 0.05 was considered as statistically significant

and D1. In contrast, there was no significant correlation between *SYT-SSX1* and mean expression of any of the other proteins.

Cyclin D1 is accumulated in mid-G1 phase, and over expression of it has been reported to shorten the G1 phase and cause more rapid entry into S phase (Quelle *et al*., 1993). Cyclin A is essentially expressed in the S phase, in which DNA replication occurs, but is also involved in the G2-M phase transition (Sherr, 1996). Over expression of cyclin A or D1 has been demonstrated to be associated with tumour progression and/or poor prognosis in a variety of human malignancies, including soft tissue sarcomas, breast, esophageal, pancreatic and colorectal carcinomas (Kim *et al*., 1998; Shamma *et al*., 1998; Handa *et al*., 1999; Huuhtanen *et al*., 1999; Bukholm *et al*., 2001).

Since the cyclinA/CDK2 complex is required for DNA replication during S phase and initiation of mitosis, the expression level of cyclin A is usually closely linked to the proliferation indices (Bukholm *et al*., 2001; Handa *et al*., 1999; Huuhtanen *et al*., 1999). This fact taken together with our finding that cyclin A is higher expressed in *SYT-SSX1* cases, suggests that the tumour cells of this fusion type harbour a higher proliferative activity compared to the *SYT-SSX2* cells. This would also be consistent with the observations that the *SYT-SSX1* cases exhibited comparatively faster onset of metastatic disease (Kawai *et al*., 1998; Nilsson *et al*., 1999).

Cyclin D1, which is synthesized and assembled with either CDK 4 or CDK 6 in early and mid-G1 phase, is the major driving event in the transition from the G1 to S phase of the cell cycle (Sherr, 1995). Therefore, the increased expression of cyclin D1 in the *SYT-SSX1* tumours may reflect an increased transfer of cells from G1 to S. Actually, most of the cases with an high level of cyclin D1 also exhibited an increased expression of cyclin A (see Table 1).

Bcl-2, which belongs to a family of key regulators for protection of tumour cell apoptosis (Adams and Cory, 1998), was first found in human follicular lymphoma with the translocation t(14; 18)(q32; p21), juxtaposing the IgH-joining region with Bcl-2 gene from 18q21 and thereby constitutively activating the Bcl-2 expression. In line with a study by Mancuso *et al*. (2000), we found that the expression of Bcl-2 was strongly expressed in the majority of synovial sarcomas. This event may be important for the malignant phenotype of synovial sarcoma. However, we can conclude that the Bcl-2 expression level is independent of *SYT-SSX* fusion type.

Some studies have shown that over expression of cyclin E is associated with unfavourable prognosis in certain types of tumours (Mishina *et al*., 2000; Nielsen *et al*., 1996). We could, however, not find any correlation between cyclin E and the two fusion types.

P27 is a key inhibitor in cell cycle regulation and belongs to the CIP1/KIP1 family. P27 is able to inhibit several cyclin/CDKs, whose degradation is required for G1 to S phase transition (Hunter and Pines, 1994; Sherr, 2000). Consistently, we found that p27 expression was comparatively weak in the synovial sarcoma samples. This profile would favour the malignant growth of this tumour type. However, similar to the case with Bcl-2 and cyclin E we did not find any link between p27 and the *SYT-SSX* fusion types.

So far the exact function of the fusion protein and wild type *SYT* and *SSX* proteins is not clear. The C-terminal of *SYT* protein is rich in the glutamine, proline, glycine and tyrosine residues and seems to be involved in transcriptional activity. In contrast, *SSX* possesses a repressor domain located in the C-terminus, which is retained in the fusion protein (Thaete *et al*., 1999). Thus, the *SYT-SSX* fusion protein contains both potential transcriptional and repressor domains. Even though *SYT-SSX1* and *SYT-SSX2* show high homology, 13 amino acids differ in the *SSX*-part (de Leeuw *et al*., 1995). Seemingly, this difference may be enough to alter the functional capacity of the *SYT-SSX1* and *SYT-SSX2* proteins.

Taken together, our present results, demonstrating a link between fusion types and cyclin A and D1, provide further support to the notion that *SYT-SSX* influences the cell cycle machinery, as well as this influence may be differential with regard to type of *SYT-SSX* fusion.

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