Pathol. Oncol. Res. (2011) 17:357–367
DOI 10.1007/s12253-010-9334-y

RESEARCH

The Effect of SYT-SSX and Extracellular Signal-Regulated Kinase (ERK) on Cell Proliferation in Synovial Sarcoma

Wenjuan Cai · Yan Sun · Wei Wang · Chunrong Han · Mamoru Ouchida · Wenbin Xia · Xiulan Zhao · Baocun Sun

Received: 14 January 2010 / Accepted: 4 November 2010 / Published online: 14 January 2011

Abstract The character of Synovial sarcoma is the chromosomal translocation t(X; 18)(p11.2;q11.2), which results in the fusion of the SYT gene with a SSX gene. There is little study that could fully elucidate the mechanism of pathogenesis of this fusion transcript. This study is designed to gain more insight into the function of this fusion gene. We evaluated the whole genome expression in SYO-1 cells inhibited as a result of specific small interfering RNA for SYT-SSX. Cell proliferation and apoptosis were analyzed by flow cytometer and MTT. The proteins correlated with proliferation were also detected using western blot. TUNEL and Immunohistochemical stain assessment were also carried out on TMA of SS tissues. The mRNA level reduced over 90% caused by SYT-SSX specific siRNA. Five pathways were employed, that ERK1/2 pathway was differential significantly (p= 0.043218). Meanwhile, down-regulation of SYT-SSX fusion gene expression would inhibit the proliferation of SS cell and the survival rate decreased (34.1%), while apoptotic rate increased (10.92%). After transfected with SYT-SSX-specific siRNA it caused a block in G1/G0 phase (31.99%) of SYO-1 cells compared with control cells. The protein level of ERK1/2, p-ERK, and cyclin D1 altered in same trend with expression of SYT-SSX. In TMA stain assessment, SYT-SSX positive group with high ki-67 LI expressed more cyclin D1and CDK4 than the SYT-SSX negative group. High ki-67 LI was detected in cases with p-ERK expression. Meanwhile, cyclin D1 and CDK4 were shown to be more expressed in tumor cells with p-ERK expression. Our results suggest that the fusion gene SYT-SSX should be considered to play important role on SS cell growth via ERK pathway. This study may be valuable for understanding the pathogenic role and molecular mechanism of the fusion gene SYT-SSX in synovial sarcoma through the proposed genome-wide approach. Furthermore, the research would open up the possibility of using SYT-SSX and ERK as a therapeutic target.

Keywords Synovial sarcoma · Cell proliferation · Fusion gene

Wenjuan Cai, Yan Sun, and Baocun Sun contributed equally to this work.

W. Cai · Y. Sun · W. Wang · C. Han · W. Xia · B. Sun (✉)
Department of Pathology, Tianjin Cancer Institute & Hospital,
Tianjin Medical University,
Tianjin, People's Republic of China
e-mail: baocunsun@gmail.com

W. Cai · W. Wang · C. Han · X. Zhao · B. Sun
Department of Pathology, Tianjin Medical University,
Tianjin 300060, People's Republic of China

W. Cai
Department of Pathology, Tianjin First Center Hospital,
Tianjin, People's Republic of China

W. Wang
Department of Otorhinolaryngology, Tianjin First Center Hospital,
Tianjin, People's Republic of China

M. Ouchida
Department of Molecular Genetics, Graduate School of Medicine and Dentistry, Okayama University,
Okayama, Japan

Introduction

Synovial sarcoma (SS) accounts for about 10% of all soft tissue sarcomas and occurs mainly in children and young adults. It is a highly malignant soft tissue tumor. More than a quarter of patients succumb to synovial sarcoma in the

first 5 years after diagnosis despite of the best available management [1]. Although the tumor is named synovial sarcoma because of its histologic resemblance to normal synovial tissue, its exact histogenesis or line of differentiation remains unclear [2].

It has recently been manifested that the chromosomal translocation t(X; 18)(p11.2;q11.2) was the genetic character of SS, resulting in the fusion of the SYT gene with a SSX gene. [3]. Moreover, SYT-SSX fusion protein was believed to be an early genetic event and play critical roles in tumorigenesis of SS [3, 4]. However, the function of the SYT-SSX and the underlying molecular mechanisms remain poorly understood. Considering SYT-SSX fusion protein contains both transcriptional activating and repressor domains, SYT-SSX is functionally linked to the transcriptional regulatory machinery [3]. Nagai et al. reported that the suppression and activation of down-stream genes of SYT-SSX might play central roles in the development of synovial sarcoma [5]. To gain more insight into the function of this fusion gene, we analyzed changes of the whole genome expression after SYT-SSX was blocked in synovial sarcoma cell line (SYO-1) and got some down-stream genes of SYT-SSX in this study.

Some studies initially showed a potential link between SYT-SSX and tumor cell proliferation in SS [6, 7]. Nevertheless, it has not been elucidated about the concrete mechanism. Only Xie et al. [8, 9] reported that SYT-SSX might affect the growth of SS cells by controlling the expression of cyclin D1. Therefore, in the present study, the influence of SYT-SSX on proliferation, apoptosis and cell cycle of tumor cells were studied in SS cells and tumor tissues. Besides, the function of the down-stream genes of SYT-SSX involved in cell proliferation was also analyzed so that we could partly suggest the mechanism of SYT-SSX and its down-stream genes in regulating cell proliferation in SS.

Materials and Methods

RNA Interference for SYT-SSX

A 19-nucleotide target sequence derived from human SYT-SSX mRNA (Genbank accession NO. NM_005637.2) was designed by siRNA Wizard software of the Invitrogen Corporation. Two distinct siRNA duplexes for SYT-SSX were synthesized by Takara Bio Inc. (Dalian, PR China). The sequences of the siRNA for SYT-SSX were listed in Table 1. BLAST (Basic Local Alignment Search Tool) searches in the human genome database were carried out to ensure that the sequences would not match with other gene transcripts. The pBAsi-hU6 Neo DNA plasmid (Takara Bio Inc., Dalian, PR China) was used as the vector.

Table 1 The sequences of the siRNAs targeting SYT-SSX and control siRNA

	Sequences of the siRNA
siRNA1	5'-ACAGACATGTTGCCGCCCA-3'
	5'-TGGGCGGCAACATGTCTGT-3'
siRNA2	5'-TCAGGTGCATAGCAAGTGA-3'
	5'-TCACTTGCTATGCACCTGA-3'
Control siRNA	5'-AACCACGCTCAGATCGGAA-3'
	5'-TTCCGATCTGAGCGTGGTT-3'

The human synovial sarcoma cell line SYO-1 (a generous gift from Dr Mamoru Ouchida), expressed SYT-SSX2 fusion transcript [10], was routinely cultured in DMEM (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum(Hyclone, Utah,USA) and penicillin-streptomycin. SYO-1 cells were plated in six-well tissue culture dishes in maintenance media and incubated for 24–48 h before transfection. On the day of transfection, media were replaced with serum-free and antibiotic-free Opti-MEM® (Invitrogen Life Technologies, California, USA). The transient transfections were performed using 4.0 μg DNA complex with 10 μl Lipofectamine™ 2000 (Invitrogen Life Technologies, California, USA) at 37°C, 6 h. After the transfection, the media were replaced with serum-containing maintenance media and the cells were incubated for 72 h. Negative control siRNA (scrambled siRNA sequences) and Lipofectamine™ without DNA were also used, which served as controls for specificity.

Total cellular RNAs were isolated using Trizol Reagent (Invitrogen Life Technologies, California, USA) and cDNA was synthesized with a reverse transcription kit (Takara Bio Inc, Dalian, PR China). The RT-negative control was included. Then quantitative real-time PCR (qRT-PCR) was performed with the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, California, USA) using SYBR Green Premix Ex Taq™ (Takara Bio Inc, Dalian, PR China) in order to manifest the inhibiting effect of SYT-SSX. The qRT-PCR reaction was performed as the protocol of manufactory. The qRT-PCR conditions were 95°C for 10 s initial denaturation followed by 40 cycles of 95°C for 30 s, 60°C for 90 s. The melting curves were generated at the end of the run. The GAPDH was analyzed as the reference (housekeeping) gene. The primers for GAPDH and SYT-SSX were shown in Table 2. Moreover, each measurement was performed in triplicate. Data were analyzed with GeneAmp 5700 SDS Software (Applied Biosystems, California, USA) and raw cycle threshold (Ct) values were obtained for each sample. These Ct values were then expressed as a fraction of the control transcript levels using the delta–delta Ct (DDCt) method incorporat-

Table 2 The primers for real-time RT-PCR

	Sequences of primers
SYT-SSX	5'-AGCAGAGGCCTTATGGATATGA-3
	5'-CATGCCCATGTTCGTGAAA-3'
ERK1	5'-GCAAGCACTACCTGGATCAGCTC-3'
	5'-TCGGGCCTTCATGTTGATGATA-3'
ERK2	5'-TGTTCCCAAATGCTGACTCCAA-3'
	5'-TCGGGTCGTAATACTGCTCCAGATA-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'
	5'-GAAGATGGTGATGGGATTTC-3'

ing the GAPDH levels as the internal standard. The DDCt values were normalized to controls and expressed as a fold difference: 2E(–DDCt) [11].

Microarray Analysis

The microarray analysis was performed on a platform of whole genome microarray, Human OneArray™ microarray (Phalanx Biotech Group, Hsinchu, Taiwan). Total RNA from SYO-1 cells expressing SYT-SSX or depleted of SYT-SSX by siRNA treatment was changed to cRNA with incorporation of Cy5-CTP (experimental samples), or Cy3-CTP (control samples). The labeled cRNA was hybridized onto Human OneArray™ microarray triply contained 32,050 DNA oligonucleotide probes. GenePix pro V6.0 was used to read the raw intensity of the image. Expression of genes was considered to be up-regulated at least with FoldChange >=1.5 and down-regulated at least with FoldChange <=0.67 by the RNA interference. All microarray data were submitted to the Database of Annotation, Visualization and Integrated Discovery (DAVID) 2008 (National Institute of Allergy and Infectious Diseases (NIAID), NIH, http://david.abcc.ncifcrf.gov/). These differential expressed genes were further analyzed by GO (gene oncology) analysis on the web of DAVID, which is the web-based data mining environment for gene sets.

qRT-PCR for Some Down-stream Genes of SYT-SSX

Some down-stream genes of SYT-SSX chosen from the microarray analysis and involved in cell proliferation were further verified. Their expression was compared in SYT-SSX-blocked SYO-1 and control SYO-1 cells by qRT-PCR. The method mentioned above. The primers for ERK1/2, which were down-stream genes of SYT-SSX, were listed in Table 2.

Western Blot for Some Down-stream Genes of SYT-SSX

The protein expression of some related down-stream genes was detected by western blot. Protein lysates were prepared from each sample, using cold protein extraction buffer. Protein concentrations for every sample were determined with the BCA protein assay. Lysates were then boiled and loaded into a 10% SDS-PAGE gel. Total protein (25 μg) was analyzed for the expression of ERK1/2, phosphorylated ERK1/2 (p-ERK), cyclin D1, and CDK4 (antibodies from Abcam, MA, USA). A monoclonal antibody for β-actin was used to ensure equal loading and normalization of protein levels. As secondary antibodies, horseradish peroxidase–labeled anti-rabbit antibodies (Santa Cruze, California, USA) were used, followed by enhanced chemiluminescence (Thermo scientific, MA, USA) detection. The density of the bands was assessed using the Quantity One software (Bio-Rad Laboratories, California, USA).

Cell Proliferation, Cell Cycle and Apoptosis Analysis

Cell proliferation was measured using the methyl thiazolyl blue tetrazolium bromide (MTT, Sigma, Missouri, USA) colorimetric dye assay. 1×10^5 cells per well were seeded into 96-well plates in quintuplicate. SYO-1 cells were transfected with either control siRNA or SYT-SSX-specific siRNA vector. Only lipofectemine™ treated cells were also used as control. Twenty-four hours, 48 h, 72 h after transfection, 20 μl MTT (5 mg/ml in PBS) was added to each well and the cells were incubated at 37°C. After 4–6 h, 150 μl cell lysis reagent (DMSO, Sigma, Missouri, USA) was used, and absorbency was monitored at 570 nm with an EL-311SX enzymelinked immunosorbent assay reader (Bio-Tek Instruments, Vermont, USA). The experiment was replicated three times.

Fixed cells were treated with 25 μg/ml RNase A at 37°C for 30 min and then stained with propidiumiodide (PI) (50 μg/ml, Sigma, Missouri, USA) solution for 30 min in the dark. Cell cycle analysis was conducted by fluorescence-activated cell sorting with a Becton-Dickinson flow cytometer. Histograms generated by fluorescence-activated cell sorting were assessed by ModFit Cell Cycle Analysis Software (Verity Software House Inc., USA) to determine the percentage of cells in each phase.

Apoptotic cells were measured with an Annexin V/FITC kit (BD Biosciences, San Diego, California, USA) according to the manufacturer's instructions and analyzed by flow cytometer after transfection. Viable cells were negative for both PI and annexin V, while apoptotic cells were positive for annexin V. The apoptotic percentage of 10,000 cells was determined.

TUNEL and Immunohistochemical Assessment on Tissue Microarray

Seventy-four cases were selected into the study, including 54 SYT-SSX positive SSs tested by RT-PCR, 4 SYT-SSX

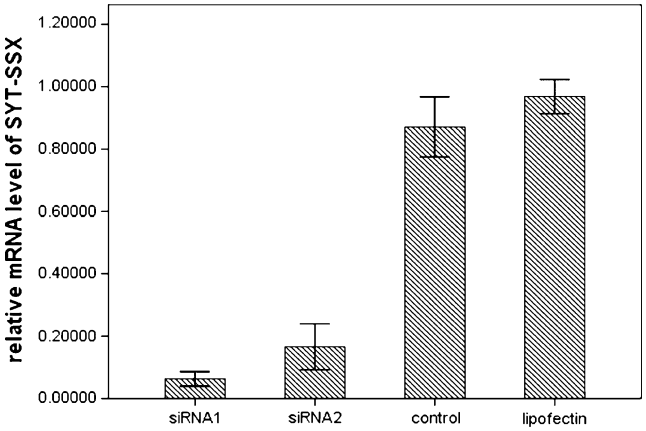

Fig. 1 The relative mRNA level of SYT-SSX in siRNA transfected SYO-1 cells relative to that in control cells after being normalized to GAPDH mRNA. SYT-SSX mRNA level transfected with siRNA1 (0.06±0.02), with siRNA2(0.17±0.07) *P<0.05

negative SSs (diagnosed as SS according to typical clinical context, histologic aspect and immunohistochemical profile), and 16 non-SSs (four malignant melanoma, four Ewing's sarcomas, four malignant peripheral nerve sheath tumor and four hemangio-peritheliomas).

A tissue microarray (TMA) was constructed using a manual tissue arrayer (Beecher Instruments Inc, Sun Prairie, WI, USA). Two typical areas from different locations in each case were selected according to HE slides and marked in the paraffin blocks. Then, two 0.6-mm cores from the two typical areas were taken into the array. Moreover, two positive (SS with SYT-SSX fusion gene confirmed by sequencing) and two negative (normal colon mucosa without SYT-SSX confirmed by sequencing) controls were included in the TMA. The cases were ordered according to the pathological number instead of categories

Visualization of DNA fragmentation, a marker of apoptosis, was performed by the TUNEL method using the In Situ Apoptosis Detection Kit (Zymed Laboratories Inc., San Francisco, CA, USA) according to instructions. In each case, at least 5,000 tumor cells were examined in the most evenly and distinctly labelled areas (ones around necrosis were avoided) at high-power field (×400), and the number of TUNEL-positive tumor cells was counted. The apoptosis index (AI) was calculated as the percentage of TUNEL-positive tumor cells in examined tumour cells.

Immunohistochemistry staining was performed on TMA. The monoclone antibodies for ki-67(clone Ki-67), cyclin D1(clone A-12), CDK4(clone DCS-35) and p-ERK(clone E-4) were from Santa Cruze biotechnology, inc. The Ki-

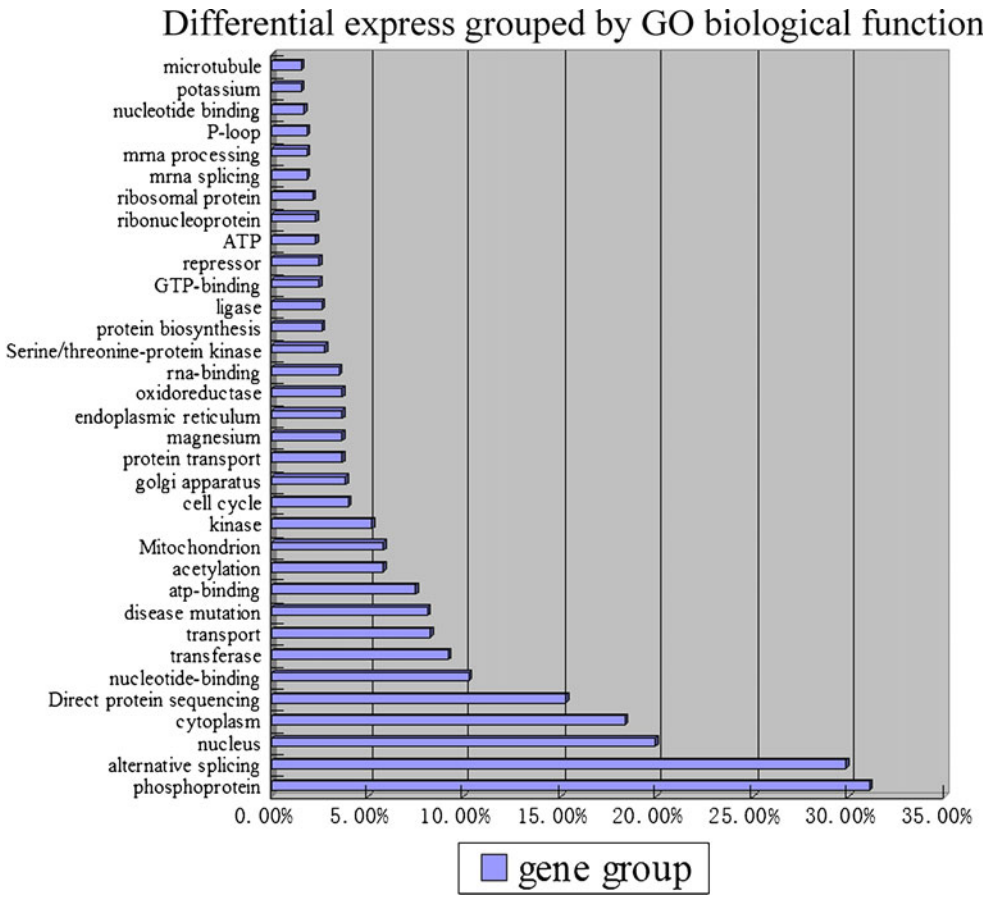

Fig. 2 Differential expression grouped by GO biological function

Table 3 Biocarta pathway analysis with all changed genes

Category term	Count	%	*P* value
h_erk Pathway: Erk1/Erk2 Mapk signaling pathway	11	1.68	0.043218*
h_her2 Pathway: Role of ERBB2 in signal transduction and oncology	4	0.63	0.067049
h_gh Pathway: growth hormone signaling pathway	4	0.63	0.083721
h_mapk Pathway: MAPKinase signaling pathway	8	1.26	0.091814
h_keratinocyte Pathway: Keratinocyte differentiation	5	0.79	0.098157

*P-value 0.05 was used as a cut-off

67-labelling index (LI) was estimated by counting the percentage of Ki-67-positive cell nuclei per 1,000 tumour cells in the region with the greatest density of staining. The expressions of cyclin D1, CDK4, and p-ERK were analyzed using the German semi-quantitative scoring system in considering the staining intensity and area extent. The intensity of marker expression was quantified using the following scores: 0 = negative, 1 = weakly positive, 2 = moderately positive, 3 = strongly positive. The extent of marker expression was quantified by

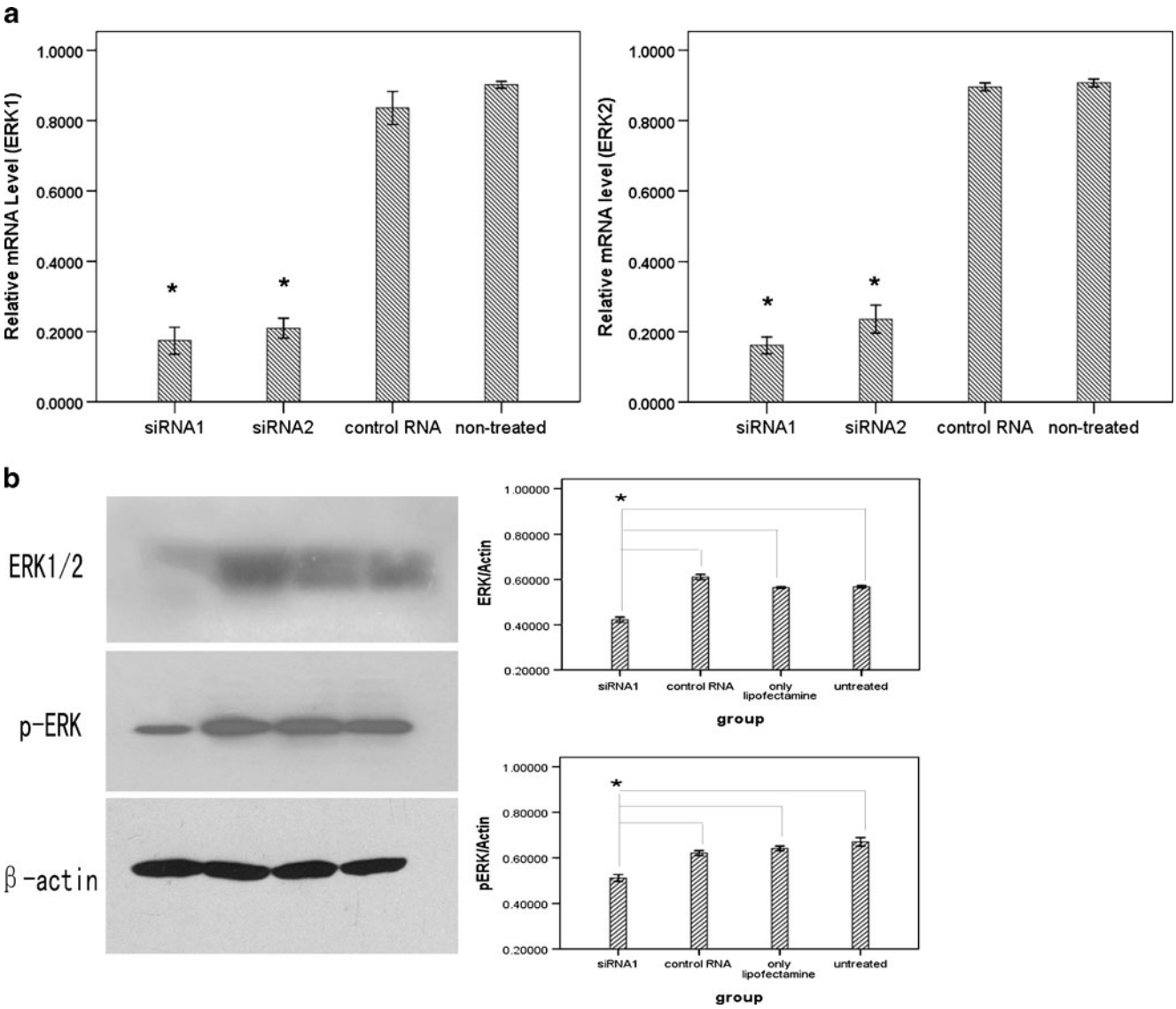

Fig. 3 **a** Effects of down-regulation of SYT-SSX on ERK1/2 expression. The relative mRNA level of ERK1(0.17±0.04) and ERK2(0.21±0.03) were significantly lower than that of control RNA and normal cells.*P<0.05. **b** The protein level of ERK1/2 and p-ERK detected by western blot. The density of band with siRNA1 was decreased compared with the control cells. ERK(0.42±0.01) p-ERK (0.51±0.01) *P<0.05

evaluating the percentage of the positive staining areas (0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–80%, and four points = 81–100%). The final immunoreactive score was determined by multiplying the intensity of positivity and the area extent of positivity scores, yielding a range from 0 to 12. The expression of p-ERK was definited to be positive when the final immunoreactive score was not less than 4 [12, 13].

Statistics

Statistical comparisons were made by using ANOVA with subsequent application or *t* test where appropriate. *P* value of less than 0.05 was considered statistically significant. All data were analyzed with SPSS 13.0 software (SPSS Inc., Chicago, USA).

Results

RNA Interference in SYO-1

The SYT-SSX expression in SYO-1 transfected with two distinct siRNAs, negative control siRNA and lipofectamine™ was shown in Fig. 1. Compared with the cells only treated with lipofectamine™, the expression of SYT-SSX reduced about 93.7% and 83.4% in the cells transfected with SYT-SSX-specific siRNA1 and siRNA2, respectively. However, there was no obvious difference of SYT-SSX expression between cells transfected with negative control siRNA and ones only treated with lipofectamine™.

Microarray Analysis

Compared with gene expression in cells transfected with negative control siRNA, the expression of 375 genes was up-regulated and of 418 genes was down-regulated in cells transfected with SYT-SSX-specific siRNA1 ($P<0.05$). Among the 793 genes with significant change, 653 genes could be assigned into different functional categories with DAVID gene functional category analysis (Fig. 2). Subsequently, five pathways hit by biocarta pathway analysis were indicated, including ERK1/2 pathway ($P=0.043$, Table 3).

qRT-PCR and Western Blot

The expression of ERK was confirmed to be significantly deceased after blocking SYT-SSX (ERK1 mean=17.42%, ERK2 mean=16.15%) by qRT-PCR (Fig. 3a). Moreover, protein expression of ERK and p-ERK detected by western blot was equal to the results of qRT-PCR (Fig. 3b).

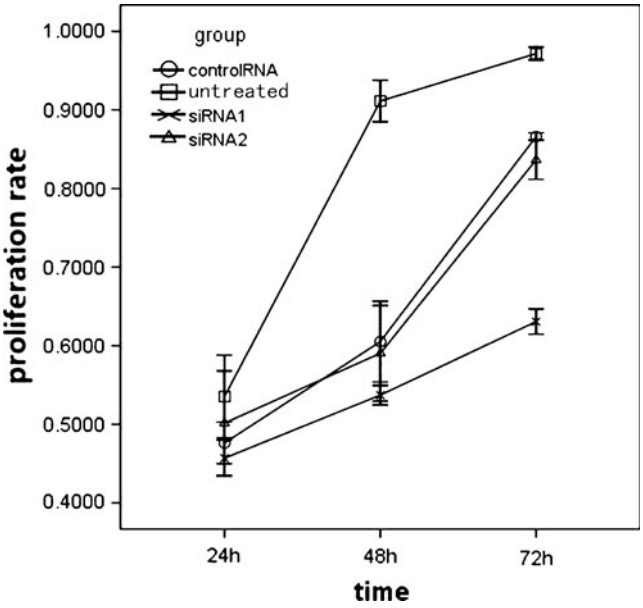

Fig. 4 Suppressed SYT-SSX expression inhibits cell proliferation. The survival rate of SYO-1 cells transfected with SYT-SSX-specific siRNA was most significantly reduced compared with that of control SYO-1 cells at 72 h. Cell proliferation was determined by MTT assay

Proliferation and Apoptosis Analysis in SYO-1

The growth of SYO-1 cells was observed at 24 h, 48 h, 72 h after transfection. As shown in Fig. 4, the proliferation rate of SYO-1 cells transfected with specific siRNA1 (mean=0.631) was most significantly reduced compared with that of SYO-1 cells only treated with lipofectamine™ (mean=0.972) at 72 h. While the proliferation rate of cells transfedcted with siRNA2 remained largely unchanged. The proliferation rate of cells with siRNA1 was the lowest at 72 h

In comparison with control SYO-1 cells treated with lipofectamine™, SYT-SSX-specific siRNA1 and siRNA2 caused an increase in the percentage of G1/G0 phase cells, of about 31.99% and 22.53% accompanied by a significant decrease in the percentage of G2/M (6.31% and 0.6%)and S phase cells(25.84% and 18.00%) respectively (Fig. 5a). In addition, cell cycle related proteins, cyclin D1 and CDK4 were detected in SYO-1 cells after transfection with specific siRNA1 and negative control siRNA. The expression of cyclinD1 in siRNA1 transfected SYO-1 cells was significantly lower than that in negative control siRNA tranccripted cells and cells untreated (Fig. 5b). But there was no notable change of CDK4 expression in SYO-1 cells treated with different siRNAs.

Furthermore, in the cell apoptosis test with flow cytometer, it was shown the apoptotic rate in SYT-SSX-specific siRNAs transfected cells obviously increased to 10.92% and 9.77%. That in negative control siRNA

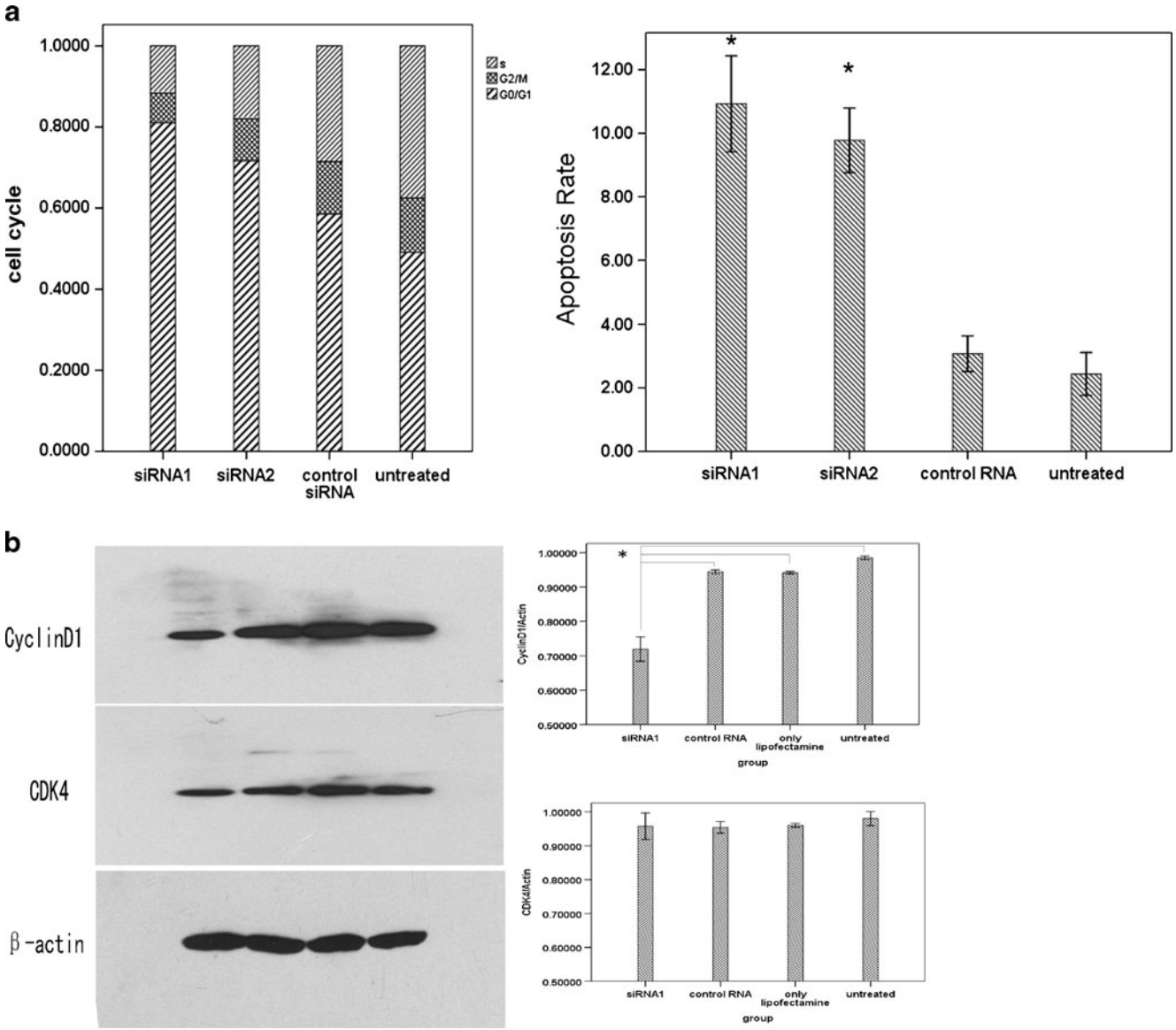

Fig. 5 **a** Assessment of cell cycle and apoptosis by flow cytometer. The distribution of cell cycles in SYO-1 cells after transfection. The apoptosis rate with siRNA increased significantly compared with control cells. * $P<0.05$. **b** expression of cyclin D1 and CDK4 in SYO-1 cells. SYO-1 cells were transfected with siRNA expressing plasmids or control siRNA expressing plasmid. β-actin was used to ensure equal loading and normalization of protein levels. * $P<0.05$

transfected cells and in cells treated with lipofectamine™ were 3.07% and 2.43% (Fig. 5a).

Proliferation and Apoptosis Analysis in Tumor Tissues

CyclinD1, CDK4, and ki-67 were expressed in the nucleus of tumor cells, while the expression of p-ERK was observed on membrane of tumor cells (Fig. 6). Cases positive for SYT-SSX showed higher ki-67 LI (mean=23.13) than SYT-SSX negative ones (mean=16.35) (Table 4). In comparison of cases not detected SYT-SSX, expression of cyclinD1 (mean=3.74/2.18), CDK4 (mean=5.42/4.05) and p-ERK (mean=4.29/3.18) showed a trend to be more expressed in cases with SYT-SSX ($P<0.05$) (Table 4). TUNEL-positive tumor cells with homogeneously stained condensed nuclei were found scattered among viable tumour cells. Although the AI means in SYT-SSX positive SSs was higher than that in SYT-SSX negative cases, there were not significant difference between TUNEL AI according to expression of SYT-SSX (Table 4). At the threshold of four scores for p-ERK IHC analysis, cases were divided into two groups: negative ($n=38$) and positive ($n=36$). Subsequently, a higher ki-67 LI (mean=27.64/12.71) were shown in p-ERK positive group. Moreover, cyclinD1 (mean=3.94/2.72) and CDK4 (mean=6.28/3.88) were more

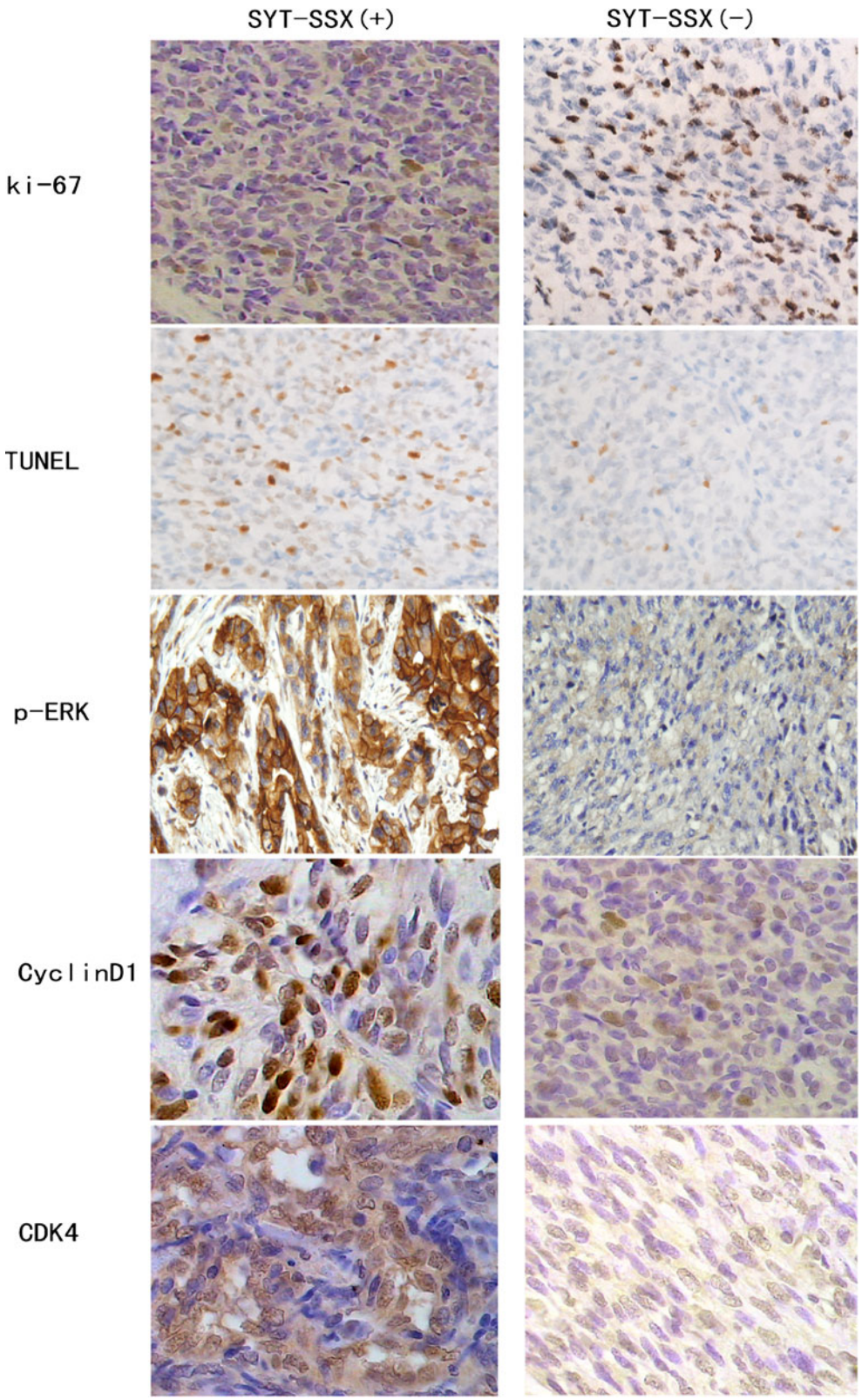

Fig. 6 The ki-67 LI and TUNEL AI were differential significantly between groups SYT-SSX positive and negative. Expression of cyclinD1 and CDK4 in nucleus of cells of SS tumor cells, expression of p-ERK on membrane of tumor cells (SP; original magnification ×400)

expressed in p-ERK positive group than those in negative group (Table 5). There were not significant differences between TUNEL AI according to expression of p-ERK.

Discussion

The translocation t(X;18)(p11.2;q11.2), giving rise to the SYT-SSX fusion genes, has been shown to be consistent

Table 4 Ki-67 LI, TUNEL AI and expression of p-ERK, cyclinD1 and CDK4 in *SYT-SSX* positive or negative group (n=74)

	SYT-SSX		t	P
	Positive(n=54)	Negative(n=20)		
Ki-67 LI	23.13±11.44	16.35±10.29	2.322	0.023*
TUNEL AI	1.99±1.78	1.70±1.26	0.667	0.507
p-ERK	4.29±1.91	3.18±1.62	2.308	0.024*
cyclinD1	3.74±2.05	2.18±1.58	0.174	0.003*
CDK4	5.42±2.44	4.05±2.44	0.838	0.036*

*P-value 0.05 was used as a cut-off

with and specific for SS [3]. Fusion proteins generated by such translocations were often an early genetic event and play critical roles in tumorigenesis [3, 4]. However, the function of the SYT-SSX and the underlying molecular mechanisms remains poorly understood. Considering SYT-SSX fusion protein contains both transcriptional activating and repressor domains, SYT-SSX is functionally linked to the transcriptional regulatory machinery. Nagai et al. believe that the suppression and activation of downstream genes of SYT-SSX may play central roles in the development of synovial sarcoma [5].

Suppressing the SYT-SSX fusion gene in synovial sarcoma cells has been reported [9, 14]. We used specific siRNA to block the expression of SYT-SSX and investigated the effect of SYT-SSX depletion on cell proliferation. In present study, we constructed that plasmid-based RNAi that had the function silence SYT-SSX gene expression in human synovial sarcoma cells. At the same time, two siRNA duplexes were synthesized to avoid the off-target effect. Both siRNA-1 and siRNA-2 were valid in blocking SYT-SSX, however, the inhibiting effect of the former was better than that of the latter. The expression of SYT-SSX was inhibited over 90% on mRNA level after siRNA-1 transfected. But detection on protein level could not be performed for there was not SYT-SSX protein specific antibody. Therefore, siRNA-1 was used for subsequent microarray analysis.

Overall, the microarray analysis showed 793 differentially expressed genes which changed at least ±1.5 fold. Among them, 375 transcripts were up-regulated, and 418 were down-regulated. Those genes were involved in phosphoprotein, transcription regulation, alternative splicing, direct protein sequencing, nucleotide-binding, transport, cell cycle, kinase and so on. Then pathway analysis indicated 11 differentially expressed genes (1.68%) were included in ERK pathway. The following qRT-PCR and western blot detection for ERK also manifested down-regulation of SYT-SSX significantly reduced both ERK and p-ERK expression. Likewise, the expression of p-ERK in SYT-SSX-positive SSs was significantly higher than that in SYT-SSX-negative tumors. From those results, we hypothesized initially that SYT-SSX might play an important role via ERK pathway. ERK is a miscellaneous kinase and can phosphorylate more than 100 different substrates [15]. Therefore activation of ERK may affect a broad array of cellular functions, including proliferation, survival, apoptosis, motility, transcription, metabolism and differentiation, and is in part responsible for oncogenesis [15].

In the present study, the cell growth curve showed a distinguished diversity, which indicated the survival rate of SS cell would be reduced significantly after blocking SYT-SSX. Consistent with results in SYO-1 cells, ki-67 LI in SYT-SSX positive SSs was obviously higher than that in SYT-SSX negative cases. The above results indicate SYT-SSX maybe affect the proliferation of tumor cells in SS. Meanwhile, SYO-1 cells were generally arrested in the G0/G1 phase after treatment with SYT-SSX siRNA. Although SYT-SSX was showed to have effect on apoptosis rate in SYO-1 SS cells by flow cytometer analysis, there was not significant difference of AI between SYT-SSX positive and negative cases. Because the condition in bodies is complicated, apoptosis will be regulated by many mechanisms. SYT-SSX is only one of many factors influencing apoptosis.

It was well established that a high expression of cyclin D1 is important for tumor cell proliferation and oncogenesis [16]. Control of cyclin D1 may occur at the transcriptional and translational level [17–19], but prompt and severe changes in cyclin D1 levels are usually regulated via protein degradation [20–22]. There was a significantly decrease of cyclin D1 protein level in present study after blocking SYT-SSX. In TMA analysis, expression of cyclin D1 in SYT-SSX-positive cases was significantly higher than that in SYT-SSX-negative ones. The role of SYT-SSX in expression of cyclin

Table 5 Ki-67 LI, TUNEL AI and expression of cyclinD1 and CDK4 in p-ERK positive or negative group (n=74)

	Expression of p-ERK		t	P
	Positive (n=36)	Negative (n=38)		
Ki-67 LI	27.64±11.47	12.71±6.93	5.464	0.000*
TUNEL AI	1.95±1.83	1.87±1.38	0.192	0.848
cyclinD1	3.94±2.11	2.72±1.81	2.672	0.009*
CDK4	6.28±2.45	3.88±1.94	4.647	0.000*

*P-value 0.05 was used as a cut-off

D1, as demonstrated in this study, may be important for proliferation of synovial sarcoma. In CDK4 expression analysis on TMA, significant difference was observed. It is regulated association with D-type cyclins, activating T172 phosphorylation [23]. However in present study, the protein CDK4 level did not alter significantly in western blot test. It remains possible that in complicated body conditions, Ras/Raf/MEK/ERK and other pathways (such as mTOR) might cooperate by activating different CDK4 kinases (and/or inactivate different CDK4 phosphatases).

Several observations suggested that Ras/Raf/MEK/ERK pathway was important for growth factor-stimulated cell-cycle re-entry [24]. ERK1/2 pathway inhibitors PD98059 and U0126 inhibit expression of cyclin D and induce G1 cell cycle arrests via the ERK1/2 pathway [25]. The inhibition of CDK4 activity is believed to result from down-regulation of cyclin D [25] and/or accumulation of the CDK inhibitor $p27^{kip1}$[26]. The SYT-SSX expression caused a significant changed expression of ERK in SYO-1 cells and SSs tissue in present study. In TMA analysis, expression of cyclin D1 and CDK4 showed same trend to be more expression as consequence of p-ERK expression. The present study thus provided the evidence that SYT-SSX might signal the cell cycle progression through adjusting of the expression of ERK, which appears as the crucial node integrating the SYT-SSX and cyclinD/CDK4.

In summary, our results suggest that the fusion gene SYT-SSX should be considered to play important role on SS cell growth maybe via ERK pathway. Drugs for ERK pathway might be expected to have effects on SS patients. As a result, the potential therapeutic area of human synovial sarcoma would be enlarged.

Acknowledgment The authors thank the National Natural Science Foundation of China (grant number 30572097) and Tianjin Education Committee of China (grant number 20080120) for the financial support. The authors appreciate Dr. Mamoru Ouchida (Department of Molecular Genetics, Graduate School of Medicine and Dentistry, Okayama University) for the generous gift of SYO-1.

Disclosure Statement No potential conflicts of interest were disclosed.

Financial Support The National Natural Science Foundation of China (grant number 30572097); Tianjin Education Committee of China (grant number 20080120).

References

1. Lewis JJ, Antonescu CR, Leung DH et al (2000) Synovial sarcoma: a multivariate analysis of prognostic factors in 112 patients with primary localized tumors of the extremity. J Clin Oncol 18(10):2087–2094
2. Clark J, Rocques PJ, Crew AJ et al (1994) Identification of novel genes, SYT and SSX, involved in the t(X;18)(p11.2;q11.2) translocation found in human synovial sarcoma. Nat Genet 7 (4):502–508
3. dos Santos NR, de Bruijn DR, van Kessel AG (2001) Molecular mechanisms underlying human synovial sarcoma development. Genes Chromosom Cancer 30(1):1–14
4. Ladanyi M (2001) Fusions of the SYT and SSX genes in synovial sarcoma. Oncogene 20(40):5755–5762
5. Nagai M, Tanaka S, Tsuda M et al (2001) Analysis of transforming activity of human synovial sarcoma-associated chimeric protein SYT-SSX1 bound to chromatin remodeling factor hBRM/hSNF2 alpha. Proc Natl Acad Sci USA 98 (7):3843–3848
6. Nilsson G, Skytting B, Xie Y et al (1999) The SYT-SSX1 variant of synovial sarcoma is associated with a high rate of tumor cell proliferation and poor clinical outcome. Cancer Res 59(13):3180–3184
7. Inagaki H, Nagasaka T, Otsuka T, Sugiura E, Nakashima N, Eimoto T (2000) Association of SYT-SSX fusion types with proliferative activity and prognosis in synovial sarcoma. Mod Pathol 13(5):482–488
8. Xie Y, Skytting B, Nilsson G et al (2002) 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. Oncogene 21(37):5791–5796
9. Xie Y, Tornkvist M, Aalto Y et al (2003) Gene expression profile by blocking the SYT-SSX fusion gene in synovial sarcoma cells. Identification of XRCC4 as a putative SYT-SSX target gene. Oncogene 22(48):7628–7631
10. Kawai A, Naito N, Yoshida A et al (2004) Establishment and characterization of a biphasic synovial sarcoma cell line, SYO-1. Cancer Lett 204(1):105–113
11. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25(4):402–408
12. Siami K, McCluggage WG, Ordonez NG et al (2007) Thyroid transcription factor-1 expression in endometrial and endocervical adenocarcinomas. Am J Surg Pathol 31(11):1759–1763
13. Remmele W, Schicketanz KH (1993) Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs. subjective grading (IRS). Pathol Res Pract 189(8):862–866
14. Xie Y, Skytting B, Nilsson G et al (2002) SYT-SSX is critical for cyclin D1 expression in synovial sarcoma cells: a gain of function of the t(X;18)(p11.2;q11.2) translocation. Cancer Res 62(13):3861–3867
15. Ramos JW (2008) The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. Int J Biochem Cell Biol 40 (12):2707–2719
16. Yu Q, Geng Y, Sicinski P (2001) Specific protection against breast cancers by cyclin D1 ablation. Nature 411(6841):1017–1021
17. Bose S, Chandran S, Mirocha JM, Bose N (2006) The Akt pathway in human breast cancer: a tissue-array-based analysis. Mod Pathol 19(2):238–245
18. Hsu MK, Qiao L, Ho V et al (2006) Ethanol reduces p38 kinase activation and cyclin D1 protein expression after partial hepatectomy in rats. J Hepatol 44(2):375–382
19. Ramdass B, Maliekal TT, Lakshmi S et al (2007) Coexpression of Notch1 and NF-kappaB signaling pathway components in human cervical cancer progression. Gynecol Oncol 104(2):352–361
20. Liu Y, Xi L, Liao G et al (2007) Inhibition of PC cell-derived growth factor (PCDGF)/granulin-epithelin precursor (GEP) decreased cell proliferation and invasion through downregulation of cyclin D and CDK4 and inactivation of MMP-2. BMC Cancer 7:22

21. Watanabe M, Miyajima N, Igarashi M, Endo Y, Watanabe N, Sugano S (2008) Sodium phenylacetate inhibits the Ras/MAPK signaling pathway to induce reduction of the c-Raf-1 protein in human and canine breast cancer cells. Breast Cancer Res Treat
22. Recchia AG, Musti AM, Lanzino M et al (2009) A cross-talk between the androgen receptor and the epidermal growth factor receptor leads to p38MAPK-dependent activation of mTOR and cyclinD1 expression in prostate and lung cancer cells. Int J Biochem Cell Biol 41(3):603–614
23. Bockstaele L, Coulonval K, Kooken H, Paternot S, Roger PP (2006) Regulation of CDK4. Cell Div 1:25
24. Lobenhofer EK, Huper G, Iglehart JD, Marks JR (2000) Inhibition of mitogen-activated protein kinase and phosphatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ 11(2):99–110
25. Squires MS, Nixon PM, Cook SJ (2002) Cell-cycle arrest by PD184352 requires inhibition of extracellular signal-regulated kinases (ERK) 1/2 but not ERK5/BMK1. Biochem J 366(Pt 2):673–680
26. Motti ML, De Marco C, Califano D et al (2007) Loss of p27 expression through RAS–>BRAF–>MAP kinase-dependent pathway in human thyroid carcinomas. Cell cycle 6(22):2817–2825