

# Molecular alterations of monophasic synovial sarcoma: loss of chromosome 3p does not alter RASSF1 and MLH1 transcriptional activity

L. Pazzaglia<sup>1</sup>, M.S. Benassi<sup>1</sup>, P. Ragazzini<sup>1</sup>, G. Gamberi<sup>1</sup>, F. Ponticelli<sup>1</sup>,  
A. Chiechi<sup>1</sup>, C.M. Hattinger<sup>1</sup>, L. Morandi<sup>4</sup>, M. Alberghini<sup>2</sup>, L. Zanella<sup>2</sup>, P. Picci<sup>1</sup> and M. Mercuri<sup>3</sup>

<sup>1</sup>Laboratory of Oncologic Research, <sup>2</sup>Department of Pathology and

<sup>3</sup>5th Orthopaedic Division, Rizzoli Orthopaedic Institute, Bologna, Italy and <sup>4</sup>Department of Pathology, Bellaria Hospital, Bologna, Italy

**Summary.** Differential diagnosis of monophasic synovial sarcoma requires the detection of specific biological markers. In this study we evaluated the presence of molecular alterations in 15 monophasic synovial sarcomas. Multiple changes affecting chromosome arms were detected by CGH-array in all microdissected cases available, and an association between gain or loss of specific regions harbouring cancer progression-associated genes and aneuploid status was found. The most frequent alteration was loss of 3p including 3p21.3-p23 region that, however, did not involve the promoter regions of the corresponding genes, RASSF1 and MLH1. Using Real-Time PCR, mRNA levels of both resulted moderately high compared to normal tissue; however, the weak to absent protein expression suggests RASSF1 and MLH1 post-transcription deregulation. Moreover, immunohistochemical analysis revealed that both mesenchymal and epithelial antigens were present in diploid tumours. These findings confirm the genetic complexity of monophasic synovial sarcoma and underline the need to integrate different analyses for a better knowledge of this tumour, essential to investigate new diagnostic and prognostic markers.

**Key words:** Synovial sarcoma, Laser capture microdissection, CGH-microarray, Real-Time PCR, Gene expression

## Introduction

Synovial sarcoma (SS) is a clinically and morphologically well defined entity, that displays biphasic differentiation, with variable glandular

formations, and characterized by a specific chromosomal translocation t(X:18)(p11.2;q11.2) (Fisher et al., 2002). No origin from a specific epithelium has ever been demonstrated, and thus SS belongs to the category of soft tissue tumours of uncertain histogenesis, and represents about 8.4% of soft tissue sarcomas (Weiss and Goldblum, 2001). It occurs mostly in adolescents and young adults, from the second to fifth decade of life, but all ages can be affected (Kempson et al., 1998). Microscopically, SS is characterized by four main histologic subtypes: biphasic, monophasic fibrous, monophasic epithelial and poorly differentiated, depending on the predominance of the two cellular components of the tumour. Although it has been traditionally considered a high grade sarcoma, recent advances in the treatment of these malignancies has considerably improved survival of patients (Weiss and Goldblum, 2001). The most important clinical factors improving prognosis are: young age of the patient (15 years or younger), the size of the tumour (5 cm or smaller), its location in the distal extremity and the low tumoural stage.

Molecular analysis of SS has discovered that the recurrent (X;18)(p11.2;q11.2) translocation is a SS-specific genetic alteration resulting in SYT-SSX1, SYT-SSX2 or SYT-SSX4 fusion transcript, useful in the molecular diagnosis of these tumours (Ladany and Bridge, 2000). Although the presence of the same type of fusion transcript in both epithelial and mesenchymal components emphasizes the hypothesis that SS has a monoclonal origin (Kasai et al., 2000), secondary genetic aberrations may influence the tumour growth rate and clinical outcome (Skytting et al., 1999). In particular, a previous study on 67 synovial sarcomas showed that the most frequent aberrations affected 8q, 12q, 13q and 3p chromosomes (Szymanska et al., 1998), and recent genome-wide analyses of gene expression profile suggested classifying SS according to their genetic alterations, which are the potential targets for

molecular therapy (Allander et al., 2002).

Deletion of 3p is frequent in many adult cancers including lung, breast, ovary, testicular, and head and neck carcinomas (Kok et al., 1997). Particularly, loss at 3p21.3-p23 is a very early event in primary cancer development, implying the presence of tumour suppressor genes in this location (Sanchez et al., 1994). RASSF1, a new RAS effector homologue gene, is located in 3p21.3 (Lerman and Minna, 2000) and frequently is inactivated in tumours by methylation of promotor (Aganthangelou et al., 2001). Two distinct GC-rich promotors produce RASSF1A and RASSF1C transcripts (Damman et al., 2000). RASSF1A interacts with the proapoptotic protein kinase MTS1, suggesting its involvement in RAS-regulated apoptotic pathway (Khokhlatchev et al., 2002) and a recent study shows that RASSF1A could inhibit cell cycle progression (Shivakumar et al., 2002).

MLH1 is also located in the 3p21.3-p23 region and this mismatch repair gene plays a fundamental role in the correction of replication errors, leading to cancer development when mutated (Balogh et al., 2003).

In the present study we assessed molecular aspects of a series of grade 3 monophasic SS, in the attempt to characterise the biological parameters indicative of high aggressiveness of the tumour. Since numerous genomic aberrations found in our series of monophasic synovial sarcomas included alterations in the 3p21.3-p23 chromosome region, we investigated the expression of RASSF1 and MLH1 genes in order to study their role in the complex development of this tumour.

## Materials and methods

### Tumor specimens

Specimens of primary grade 3 spindle monophasic SS of the extremities diagnosed at the Rizzoli Institute by

a group of expert pathologists, were obtained from 15 patients before chemo- and/or radiotherapy. Diagnosis was based on hematoxylin-eosin stained samples according to histopathological criteria (Weiss and Goldblum, 2001). Histological grade was determined using the FNCLCC grading system (Guillou et al., 1997). All samples were positive for SYT-SSX1 or SYT-SSX2 fusion types. For each specimen frozen and paraffin-embedded material was available as was both tumour and adjacent normal tissue. In all specimens the percentage of tumour cells estimated after hematoxylin-eosin staining of tissue sections contiguous to those used for the study was equal or more than 90%. For CGH-microarray analysis in only eight cases the quality of the material was eligible. Clinical and pathological characteristics of patients are reported in the Table 1.

### Immunohistochemistry and flow cytometry

Immunohistochemistry (IHC) was performed to test SS antigenicity. 5 micron sections from archival formalin-fixed, paraffin-embedded material, deparaffinized and rehydrated, were immunostained with monoclonal antibodies (Table 2). The detection of immunoreaction was performed with streptavidin-biotin alkaline phosphatase/red/rabbit/mouse detection system (DAKO Glostrup DM).

Expression level of MLH1 and RASSF1 protein was assessed on paraffin-embedded tumour sections with avidin-biotin-peroxidase complex method (BIOMEDA, Foster City CA). Primary antibodies used are shown in the Table 2. Negative controls were performed by omitting the primary antibody. According to the percentage of positive cells samples were scored as negative (no positive cells), focal positive ( $\leq 50\%$  positive cells), or positive ( $>50\%$  positive cells).

DNA content was assessed by flow cytometry. Cytofluorimetric analysis was performed on nuclear

**Table 1.** Clinicopathologic characteristics and clinical outcome of 15 monophasic synovial sarcoma patients.

CASE No.	GENDER	AGE, years	SITE	GRADE	TUMOR SIZE, cm	OUTCOME	FOLLOW-UP MONTHS
1	F	57	leg	3	> 5	DOD	8
2	F	55	leg	3	> 5	DOD	2
3	M	46	leg	3	< 5	NED	28
4	F	45	leg	3	< 5	DOD	45
5	M	39	arm	3	> 5	DOD	14
6	F	74	leg	3	> 5	NED	65
7	M	49	leg	3	> 5	DOD	15
8	F	46	leg	3	> 5	NED	27
9	F	29	leg	3	< 5	NED	33
10	M	61	arm	3	> 5	NED	12
11	M	29	arm	3	< 5	NED	132
12	F	42	leg	3	< 5	DOD	202
13	M	23	leg	3	< 5	DOD	30
14	M	19	leg	3	< 5	DOD	42
15	F	75	leg	3	> 5	DOD	54

F: Female; M: male; NED: non evidence of disease; DOD: died of disease.

## Chromosome alterations in monophasic synovial sarcoma

suspension obtained by trimming cryopreserved tissue according to Vindelov modified method (Vindelov et al., 1986) using Cycle Test Plus Kit (BD, Becton Dickinson, Palo Alto CA). Analysis was performed on 20000 nuclei per sample using a B.D. FACSCAN FLOW CYTOMETER (BD) and the data obtained were analysed with the Cellfit Program. Ploidy evaluation of the sample was performed by referring to the DNA distribution of a normal sample processed parallel to the tested sample. DNA Index (D.I.) was obtained from the ratio between G0/G1 peak of each population present in the specimen and G0/G1 peak of the reference sample. D.I. =1 diploid sample; D.I. ≠ 1 aneuploid sample.

### Laser microdissection and DNA extraction

Paraffin sections were deparaffinized in xylene and rehydrated through graded alcohol series to distilled water. Sections were stained using eosin Y.

Nonmalignant and tumour cells ( $\approx$  2000) were isolated from the slides using laser capture microdissection (LCM) (PixCell II laser capture microdissection system; Arcturus Engineering Inc. Mountain View, CA) (Emmert-Buck et al., 1996). Cells were captured using a 30  $\mu$ m pulse to focally melt a thermoplastic membrane attached to a transparent flat cap. After LCM, the cap containing the captured tissue was placed on a 0.5 ml standard Eppendorf microfuge tube.

Isolation of DNA was performed using QIAamp

DNA Micro Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. Only eight of 15 cases were eligible for CGH array analysis.

### DOP-PCR

DOP-PCR was performed using universal primer 6-MW (5'-CCGACTCGAGNNNNNNATGGG-3') on a thermocycler (GENENCO PTC-200, St. Bakersfield, CA) as previously described (Telenius et al., 1992). Nine initial cycles were performed at low stringency conditions (denaturation at 94°C for 30 seconds (s), annealing at 30°C for 180 s ramp from 30°C to 72°C for 210 s and extension at 72°C for 180 s) followed by 29 cycles in high stringency conditions (denaturation at 94°C for 30 s, annealing at 62°C for 90 s and extension at 72°C for 120 s; a final extension for 480 s at 72°C followed).

### CGH-microarrays

CGH-Microarrays experiments were performed with Vysis GenoSensor System (Vysis Inc., Downers Grove, IL) according to the manufacturer's protocol. The microarray contains target clone DNA (P1, PAC or BAC clone) representing regions that are important in cytogenetics and oncology. 100 ng of tumour and normal DNA (PCR product) were labeled by random priming reaction, with Cy3-dCTP and Cy5-dCTP respectively. (Amersham Pharmacia Biotech, Buckinghamshire UK).

**Table 2.** Antibodies used for IHC.

ANTIBODY	SOURCE	DILUTION	PRETREATMENT	POSITIVE CONTROL
Monoclonal mouse anti vimentin clone V9	DAKO	1:200	No	Internal control
Monoclonal mouse anti-human cytocheratin clone MNF116	DAKO	1:100	Proteinase K	Prostate
Anti human epithelial membrane antigen clone E29	DAKO	Prediluted	No	Kidney
Polyclonal goat anti RASSF1 (n-15)	SANTA CRUZ	1:100	No	Aneurysmatic bone cyst
Monoclonal mouse anti MLH1 clone 14	CALBIOCHEM	1:10	No	Aneurysmatic bone cyst

**Table 3.** Primers and probe used for Quantitative Microsatellite Analysis

	FORWARD PRIMER	REVERSE PRIMER
Locus		
D3S1578	GCCAACACACATTAATCACATA	GGGGCCAAAATCTGCT
D3S1588	TATCCCAGAGATATTTCTTAGC	ACTGCTCTATCCAGGACACA
D3S1612	TCTTTTAGTCAGCAGTTATGTC	CCCATTAAGAAATGTTACTCTAC
Locus reference pool		
D5S643	TGGGCGACAGAGCCATC	TGTGGTGTGCCATTTATTGACT
D9S1794	GAATTGCTTGAACCTGGG	TCTGTGATCTTAGTTTGGGG
D13S1238	CTCTCAGCAGGCATCCA	GCCAACGTAATTGACACCA
D14S988	TGGTGATTGGATATCACTGG	ATGTTATGTAAGGTTTTGTTTTGTT
D19S926	TCTGGTGAGAATTCCTAAGTAGTTC	GGCCTTATGCGTGAGTAGTT
D22S922	TATCTTGATGGTGGTGTGG	TTCCTCAGTTTTACCTGTGCT

Probe 5'FAM-TGTGTGTGTGTGTGTGTGTGTG-3'TAMRA

Hybridization to Genosensor TM Array 300 v.1.0 chip (Vysis Inc., Downers Grove, IL) were performed in a 37°C dry air incubator for 72 hours. After hybridization micro-array chips were rinsed 3 fold for 10 minutes (min) in 50% 2XSSC/formamide solution at 40°C; washed 4x5 min in 1XSSC at room temperature and for 5 min in distilled water. Microarrays were counterstained with the 4,6 diamidino-2-penylindole (DAPI) IV mounting solution and were analyzed using GenoSensor Reader System (Vysis Inc., Downers Grove, IL).

#### Quantitative Microsatellite Analysis (QuMA)

We assessed the copy number of DNA, obtained from microdissected sections, at three loci in 3p21.3-3p23 region using quantitative real time PCR assay described by Ginzinger modified (Ginzinger et al., 2000).

Analysis was carried out using TaqMan PCR system performed on ABI7900 instrument (Applied Biosystems, Foster City, CA). We used internal probes carrying donor and acceptor fluorescence molecules complementary to CA repeats in microsatellite markers and two relative primers (Table 3).

Copy numbers were detected relative to a pooled reference consisting of six primer sets (D5S643, D9S1794, D13S1238, D14S988, D19S926, D22S922) selected from regions where few aberrations have been reported in CGH analysis of this tumour (Szymanska et al., 1998).

PCR was conducted in duplicate with 25 µl reaction of 1X PCR Buffer (Roche Applied Science, Penzberg-Germany), 200 µM deoxynucleotide triphosphate A, T and C, 50 µM deoxynucleotide triphosphate G (Promega, Madison WI), 1X ROX Reference Dye (Invitrogen, Carlsbad CA) 150 µM 7-Deaza-2'-doxy-guanosine-5'-triphosphate (Roche Applied Science, Penzberg-Germany), 1X GC-RICH Solution, 4 µM GT probe, 15 µM primers, 1,25 unit/µl TaqStart (Roche Applied Science, Penzberg-Germany) with 5ng of genomic DNA. Cycling parameters were as follows: 1 cycle of 95°C for 10 min and 40 cycles of 95°C for 20 s, 55°C for 20 s, 72°C for 45 s.

We used a Tolerance Interval (T.I), as described (Ginzinger et al., 2000), to calculate if the obtained DNA copy number from tumour DNA was significantly different from normal DNA.

#### RNA extraction and reverse transcription

Total RNA was extracted from normal and tumour frozen tissue (≈ 150 mg) using TRIzol Reagent (Invitrogen, Carlsbad CA) according to the manufacturer's protocol and stored at 80°C in RNasecure reagent (Ambion, Inc, Austin TX). Concentration of total RNA was measured with spectrophotometer and the 260/280 ratio of RNA was ≈ 1.6. Purity and quality were identified by a denatured gel electrophoresis. Two out of 15 cases were excluded

because of the bad quality of RNA.

Reverse transcription of mRNA was carried out in 100 µl final volume from 400 ng total RNA using High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to manufacture's instructions.

#### Real-time RT-PCR

RT-PCR was performed using an ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA). Expression of target genes MLH1 and RASSF1 was quantified using TaqMan Expression Assays (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. PCR mixture contained 1,25 µl Target Assay Mix 20X or Endogenous Control Assay Mix 20X, 25ng cDNA diluted in 11,25 µl of Rnase free water (Qiagen, Valencia, CA), 12,5 µl Taq Man Universal Master Mix 2X (Applied Biosystems, Foster City, CA) in 25 µl reaction. Following activation of UNG (uracil-N-glycosylase) for 2 min at 50°C and activation of Taq enzyme (95°C for 10 min) genes were amplified by 45 cycles (95°C for 5 s, 60°C for 1 min). For calculation of gene expression we used the 2-ΔΔCT comparative method (Winer et al., 1999). Expression of target genes was normalized to a housekeeping GAPDH gene (TaqMan Expression Assays-Applied Biosystems, Foster City, CA) and normal cDNA from the same patients was used as calibrator.

## Results

### Differentiation markers and DNA content

Presence of positive immunostaining for vimentin was confirmed in all spindle cell SS (≥ 50% of cells). A focal epithelial differentiation (≤ 50% of cells ) was present in 12 out of 15 samples, with scattered positive-

**Table 4.** Vimentin, cytokeratin and human epithelial membrane antigen (EMA) in 15 monophasic synovial sarcoma.

CASE	VIMENTIN	CYTOKERATIN	EMA	PLOIDY
1	foc pos	neg	pos	diploid
2	pos	foc pos	foc pos	diploid
3	foc pos	foc pos	foc pos	diploid
4	pos	neg	neg	aneuploid
5	pos	neg	neg	aneuploid
6	pos	neg	pos	diploid
7	pos	neg	neg	aneuploid
8	foc pos	foc pos	foc pos	aneuploid
9	pos	foc pos	neg	diploid
10	foc pos	neg	foc pos	diploid
11	pos	neg	foc pos	diploid
12	pos	neg	foc pos	aneuploid
13	pos	foc pos	pos	na
14	foc pos	foc pos	foc pos	diploid
15	pos	foc pos	foc pos	diploid

neg: negative; foc pos: focally positive; pos: positive; na: not available.

## Chromosome alterations in monophasic synovial sarcoma

cells for cytokeratin and/or epithelial membrane antigen (EMA) (Table 4).

DNA ploidy analysis (Table 4) revealed 5 aneuploid and 9 diploid cases, the latter always positive to epithelial antigens. DNA content had no influence on clinical outcome of patients with grade 3 SS.

### CGH-Microarray

CGH-microarray, by Genosensor TM Array 300

v.1.0 chip (Vysis) that contains the most important chromosome regions in oncology, showed frequent and complex alterations in all 8 SS analysed (Table 5).

Threshold set corresponded to mean hybridization ratio between tumor and normal is >1.25 for gains and <0.75 for losses. Most frequent gains were detected at 17p12-p11.2(FLI, TOP3A) loci, while losses involved mainly chromosome regions 2q23-q27 (TBR1), 3p25 (RAF1), 3p21.3-p23 (MLH1; RASSF1), 11p15.5 (HRAS) and 12ptel (SHGC-5557). Concerning 3p

**Table 5.** Most frequent DNA copy number gains and losses in 8 monophasic synovial sarcoma.

		DESCRIPTION	GENBANK NUMER ACCESSION
<b>Gains</b>			
PTGS2(COX2)	1q31.1	prostaglandin-endoperoxide synthase 2	AL033533
ITGA4	2q31-q32	Integrin, alpha 4	AC020595
DDX15	4p15.3	DEAH (Asp-Glu-Ala-His) box polypeptide 15	AC027517
C84C11/T3	5p tel	sub telomeric	NA
PIM1	6p21.2	pim-1 oncogene	AL353579
G31341	7p tel	sub telomeric	AC093614
RFC2,CYLN2	7q11.23	replication factor C (activator 1) 2	AC005015
CDK6	7q21-q22	Cyclin-dependent kinase 6	AC004128
SERPINE1	7q21.3-q22	Serine proteinase inhibitor	AC004876
PDGRL	8p22-p21.3	platelet-derived growth factor receptor-like	AF165145
MYC	8q24.12-q24.13	v-myc myelocytomatosis viral oncogene homolog	NA
WI-6509	11q tel	sub. telomeric	AP003025
PACE4C	15q tel	sub telomeric	AQ263469
FRA16D	16q23.2	fragile site, aphidicolin type, common, fra(16)(q23.2)	NA
LZ16	16q24.2	ankyrin repeat domain 11	AC023256
WI-14673	17p tel	sub telomeric	AC015853
LLGL1	17p12-17p11.2	lethal giant larvae homolog 1 (Drosophila)	AC020567
FLI, TOP3A	17p12-17p11.2	flightless I homolog (Drosophila)	NA
PPARBP(PBP)	17q12	PPAR binding protein	AC009283
SHGC-103396	17q tel	sub telomeric	AQ357495
FRA18A(D18S978)	18q12..3	fragile site, aphidicolin type, common, fra(18)(q12.2)	AP001639
CTDP1,SHGC-145820	18q tel	sub telomeric	AP001641
MKKS, SHGC-79896	20p12.1-p11.23	McKusick-Kaufman syndrome	NA
PCNT2(KEN)	21q tel	sub telomeric	AQ618375
BCR	22q11.23	breakpoint cluster region	NA
DXS580	Xp11.2	microsatellite	Z83745
<b>Loss</b>			
PRKCZ	1p36.33	protein kinase C, zeta	AC068198
NRAS	1p13.2	neuroblastoma RAS viral (v-ras) oncogene homolog	NA
TBR1	2q23-q37	T-box, brain 1	AC009487
RAF1	3p25	v-raf-1 murine leukemia viral oncogene homolog 1	NA
MLH1	3p21.3-p23	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	AC006583
D4S2930	4q tel	sub telomeric	NA
6QTEL54	6q tel	sub telomeric	NA
ABCB1(MDR1)	7q21.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	NA
stSG48460	7q tel	sub telomeric	AQ897338
MOS	8q11	v-mos moloney murine sarcoma viral oncogene	AQ009368
E2F5	8p22-q21.3	E2F transcription factor 5, p130-binding	AC011773
HRAS	11p15.5	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	NA
D11S461	11q12.2	microsatellite	NA
SHGC-5557	12p tel	sub telomeric	AC005844
GLI	12q13.2-q13.3	glioma-associated oncogene homolog (zinc finger protein)	NA
IGH(SHGC-36156)	14q tel	sub telomeric	AZ579057
ZNF217(ZABC1)	20q13.2	zinc finger protein 217	NA
20QTEL14	20q tel	sub telomeric	NA

Alterations found in 3 or more cases; NA: no available.

chromosome, numerous alterations were detected in all cases studied with a majority of losses. In only two cases gains were found in the 3p21.3-p23 region (Table 6).

An interesting association was found between aneuploid status and gain of 17q21-q22 (TOP2A), 22q11-23 (BCR), and loss for 1p36.33 (PRKCZ), 5q21-q22 (APC) 14q13 (PNN).

#### QuMA

Tolerance Interval (T.I.) was calculated as 0.4-4.4 using the mean and SD of difference cycle threshold ( $\Delta$ CT) values from 3 microsatellite loci in the 3p21.3-p23 region and pooled for 4 unrelated DNA. By comparative method (Ginzinger et al., 2000), the  $2^{-\Delta\Delta CT} \times 2$  value (DNA copy number), obtained from the tumour sample was considered normal if within the T.I. DNA copy numbers  $> 4.4$  was scored as a gain and  $< 0.4$  was scored as loss (Table 7).

Seven of 8 monophasic SS confirmed genetic abnormalities in 3p21.3-3p23 with losses in 6/8 and gains in 2/8 tumours showing concordance with CGH-array data in 7 of 8 cases.

#### MLH1 and RASSF1 RNA expression

Evaluation of RNA expression by Real-Time PCR of 13 monophasic synovial sarcoma was performed according to the  $2^{-\Delta\Delta CT}$  comparative method. Each gene was considered more expressed, when the value was  $< 1 \pm SD$  and less expressed when the value was  $> 1 \pm SD$ . The value 1 corresponds to fluorescence emission in each amplification reaction for target and reference in normal tissue. A high expression of MLH1 and/or RASSF1 genes was seen in 11 tumor tissue samples compared to correspondent normal tissue. (Table 8). Co-overexpression of both genes was seen in 9 out of 13 cases while two cases showed loss of either one or the other. Expression of RASSF1 mRNA presented a high variability of values ranging from 1.3 to 38.58 while the MLH1 mRNA expression was less changeable with values from 1.59 to 14.12.

#### MLH1 and RASSF1 protein expression

RASSF1 and MLH1 protein analyses were performed by immunohistochemistry in all specimens. As shown in table 9, 14/15 cases resulted positive for RASSF1 (Fig. 1). Immunoreactivity ranged from moderate to weak with a dishomogeneous distribution. Higher intensity expression of RASSF1 protein was associated with higher mRNA levels. On the contrary, MLH1 immunoreactivity was minimal or not detected in any samples studied.

No correlations were found between biological parameters and clinical data of the patients patients.

#### Discussion

Differential diagnosis of monophasic synovial sarcoma from other spindle cell sarcomas like leiomyosarcoma and malignant peripheral nerve sheath tumour requires the detection of biological markers such as specific chromosomal translocation t(X;18)(p11.2;q11.2). However, the discordant results obtained on prognostic significance of SYT-SSX fusion transcripts (Ladanyi et al., 2002; Guillou et al., 2004) and the lack of specific therapies for this rare and complex tumor type direct to further studies for detection of molecular targets useful for prognosis and treatment (Van de Rijn and Rubin, 2002). Previous studies identified a distinct gene expression profile by c-DNA microarrays in SS compared to other soft tissue sarcomas, suggesting a new classification method (Allander et al., 2002; Nielsen et al., 2002).

In order to compare the molecular profile with histopathological aspects we analysed a homogeneous series of monophasic synovial sarcomas from non-treated patients with complete histological and clinical data. DNA ploidy analysis seems to predict clinical outcome of patients with soft tissue sarcoma (Mankin et al., 2002) and we found that the tumors with aneuploid status of DNA presented specific chromosome aberrations frequently involved in the tumor development. Infact our data in monophasic SS agrees

**Table 6.** Aberrations of chromosome 3p analysed by GenoSensor TM array 300v1.0 chip (Vysis).

	3PTEL25 3ptel	3PTEL01 3ptel	VHL3 p25-p26	RAF1 3p25	THRB 3p24.3	MLH1 3p21.3p23	RASSF1 3p21.3	FHIT 3p14.2	p44S10 3p14.1	D3S1274 3p12-3p13
case 1				Red			Green			
case 2				Red		Red				
case 3				Red				Red		
case 4									Red	
case 5	Red			Green		Green				Green
case 6						Red				
case 7							Red			
case 8				Red		Red				

Green: gain; Red: loss

## Chromosome alterations in monophasic synovial sarcoma

with previous studies on solid tumors that found amplification and overexpression of TOP2A gene in breast and gastric carcinoma (Varis et al., 2002; Murthy et al., 2005) and loss of APC in colon cancer (Rupnarain et al., 2004).

Moreover, in accordance with Szymanska (Szymanska et al., 1998), we found losses of chromosome 3p in all SS studied. Chromosome 3p is one of the most frequently altered in carcinoma (Kok et al., 1997) and the high incidence of loss of

heterozygosity at 3p21.3-p23 in many sporadic human cancers have suggested that this locus harbours one or more critical tumours suppressor genes (Agathangelou et al., 2003). In our series CGH-array, confirmed by QUMA analysis on the 8 cases with available DNA,

**Table 7.** Relative DNA copy number at each of target loci determined by Quantitative Microsatellite Analysis in 8 monophasic synovial sarcoma.

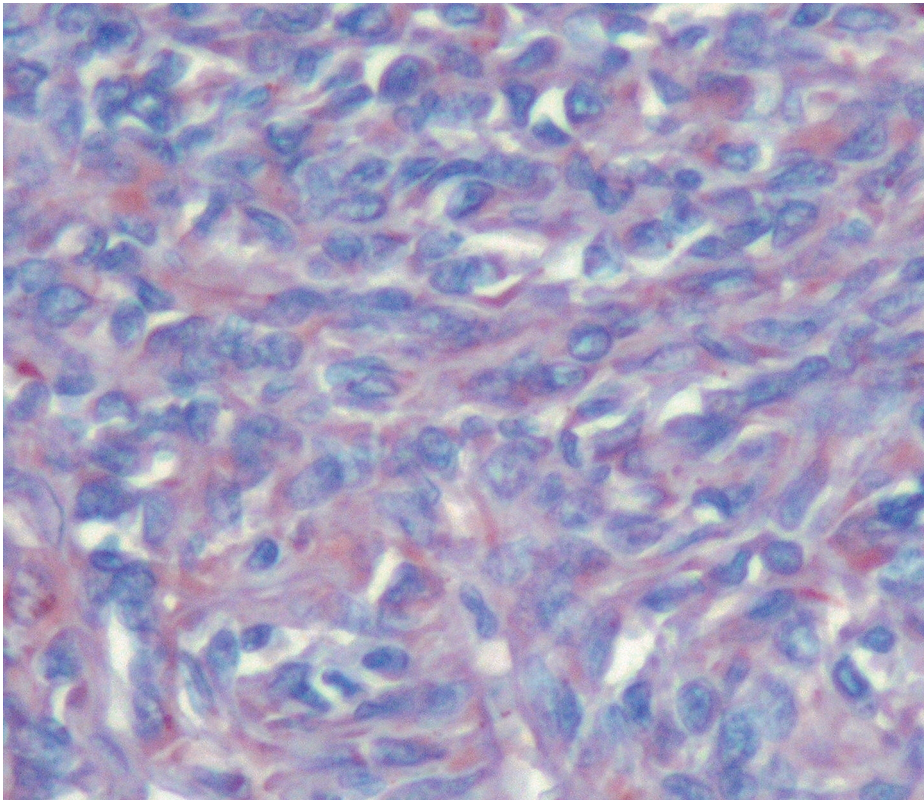
	3p21.3-3p23		3p21.3	
	D3S1612	D3S1578	D3S1588	
Case 1	0,28	5,46	3,14	
Case 2	0,04	0,54	1,04	
Case 3	0,82	2,30	NA	
Case 4	0,76	NA	0,26	
Case 5	5,32	NA	2,34	
Case 6	0,12	0,8	2,58	
Case 7	2,94	0,36	0,76	
Case 8	0,02	NA	0,66	

Tolerance Interval: loss < 0,4-4,4 > gain; NA: not available

**Table 8.** mRNA expression by Real-Time PCR of 13 monophasic synovial sarcoma.

Case	RASSF1		Case	MLH1	
	2- DDCT	SD±		2- DDCT	SD±
1	0.75	0.32	1	0.55	0.28
2	2.45	0.43	2	1.87	0.43
3	1.53	0.05	3	1.12	0.47
4	6.04	0.14	4	1.88	0.08
5	4.97	0.17	5	1.81	0.36
6	20.46	0.15	6	9.88	0.28
7	38.58	0.09	7	14.12	0.17
8	0.53	0.08	8	1.1	0.12
9	5.37	0.22	9	3.64	0.23
10	10.97	0.1	10	6.13	0.1
11	1.3	0.21	11	2.6	0.31
13	7.52	0.24	13	1.59	0.2
14	4.92	0.23	14	3.24	0.22

Each gene was considered more expressed when the value of 2- DDCT was < 1± SD



**Fig. 1.** Positive immunoreactivity for RASSF1 protein in monophasic SS. IHC, x 20

## Chromosome alterations in monophasic synovial sarcoma

**Table 9.** RASSF1 and MLH1 protein expression in 15 monophasic synovial sarcoma evaluated by immunohistochemistry.

CASE	RASSF1	MLH1
1	pos	neg
2	pos	neg
3	pos	neg
4	neg	neg
5	foc pos	neg
6	foc pos	neg
7	pos	neg
8	pos	neg
9	pos	pos
10	pos	neg
11	foc pos	neg
12	pos	foc pos
13	pos	neg
14	pos	neg
15	pos	foc pos

neg: negative; foc pos: focally positive; pos: positive

showed 3p21.3-p23 copy number alterations with prevalence of losses not involving the promoter regions of corresponding genes. In spite of moderate or absent RASSF1 and MLH1 protein expression, relatively high mRNA levels were found in all samples, suggesting post-transcriptional deregulation mechanisms. Both RASSF1 and MLH1 are often lost in human carcinomas (Shivakumar et al., 2002; Plank et al., 2003). Recent studies found epigenetic inactivation of RASSF1 in osteosarcoma (Lim et al., 2003) and leiomyosarcoma (Seidel et al., 2005), but no previous data on SS were reported. It is known that an increased expression of RASSF1A gene may be associated with constitutive activation of RAS, resulting in an RAS-dependent apoptosis (Dammann et al., 2000; Vos et al., 2000). However, the presence of many splice variants determines controversial and not well defined interpretations regarding its action mechanism that is still under investigation.

In conclusion, our results confirm the genetic complexity of monophasic synovial sarcoma revealing the presence of specific alterations in chromosome region that harbour genes controlling the malignancy progression. Moreover, we found that alterations of the copy number in chromosome region 3p21.3-p23 do not involve the transcriptional activity of the corresponding genes that undergo post-transcriptional down-regulation.

*Acknowledgements.* This work was supported by grants from the Istituti Ortopedici Rizzoli (Ricerca Corrente), the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), the Italian Ministry of Health (Ricerca Finalizzata), the Italian Ministry for Education, University, and Research (M.U.R.S.T.), and the C.N.R. Special Project "Oncologia". The authors thank Dr Alba Ballardelli for the revision of the paper and Cristina Ghinelli, medical artist, for graphic work.

## References

- Agathangelou A., Honorio S., Macartney D.P., Martinez A., Dallol A., Rader J., Fullwood P., Chauhan A., Walker R., Shaw J.A., Hosoe S., Lerman M.I., Minna J.D., Maher E.R. and Latif F. (2001). Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene* 20, 1509-1518.
- Agathangelou A., Bieche I., Ahmed-Choudhury J., Nicke B., Dammann R., Baksh S., Gao B., Minna J.D., Downward J., Maher E.R. and Latif F. (2003). Identification of novel gene expression targets for the Ras association domain family 1 (RASSF1A) tumor suppressor gene in non-small cell lung cancer and neuroblastoma. *Cancer Res.* 63, 5344-5351.
- Allander S.V., Illei P.B., Chen Y., Antonescu C.R., Bittner M., Ladanyi M. and Meltzer P.S. (2002). Expression profiling of synovial sarcoma by cDNA microarrays. *Am. J. Pathol.* 161, 1587-1595.
- Balogh G.A., Russo I.H. and Russo J. (2003). Mutations in mismatch repair genes are involved in the neoplastic transformation of human breast epithelial cells. *Int. J. Oncol.* 23, 411-419.
- Dammann R., Li C., Yoon J.H., Chin P.L., Bates S. and Pfeifer G.P. (2000). Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat. Genet.* 25, 315-319.
- Emmert-Buck M.R., Bonner R.F., Smith P.D., Chuaqui R.F., Zhuang Z., Goldstein S.R., Weiss R.A. and Liotta L.A. (1996). Laser capture microdissection. *Science* 274, 998-1001.
- Fisher C., De Bruijn D.R.H. and Geurts Van Kessel A. (2002). Synovial sarcoma. In: *Pathology and genetics tumors of soft tissue and bone.* Fletcher C., Unni K. and Mertens F. (eds.) IARC press. pp 200-204.
- Ginzinger D.G., Godfrey T.E., Nigro J., Moore D.H. 2nd, Suzuki S., Pallavicini M.G., Gray J.W. and Jensen R.H. (2000). Measurement of DNA copy number at microsatellite loci using quantitative PCR analysis. *Cancer Res.* 60, 5405-5409.
- Guillou L., Coindre J.M., Bonichon F., Nguyen B.B., Terrier P., Collin F., Vilain M.O., Mandard A.M., Le Doussal V., Leroux A., Jacquemier J., Duplay H., Sastre-Garau X. and Costa J. (1997). Comparative study of the National Cancer Institute and French Federation of Cancer Centre Sarcoma Group grading system in a population of 410 adult patients with soft tissue sarcoma. *J. Clin. Oncol.* 15: 350-362.
- Guillou L., Benhattar J., Bonichon F., Gallagher G., Terrier P., Stauffer E., Somerhausen N. de S., Michels J.J., Jundt G., Vince D.R., Taylor S., Genevay M., Collin F., Trassard M. and Coindre J.M. (2004). Histological grade, but not SYT-SSX fusion type is an important prognostic factor in synovial sarcoma patients with synovial sarcoma: a multicenter, retrospective analysis. *J. Clin. Oncol.* 22:4040-4050
- Kasai T., Shimajiri S. and Hashimoto H. (2000). Detection of SYT-SSX fusion transcripts in both epithelial and spindle cell areas of biphasic synovial sarcoma using laser capture microdissection. *Mol. Pathol.* 53, 107-110.
- Kempson R.L., Fletcher C. and Evans H.L. (1998). Tumors of uncertain and nonmesenchymal differentiation. In: *Atlas of tumor pathology. Tumors of the soft tissues.* 3rd. Armed Forces Institute of Pathology. Washington DC. pp 472-484.
- Khokhlatchev A., Rabizadeh S., Xavier R., Nedwidek M., Chen T., Zhang X.F., Seed B. and Avruch J. (2002). Identification of a novel Ras-regulated proapoptotic pathway. *Curr. Biol.* 12, 253-256.



## *Chromosome alterations in monophasic synovial sarcoma*

- Kok K., Naylor S.L. and Busys C.H. (1997). Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv. Cancer Res.* 71, 27-92.
- Ladanyi M. and Bridge J.A. (2000). Contribution of molecular genetic data to the classification of sarcomas. *Hum. Pathol.* 31, 532-538.
- Ladanyi M., Antonescu C.R., Leung D.H., Woodruff J.M., Kawai A., Healey J.A., Brennan M.F., Bridge J.A., Neff J.R., Barr F.G., Goldsmith J.D., Brooks J.S., Goldblum J.R., Ali S.Z., Shipley J., Cooper C.J., Fisher C., Skytting B. and Larsson O. (2002). Impact of SYT-SSX fusion type on the clinical behaviour of synovial sarcoma: A multi-institutional retrospective study of 243 patients. *Cancer Res.* 62, 135-140.
- Lerman M.I. and Minna J.D. (2000). The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. *Cancer Res.* 60, 6116-6133.
- Lim S., Yang M.H., Park J.H., Nojima T., Hashimoto H., Unni K.K. and Park Y.K. (2003). Inactivation of the RASSF1A in osteosarcoma. *Oncol. Rep.* 10, 897-901
- Mankin H.J., Fondren G., Hornicek F.J., Gebhardt M.C. and Rosenberg A.E. (2002). The use of flow cytometry in assessing malignancy in bone and soft tissue sarcoma. *Clin. Orthop.* 397, 95-105
- Murthy S.K., Magliocco A.M. and Demetrick D.J. (2005). Copy number analysis of c-erb-B2 (HER-2/neu) and topoisomerase II alpha genes in breast carcinoma by quantitative real-time polymerase chain reaction using hybridization probes and fluorescence in situ hybridization. *Arch. Pathol. Lab. Med.* 129, 39-46.
- Nielsen T.O., West R.B., Linn S.C., Alter O., Knowling M.A., O'Connell J.X., Zhu S., Fero M., Sherlock G., Pollack J.R., Brown P.O., Botstein D. and Van de Rijn M. (2002). Molecular characterisation of soft tissue tumors: a gene expression study. *Lancet* 359, 1301-1307
- Planck M., Ericson K., Piotrowska Z., Halvarsson B., Rambech E. and Nilbert M. (2003). Microsatellite instability and expression of MLH1 and MSH2 in carcinomas of the small intestine. *Cancer* 97, 1551-1557.
- Rupnarain C., Dlamini Z., Naicker S. and Bhoola K. (2004). Colon cancer: genomics and apoptotic events. *Biol. Chem.* 385, 449-464.
- Sanchez Y., El-Naggar A., Pathak S. and Killary A.M. (1994). A tumor suppressor locus within 3p14-p12 mediates rapid cell death of renal cell carcinoma in vivo. *Proc. Natl. Acad. Sci. USA* 91, 3383-3387.
- Seidel C., Bartel F., Rastetter M., Bluemke K., Wurl P., Taubert H. and Dammann R. (2005). Alterations of cancer-related genes in soft tissue sarcomas: hypermethylation of RASSF1A is frequently detected in leiomyosarcoma and associated with poor prognosis in sarcoma. *Int. J. Cancer* 114, 442-447.
- Shivakumar L., Minna J., Sakamaki T., Pestell R. and White M. (2002). The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol. Cell Biol.* 22, 4309-4318.
- Skytting B.T., Szymanska J., Aalto Y., Lushnikova T., Blomqvist C., Elomaa I., Larsson O. and Knuutila S. (1999). Clinical importance of genomic imbalances in synovial sarcoma evaluated by comparative genomic hybridization. *Cancer Genet. Cytogenet.* 115, 39-46.
- Szymanska J., Serra M., Skytting B., Larsson O., Virolainen M., Akerman M., Tarkkanen M., Huuhtanen R., Picci P., Bacchini P., Asko-Seljavaara S., Elomaa I. and Knuutila S. (1998). Genetic imbalances in 67 Synovial sarcoma evaluated by comparative genomic hybridization. *Gene Chromosomes Cancer* 23, 213-219.
- Telenius H., Carter N.P., Bebb C.E., Nordenskjold M., Ponder B.A. and Tunnacliffe A. (1992). Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics* 13, 718-725.
- Van de Rijn M. and Rubin B.P. (2002). Gene expression studies on soft tissue tumors. *Am. J. Pathol.* 161, 1531-1534.
- Varis A., Wolf M., Monni O., Vakkari M.L., Korkola A., Moskaluk C., Frierson H. Jr, Powell S.M., Knuutila S., Kallioniemi A. and El-Rifai W. (2002). Targets of gene amplification and overexpression at 17q in gastric cancer. *Cancer Res.* 62, 2625-2629.
- Vindelov L.L., Christensen I.J. and Nissen N.I. (1986). A detergent - trypsin method for the preparation of nuclei for Flow Cytometric DNA analysis. *Cytometry* 3, 323-327.
- Vos M.D., Ellis C.A., Bell A., Birrer M.J. and Clark G. (2000). Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J. Biol. Chem.* 275, 35669-35672.
- Weiss S.W. and Goldblum J.R. (2001). Malignant soft tissue tumors of uncertain type. In: Enzinger and Weiss's. *Soft tissue tumors*. Fourth edition. Mosby St Louis. pp 1483-1509.
- Winer J., Jung C.K., Shackel I. and Williams P.M. (1999). Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal. Biochem.* 270, 41-49.

Accepted October 14, 2005