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Amplification of CDK4, MDM2, SAS and GLI genes in leiomyosarcoma, alveolar and embryonal rhabdomyosarcoma

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Summary. We evaluated amplification and overrepresentation of CDK4, MDM2, GLI and SAS genes of the 12q13-15 region, in a group of soft tissue sarcomas including leiomyosarcomas (LMS), alveolar rhabdomyosarcomas (ARMS) and embryonal (anaplastic and classic variants) rhabdomyosarcomas (ERMS), to ascertain genomic alterations and possible differences within histologic subtypes of rhabdomyosarcoma (RMS).

Quantitative real-time PCR was performed on DNA samples from 29 LMS, 9 ARMS, 7 anaplastic ERMS and 6 classic ERMS. Alteration of one or more of the 12q13-15 genes was revealed in 13/29 LMS (45%) and 12/22 RMS (54%) including 5/9 ARMS (56%), 5/7 anaplastic ERMS (71%) and 2/6 classic ERMS (33%). The potential importance of overproduction of protein products in neoplastic development, led us also to study a possible high expression of cdk4, mdm2 and gli proteins in immunohistochemical staining experiments on paraffin-embedded tissue samples of the same cases. Among LMS and RMS most cases with CDK4, MDM2 and GLI gene alterations also showed a simultaneous high expression of the relative protein.

In summary, these results indicate that amplification or overerepresentation of genes at 12q13-15 region involve both LMS and RMS. Moreover these genes alterations reveal predominantly in the alveolar and in the anaplastic variant of the embryonal subtype. These two seem to have a more similar behavior than anaplastic and classic embryonal that are classified in the same subtype.

Key words: CDK4, MDM2, SAS, GLI, Leiomyosarcoma, Rhabdomyosarcoma

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Introduction

Leiomyosarcoma (LMS) and rhabdomyosarcoma (RMS) are an aggressive group of malignant soft-tissue tumors that develop principally in adults and children, respectively.

LMS is a rare soft-tissue tumor that has the phenotypic features of smooth-muscle differentiation and originates, in most cases, from the vessel walls. Histologically, it is characterized by a proliferation of spindle cells, with elongated nuclei and eosinophilic cytoplasm (Phelan et al., 1962). It accounts for approximately 7% of soft-tissue sarcomas, and it is usually located in the extremities (Campanacci, 1999). Most LMS show a highly aggressive growth pattern with a high incidence of recurrences and metastases, which has been related to tumor grade. Superficial LMS have the best prognosis. Prognosis progressively worsens for LMS located in the subcutis, in deep-seated tumors and when originating in the retroperitoneum (Campanacci, 1999).

RMS account for approximately 20% of all malignant soft-tissue tumors and is the most common childhood soft-tissue sarcoma (Bale and Reye, 1975). It is still not completely clarified whether RMS originate from undifferentiated mesenchymal cells or from areas of embryonal muscle that do not follow a normal pathway of differentiation (Campanacci, 1999). RMS is a small round cell tumor that, according to histological features, has been classified in embryonal, botryoid, alveolar and pleomorphic. However, modifications of this classification are in course and no universal definition is accepted. Currently, three major histologic variants are recognized: embryonal (50-60%), alveolar (25-30%) and pleomorphic (5%), with distinct appearance and behavior. Other variants have also been recognized, such as anaplastic rhabdomyosarcoma, as reported in the International Classification of Rhabdomyosarcoma (ICR) (Newton et al., 1995). Anaplastic rhabdomyosarcoma is considered the

pleomorphic variant of rhabdomyosarcoma that occurs in children. Rhabdomyosarcomas of the embryonal type are seen mostly in children, in the age from birth to 10-15 years, and those of the alveolar type are observed mainly in adolescents and young adults. Pleomorphic rhabdomyosarcoma occurs mostly in older patients. The course of the tumor is rather fast and aggressive, and local recurrences and metastases are very frequent (Campanacci, 1999). Since prognosis appears to be dictated by histological subtypes and clinical features, an accurate histopathological evaluation is important not only to classify each neoplasm but also to predict its clinical course. The presence of anaplastic features in aggregates or diffused sheets has been shown to be an important prognostic histopathologic determinant in rhabdomyosarcoma (Kodet et al., 1993). In general, the prognosis of ARMS is worse than that of ERMS. In particular, among the ERMS, tumors showing anaplastic areas exhibit a worse clinical outcome compared with the classic ERMS, with a survival rate similar to that of ARMS (Crist et al., 1990).

The biological basis of the difference in clinical behavior of these sarcomas may be related to the different origin and/or to the genetic background of tumor cells. Studies focused on the analysis of genetic alterations in LMS and RMS have demonstrated that, like in other bone and soft-tissue sarcomas, gain or amplification of sequences located in the long arm of chromosome 12 is a rather common event suggesting, in the pathogenesis of these tumors, a possible involvement of genes located in this region (Roberts et al., 1989; Patterson et al., 1994; Nilbert et al., 1995). Amplification of genes located at 12q13-15 region has been frequently observed in several musculo-skeletal tumors, including RMS, LMS, osteosarcoma, malignant fibrous histiocytoma, fibrosarcoma, liposarcoma and malignant schwannoma (Kinzler et al., 1987; Roberts et al., 1989; Khatib et al., 1993; Reifenberger et al., 1996). Among the genes located at 12q13-15, those reported to have been more often gained or amplified are CDK4 (Matsushime et al., 1992), MDM2 (Oliner et al., 1992), SAS (Jankowski et al., 1994) and GLI (Kinzler et al., 1987; Ruppert et al., 1991). In this study, the copy number changes of the CDK4, MDM2, GLI and SAS genes were investigated by real-time polymerase chain reaction (PCR) in a series of 29 leiomyosarcoma (LMS),

Table 1. Clinical data of leiomyosarcoma patients.

CASE No.	GENDER	AGE (years)	SOURCE OF SAMPLE	DIAGNOSIS Histologic type	HISTOLOGIC GRADE FNCLCC	TUMOR SIZE	SITE	FLOLOW-UP months	OURCOME
1*	M	58	resection	LMS pleomorphic	Grade 2	> 5cm	forearm	37	NED
2*	M	64	resection	LMS pleomorphic	Grade 3	> 5cm	arm	16	DOD
3*	F	74	resection	LMS pleomorphic	Grade 2	> 5cm	pelvis	4	DOD
4*	M	47	resection	LMS pleomorphic	Grade 3	> 5cm	thorax	37	DOD
5*	M	51	resection	LMS pleomorphic	Grade 3	> 5cm	thigh	49	DOD
6*	M	53	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	20	DOD
7*	M	43	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	67	AWD
8*	F	66	biopsy	LMS pleomorphic	Grade 3	> 5cm	hamstring	15	DOD
9*	F	21	biopsy	LMS pleomorphic	Grade 2	> 5cm	thigh	169	NED
10*	M	85	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	13	DOD
11*	F	62	resection	LMS pleomorphic	Grade 2	> 5cm	thigh	13	DOD
12*	F	79	resection	LMS pleomorphic	Grade 2	< 5cm	forearm	98	AWD
13*	M	54	resection	LMS pleomorphic	Grade 3	> 5cm	leg	9	DOD
14*	F	68	resection	LMS pleomorphic	Grade 2	> 5cm	thigh	55	DOD
15*	M	47	resection	LMS pleomorphic	Grade 3	> 5cm	thorax	7	DOD
16*	F	58	resection	LMS pleomorphic	Grade 3	> 5cm	thigh	4	DOD
17*	M	38	biopsy	LMS pleomorphic	Grade 2	> 5cm	leg	57	NED
18*	F	48	resection	LMS pleomorphic	Grade 2	> 5cm	forearm	60	AWD
19°	M	44	resection	LMS pleomorphic	Grade 3	< 5cm	thigh	34	NED
20°	F	53	resection	LMS pleomorphic	Grade 3	> 5cm	thigh	19	DOD
21*	M	76	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	12	DOD
22*	F	74	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	5	DOD
23*	M	78	biopsy	LMS pleomorphic	Grade 2	> 5cm	leg	22	NED
24*	M	35	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	17	AWD
25*	M	67	biopsy	LMS pleomorphic	Grade 3	> 5cm	forearm	18	NED
26*	F	63	biopsy	LMS pleomorphic	Grade 3	> 5cm	thigh	32	DOD
27*	F	67	resection	LMS pleomorphic	Grade 3	> 5cm	thigh	21	DOD
28*	M	42	resection	LMS pleomorphic	Grade 2	> 5cm	leg	41	DOD
29*	M	21	biopsy	LMS pleomorphic	Grade 2	> 5cm	thigh	4	DOD

M: male; F: female; LMS: leiomyosarcoma; NED: no evident disease; DOD: died of disease; AWD: alive of disease; FNCLCC: Federation Nationale des Centres de Lutte contre le Cancer; *: frozen material; °: paraffin embedded material.

9 alveolar rhabdomyosarcoma (ARMS) and 13 embryonal rhabdomyosarcoma (ERMS), including 7 anaplastic ERMS and 6 classic ERMS. On the same cases, the level of cdk4, mdm2 and gli proteins were also determined by immunohistochemistry, in order to assess the relationship between increased gene copy number and increased level of protein.

Materials and methods

Tumor samples

Tumor samples derived from 51 high-grade soft-tissue sarcomas including 29 LMS, 9 ARMS, and 13 ERMS (7 of which showed anaplastic features) referred at the Istituti Ortopedici Rizzoli (Bologna, Italy) from 1982 to 1999. All LMS were pleomorphic and deep tumors, and the LMS specimens were obtained from initial biopsies or from surgical excisions of the primary tumor (Table 1). All RMS specimens were obtained from initial biopsies in absence of preoperative chemotherapy (Table 2). Thirty-two samples (27 LMS, 1 ARMS and 4 ERMS) were obtained from tumor specimens that were snap-frozen in liquid nitrogen and stored at -80 °C until analysis. The remaining 19 samples were obtained from formalin-fixed, paraffin-embedded tumor specimens.

Histopathological diagnosis was determined on hematoxylin-eosin stained tissue sections following conventional criteria (Campanacci, 1999). According to the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) system for sarcoma grading (Oliveira and Nascimento, 2001), 11/29 LMS and 18/29 LMS were grade 2 and 3 respectively (Table 1), and 16/22 RMS and 6/22 RMS were grade 2 and 3 respectively (Table 2).

DNA extraction

High molecular weight DNA was isolated from tumor samples and peripheral blood lymphocytes according to standard protocols (Sambrook et al., 1989). After isolation, to verify the integrity level, all DNA samples were electrophoresed on 1.5% agarose gel and marked by ethidium bromide staining. Then, DNA concentration of each frozen sample was determined by spectrophotometry; DNA dosage of each archivial sample was performed by quantitative PCR using a reference curve created by a series of DNA dilutions at a known concentration.

Real-Time PCR

All quantitative PCR reactions were performed on DNA, by ABI PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City CA, USA), with

Table 2. Clinical data of rhabdomyosarcoma patients.

CASE No.	GENDER	AGE years	SOURCE OF SAMPLE	DIAGNOSIS	HISTOLOGIC GRADE FNCLCC	SITE	ANAPLASIA	FOLLOW-UP months	OUTCOME
1*	F	5	biopsy	ARMS	Grade 2	thigh	/	61	DOD
2°	F	13	biopsy	ARMS	Grade 3	thigh	/	24	DOD
3°	F	25	biopsy	ARMS	Grade 2	leg	/	139	NED
4°	M	49	biopsy	ARMS	Grade 2	thigh	/	14	DOD
5°	F	32	biopsy	ARMS	Grade 3	leg	/	169	AWD
6°	M	31	biopsy	ARMS	Grade 2	forearm	/	16	NED
7°	M	19	biopsy	ARMS	Grade 2	calf	/	16	DOD
8°	M	4	biopsy	ARMS	Grade 2	forearm	/	20	DOD
9°	F	6	biopsy	ARMS	Grade 2	forearm	/	26	DOD
10*	M	10	biopsy	A. ERMS	Grade 3	arm	Focal	30	DOD
11*	M	14	biopsy	A. ERMS	Grade 2	foot planta	Focal	13	DOD
12*	F	15	biopsy	A. ERMS	Grade 2	shoulder	Diffuse	32	DOD
13°	F	2	biopsy	A. ERMS	Grade 2	thigh	Diffuse	46	NED
14°	F	1	biopsy	A. ERMS	Grade 2	thigh	Diffuse	78	DOD
15°	M	8	biopsy	A. ERMS	Grade 3	tendo Achillis	Diffuse	13	DOD
16°	M	12	biopsy	A. ERMS	Grade 2	shoulder	Diffuse	10	DOD
17°	F	16	biopsy	C. ERMS	Grade 2	leg, knee	Negative	112	DOD
18°	M	10	biopsy	C. ERMS	Grade 2	forearm	Negative	226	AWD
19*	F	12	biopsy	C. ERMS	Grade 2	thigh	Negative	5	DOD
20°	M	7	biopsy	C. ERMS	Grade 3	arm	Negative	19	DOD
21°	M	13	biopsy	C. ERMS	Grade 3	leg	Negative	14	DOD
22°	M	27	biopsy	C. ERMS	Grade 2	gluteus	Negative	9	DOD

M: male; F: female; ARMS: alveolar RMS; A. ERMS: anaplastic embryonal rhabdomyosarcoma; C. ERMS: classic embryonal rhabdomyosarcoma; NED: no evident disease; DOD: died of disease; AWD: alive with disease; FNCLCC: Federation Nationale des Centres de Lutte Contre le Cancer; *: frozen material; °: paraffin embedded material.

TaqMan technology (Orlando et al., 1998; Lehmann et al., 2000). This method of analysis is extremely sensitive, highly reproducible and can enable the exact quantification of minute amounts of nucleic acid. Moreover, the DNA to be analyzed can be significantly degraded without affective the quantitation, a factor that can be very valuable when working with clinical specimens and archivial tissue.

The PCR mixture contained 300 nM of each primer, 200 nM of probe and 10 ng of DNA in 1X TaqMan Universal PCR Master Mix in a 25 μl final reaction volume. All reagents were obtained from PE Applied Biosystems

Following activation of the UNG (uracil-N-glycosylase) (for 2 min at 50 °C) and of AmpliTaq Gold (for 10 min at 90 °C) all genes were amplified by 40 cycles for 15 seconds at 95 °C and for 1 min at 60 °C.

The sequences of the primers and hybridization probes used, are given in Table 3.

For each sample, DNA extract was analyzed in triplicate.

A negative control for each probe was included in every run in order to exclude false-positive results.

Evaluation of real-time PCR results

The principle of the TaqMan reaction has been described previously in detail (Orlando et al., 1998; Lehmann et al., 2000) .

Initial template concentration was derived from the cycle number at which the fluorescent signal crossed a threshold in the exponential phase of PCR (CT). The relative gene copy number was determined based on the threshold cycles of the gene of interest and of a reference gene. For calculation of gene amplifications this relative value, CT value (target gene) minus CT value (reference gene), is compared with the same value for calibrator. To measure oncogene amplification we used a comparative assay in which arithmetic formulas were used to achieve the result for relative quantitation. After performing a validation experiment to validate the AACT calculation, the amount of target, normalized to an endogenous reference and relative to a calibrator, was given by: 2-AACT. As endogenous single copy reference gene, the ß-actin gene was chosen and normal DNA from lymphocytes was used as calibrator.

Table 3. Oligonucleotides used in this study.

BETA-ACTINA
forward primer : 5'-AGCGCGGCTACAGCTTCA-3'
reverse primer : 5'-TTCTCCTTAATGTCACGCACGA-3'
probe : 6FAM5'-CACCACGCGGCCGAGCGGGA-3`TAMRA
CDK4
forward primer: 5'- TCTGCTACCGAGCTCCCG-3'
reverse primer : 5'-GCCAACACTCCACATGTCCA-3'
probe: 6FAM5'-AGTTCTTCTGCAGTCCACATATGCAACACCT-3'TAMRA
MDM2
forward primer : 5'-GAGCTTGGCTGCTTCTGGG-3`
reverse primer : 5'-TCGGCTTCTTGCTCCATCTT-3'
probe : 6FAM5`-CCTGTGTGGCCCTGTGTGTCGG-3`TAMRA
GLI
forward primer: 5'TTCTTCCAGAGCCCCCAAC-3'
reverse primer : 5'-GCGGCCGGGACAGAG-3'
probe : 6FAM5`-CAACTTGATTTGCCCCAAACCGCA-3`TAMRA
SAS
forward primer : 5`-CGGTGACGTAATGCGGG-3'
reverse primer : 5`-TGCGTGGCTAATCCTGGAC-3'
probe : 6FAM5'-TGATTGGCATGCAGGCCTTGGA-3`TAMRA

Immunohistochemistry

Immunohistochemistry was performed on 4-6 μm sections obtained from formalin-fixed, paraffin-embedded tumor specimens by using a streptoavidin-biotin-peroxidase complex (sABC) method (AutoProbe III, Biomeda, Foster City, CA). Tissue sections were dewaxed with xylene and gradually rehydrated with ethanol and water. After blocking of the endogenous peroxidase activity and treatment with blocking serum, slides were incubated at 4 °C overnight with the following primary antibodies: polyclonal anti-human cdk4 (C-22) (1:50 dilution rate, Santa Cruz Biotecnology, Santa Cruz, CA), monoclonal anti-human mdm2 (IF2) (1:10 dilution rate, Oncogene Research Products, Boston, MA), anti-human polyclonal gli (N-16) (1:50 dilution rate, Santa Cruz Biotecnology, Santa Cruz, CA). After incubation with secondary biotinylated antibody and sABC complex, the final reaction product was revealed by incubation with 3-amino-9-ethylcarbazole and nuclei were counterstained with hematoxylin.

Immunoreactivity for cdk4 and mdm2 was quantified by scoring the number of tumor cells showing a positive nuclear staining. Immunoreactivity for gli was quantified by scoring the number of positive tumor cells. Tumor samples showing a positive immunostaining in more than 5% of cells were classified as positive. High expression was defined when a positive immunoreactivity was present in more than 50% of cells.

In each determination, negative controls were performed by omitting the primary antibody.

Results

Real-Time PCR

All 51 tumors were screened by real-time PCR with ABI PRISM 7700 for amplification and over-representation of CDK4, MDM2, GLI and SAS genes.

Real-time PCR data are summarized in Table 4 and 5. Amplification of one or more of these genes was found in 4 out of 29 LMS (14%) and in 5 out of 22 RMS (23%), including 2/9 ARMS (22%), 3/7 anaplastic ERMS (43%). The degree of amplification ranged from 11-fold to more than 60-fold.

Overrepresentation of one or more of these genes

was found in 13/29 LMS (45%) and 15/22 RMS (68%), including 3/9 ARMS (33%), 4/7 anaplastic ERMS (57%) and 2/6 classic ERMS (33%).

LMS

In LMS (Table 4 and 6) CDK4 was amplified in 2/29 cases (7%) and overrepresented in 4/29 cases (14%). Among these, in one (case 6) CDK4 was co-overrepresented with SAS and in one (case 3) with both SAS and MDM2.

MDM2 resulted to be amplified in 1/29 LMS (3%), and overrepresented in 3/29 cases (10%) one of which (case 10) was co-overrepresented with SAS and one (case 3) with both CDK4 and SAS.

SAS was the gene most frequently alterated in LMS, showing a very high increased copy number in 1/29 cases (3%) and was overrepresented in 6/29 cases (21%). Among these, in two (cases 3 and 10) SAS was co-overrepresented with both CDK4 and MDM2 and with MDM2, respectively.

None of the 29 LMS showed evidence for GLI alterations.

RMS

Tables 5 and 6 showed that in RMS, the amplification and overrepresentation of all genes was found in a higher proportion of alveolar and anaplastic embryonal cases compared with classic embryonal cases.

CDK4 was amplified in 2/22 tumors (9%), including 1/9 ARMS (11%) (case 9) in which CDK4 was co-amplified with both SAS and GLI, and 1/7 anaplastic ERMS (14%). CDK4 was overrepresented in 5/22 cases (23%) including 2/9 ARMS (22%), one of these (case 1) showed CDK4 co-overrepresented with both MDM2 and GLI, and 3/7 anaplastic ERMS (43%). Among these, CDK4 was co-overrepresented with SAS in cases 10 and 11, and with both MDM2 and GLI in case 15.

None of the classic ERMS showed evidence for CDK4 amplification or overrepresentation.

MDM2 amplification was revealed in 2/22 RMS (9%) including 1/9 ARMS (11%) and 1/7 anaplastic ERMS (14%). Overrepresentation of MDM2 was detected in 3/22 RMS (14%). Among these, in one anaplastic RMS (case 12) MDM2 was co-amplified with

Table 4. Analysis results in leiomyosarcoma.

CASE No.	DIAGNOSIS Histologic type	CDK4		MDM2		GLI		SAS
		DNA fold increase	protein expression	DNA fold increase	protein expression	DNA fold increase	protein expression	DNA fold increase
1	LMS pleomorphic	1	Neg	1	Neg	1	Pos	1
2	LMS pleomorphic	1	Pos	1	Neg	1	High expression	1
3	LMS pleomorphic	4	High expression	4	Neg	1	Pos	5
4	LMS pleomorphic	3	High expression	1	Neg	1	Pos	2
5	LMS pleomorphic	2	High expression	1	Pos	1	High expression	2
6	LMS pleomorphic	3	High expression	1	Neg	1	High expression	5
7	LMS pleomorphic	1	Neg	1	Neg	1	High expression	2
8	LMS pleomorphic	4	High expression	1	Neg	1	Neg	1
9	LMS pleomorphic	1	High expression	8	High expression	1	High expression	1
10	LMS pleomorphic	1	Neg	3	High expression	1	Neg	5
11	LMS pleomorphic	1	Pos	1	Pos	1	High expression	1
12	LMS pleomorphic	1	Pos	1	Neg	1	Neg	1
13	LMS pleomorphic	1	Pos	1	Neg	1	High expression	3
14	LMS pleomorphic	1	Pos	1	Pos	1	Pos	2
15	LMS pleomorphic	1	High expression	1	Neg	1	High expression	1
16	LMS pleomorphic	1	Pos	1	Neg	1	High expression	1
17	LMS pleomorphic	1	High expression	2	Pos	1	Pos	62
18	LMS pleomorphic	2	Pos	1	Neg	1	Neg	3
19	LMS pleomorphic	1	High expression	1	Neg	1	Pos	3
20	LMS pleomorphic	1	Pos	1	Pos	1	Neg	1
21	LMS pleomorphic	1	Neg	1	Neg	1	Neg	1
22	LMS pleomorphic	1	Pos	1	Pos	1	Pos	1
23	LMS pleomorphic	1	Pos	1	Pos	1	Pos	2
24	LMS pleomorphic	1	Neg	1	Neg	1	Neg	1
25	LMS pleomorphic	1	Pos	1	Neg	1	Pos	2
26	LMS pleomorphic	1	Neg	1	Pos	1	Neg	2
27	LMS pleomorphic	20	High expression	1	Neg	1	Pos	1
28	LMS pleomorphic	1	Neg	22	High expression	1	Pos	1
29	LMS pleomorphic	11	High expression	2	Pos	1	High expression	1

LMS: leiomyosarcoma; Neg: negative; Pos: positive.

SAS, while was co-overrepresented with both CDK4 and SAS in case 1 among ARMS, and with both CDK4 and GLI in case 15 among anaplastic ERMS. Alterations of MDM2 were not seen in the classic group of RMS.

SAS gene was amplified in 3/22 RMS (14%), including 1/9 ARMS (11%) that showed SAS co-amplified with both CDK4 and GLI, and 2/7 anaplastic ERMS (29%). Among these, SAS was co-amplified with MDM2 in one anaplastic ERMS (case 12).

Moreover, SAS overrepresentation was revealed in 5/22 RMS (23%) including 1/9 ARMS (11%), 2/7 anaplastic ERMS (29%) and 2/6 classic ERMS (33%).

Amplification and overrepresentation of GLI gene was detected in 1/22 RMS (4%) and in 2/22 RMS (9%), including 1/9 ARMS (11%) and 2/7 anaplastic ERMS (29%), respectively. GLI gene was co-amplified with CDK4 and SAS (case 9) and co-overrepresented with both CDK4 and MDM2 in case 15.

A comparison of the types of soft-tissue sarcomas examined (Table 7), indicated that the most frequently amplified and/or overrepresented genes were CDK4 (21% of LMS, 33% of ARMS, 57% of anaplastic ERMS) and SAS (24% of LMS, 22% of ARMS, 57% of anaplastic ERMS and 33% of classic ERMS), followed by MDM2 (14% of LMS, 33% of ARMS, 29% of anaplastic ERMS) and GLI (11% of ARMS, 29% of anaplastic ERMS). Moreover, among the RMS, alteration of 12q13-15 genes was detected predominantly in ARMS and in ERMS cases characterized with focal or diffused anaplasia, compared

Table 5. Analysis results in rhabdomyosarcoma.

CASE No.	DIAGNOSIS	CDK4		MDM2		GLI		SAS
		DNA fold increase	protein expression	DNA fold increase	protein expression	DNA fold increase	protein expression	DNA fold increase
1	ARMS	4	High expression	3	High expression	2	Pos	8
2	ARMS	1	Neg	1	Neg	1	Neg	1
3	ARMS	2	Pos	39	High expression	1	Neg	1
4	ARMS	1	Pos	1	Pos	1	Pos	1
5	ARMS	3	Pos	1	Neg	1	High expression	1
6	ARMS	1	Pos	1	Pos	1	Neg	2
7	ARMS	1	Pos	7	Neg	1	High expression	1
8	ARMS	2	High expression	1	Neg	1	Pos	1
9	ARMS	31	High expression	1	Pos	11	High expression	23
10	A. ERMS	3	High expression	1	Neg	1	Pos	5
11	A. ERMS	3	Pos	1	Neg	1	Neg	4
12	A. ERMS	1	High expression	30	Pos	1	Pos	24
13	A. ERMS	1	High expression	1	Pos	1	High expression	1
14	A. ERMS	1	Neg	1	Neg	1	Pos	1
15	A. ERMS	4	High expression	7	High expression	3	Pos	13
16	A. ERMS	60	High expression	1	Pos	3	High expression	1
17	C. ERMS	1	Pos	1	Pos	1	Pos	7
18	C. ERMS	1	Pos	1	Neg	1	Pos	1
19	C. ERMS	1	Pos	1	Neg	1	Pos	1
20	C. ERMS	1	Neg	1	Neg	1	Neg	1
21	C. ERMS	2	High expression	1	High expression	2	Neg	4
22	C. ERMS	1	High expression	1	Pos	1	Pos	1

ARMS: alveolar rhabdomyosarcoma; A. ERMS: anaplastic embryonal rhabdomyosarcoma; C. ERMS: classic embryonal rhabdomyosarccomaa: Pos: positive; Neg: negative.

Table 6. Genetic analysis results.

TYPE OF SARCOMA	CDK4 GENE		MDM2 GENE		GLI GENE		SAS GENE	
	Ampl.	Overrep.	Ampl.	Overrep.	Ampl.	Overrep.	Ampl.	Overrep.
LMS (29 cases)	2 (7%)	4 (14%)	1 (3%)	3 (10%)	0	0	1 (3%)	6 (21%)
ARMS (9 cases)	1 (11%)	2 (22%)	1 (11%)	2 (22%)	1 (11%)	0	1 (11%)	1 (11%)
Anaplastic ERMS (7 cases)	1 (14%)	3 (43%)	1 (14%)	1 (14%)	0	2 (29%)	2 (29%)	2 (29%)
Classic ERMS (6 cases)	0	0	0	0	0	0	0	2 (33%)

LMS: leiomyosarcoma; ARMS: alveolar rhabdomyosarcoma; ERMS: embryonal rhabdomyosarcoma; Ampl: amplification (≥ 10 copies); Overrep: overrepresentation (from 3 to 10 copies).

with classic ERMS.

Immunohistochemistry

In order to determine if 12q13-15 gene alterations also resulted in increased levels of their relative products, immunohistochemistry for cdk4, mdm2 and gli proteins was performed on all LMS and RMS tissue specimens. Immunohistochemistry for sas protein was not carried out since no sas-specific antibody is

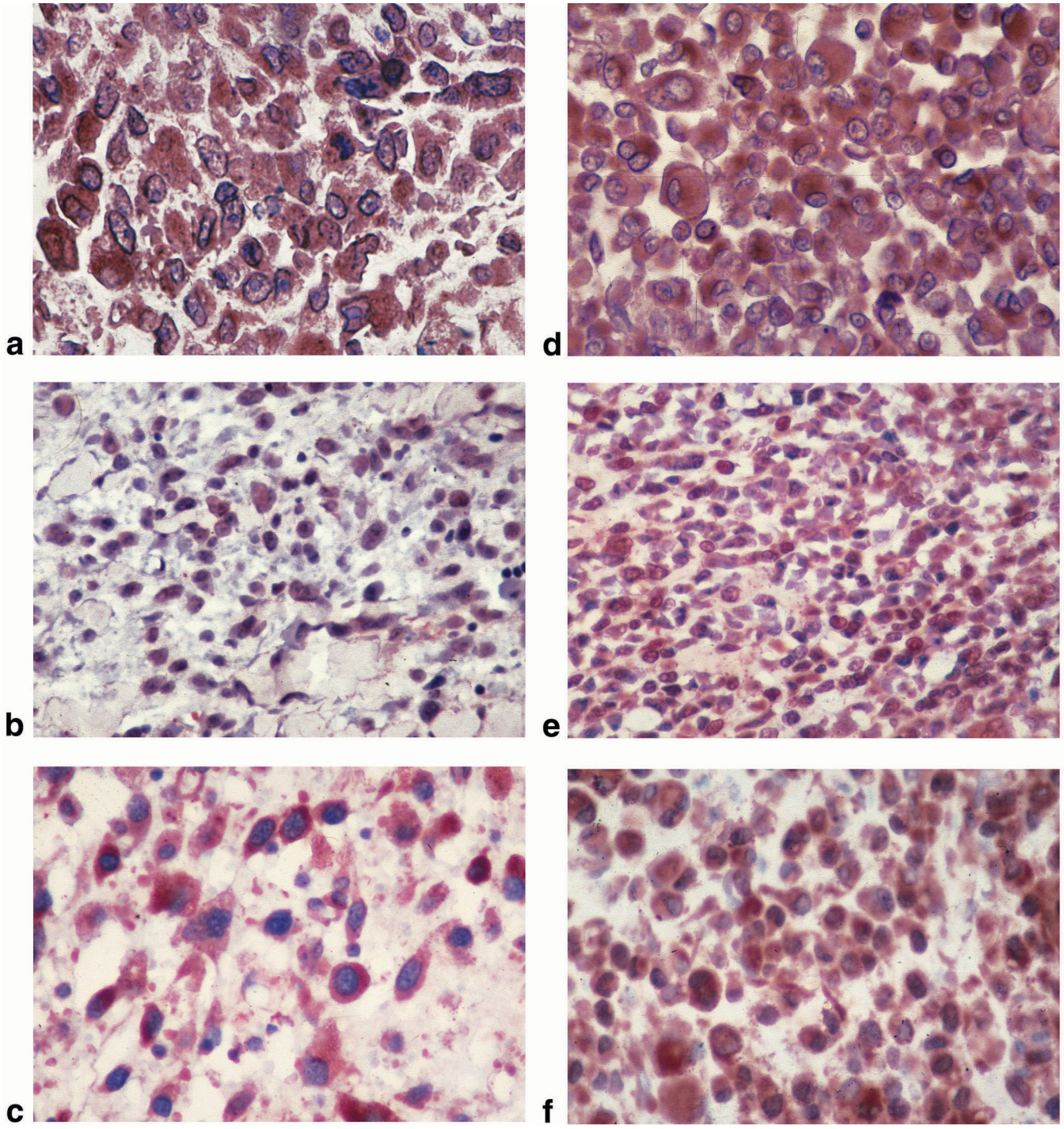

Fig. 1. Protein expression evaluated by IHC. Immunostaining for cdk4 **(a),** mdm2 **(b**) and gli (**c)** proteins in LMS. Immunostaining for cdk4 **(d),** mdm2 **(e)** and gli **(f)** proteins in RMS. Immunohistochemistry data for all samples are summarized in Tables 4 and 5.

commercially available.

Using antibodies directed against gli (Fig. 1c,f) protein, distinct cytoplasmic and/or nuclear staining was observed in LMS and RMS tumor specimens. LMS and RMS cases showed a nuclear immunoreaction of cdk4 (Fig. 1a,d) and mdm2 (Fig. 1b,e) proteins. Immunohistochemistry data are summarized in Tables 4, 5 and 8. The most frequently highly expressed proteins were cdk4 in 11/29 LMS and in 10/22 RMS, and gli in 10/29 LMS and in 5/22 RMS followed by mdm2 showing a high expression in 3/29 LMS and 4/22 RMS. In particular among the RMS, a high expression of cdk4 protein was observed in 3/9 ARMS, 5/7 anaplastic ERMS and 2/6 classic ERMS. A high expression of mdm2 protein was observed in 2/9 ARMS, 1/7 anaplastic ERMS and 1/6 classic ERMS. High expression of gli protein was observed in 3/9 ARMS and 2/7 anaplastic ERMS.

As shown in Table 8, most cases with CDK4, MDM2 or GLI gene alteration also exhibited a simultaneous high expression of the relative protein. In fact, among the 11 LMS with cdk4 high expression, 2 also showed CDK4 amplification and 4 showed CDK4 overrepresentation. In RMS, high expression of cdk4 was associated with CDK4 amplification or overrepresentation in 2 ARMS and in 3 anaplastic ERMS.

A high accumulation of mdm2 protein was revealed in 3 LMS, 2 ARMS and 1 anaplastic ERMS carrying MDM2 gene amplification or overrepresentation.

Among the RMS gli protein high expression was observed in 1 ARMS carrying also GLI gene amplification and in 1 anaplastic ERMS with GLI gene overrepresentation. Gli protein high expression was observed also in 10 LMS, although LMS showed no evidence of GLI gene alteration.

Table 7. Percentage of cases with gene alterations (amplification or overrepresentation).

TYPE OF SARCOMA	CDK4	MDM2	GLI	SAS
LMS (29 cases)	21%	14%	0	24%
ARMS (9 cases)	33%	33%	11%	22%
Anaplastic ERMS (7 cases)	57%	29%	29%	57%
Classic ERMS (6 cases)	0	0	0	33%

LMS: leiomyosarcoma; ARMS: alveolar rhabdomyosarcoma; ERMS: embryonal rhabdomyosarcoma; Amplification: (≥ 10 copies); Overrepresentation: (from 3 to 10 copies).

Discussion

Genetic alterations involving the 12q13-15 chromosomal region are common findings in musculoskeletal sarcomas, and several bone and soft-tissue malignant tumors exhibit amplification of various genes located in this region (Oliner et al., 1992; Forus et al., 1993; Ladanyi et al., 1993; Cordon-Cardo et al., 1994; Bell et al., 1999). In particular, among soft-tissue tumors, amplification of CDK4, MDM2, GLI and SAS has been demonstrated in well-differentiated liposarcoma and malignant fibrous histiocytoma (Roberts et al., 1989; Meltzer et al., 1991; Smith et al., 1992; Forus et al., 1993; Nilbert et al., 1995). In tumors originating from smooth muscle and striated muscle cells, gain of 12q13-15 chromosomal region or amplification of CDK4, MDM2, GLI and SAS have been reported in leiomyosarcoma and rhabdomyosarcoma (Patterson et al., 1994; Weber-Hall et al., 1996) .

In this study, quantitative real-time PCR analysis was performed on 29 LMS and 22 RMS. In RMS, alveolar morphology was observed in 9 cases and 13 tumors were classified as embryonal RMS. Among these, 7/13 showed the presence of varying numbers of anaplastic cells and, since the distribution of anaplastic cells varied from case to case, ERMS subtypes were divided into two subgroups: anaplastic ERMS and classic ERMS. Literature on RMS (Kodet et al., 1993) states that variants of anaplastic ERMS were associated with a more unfavorable prognosis compared with classic ERMS, and that such an unfavorable outcome for anaplastic ERMS was comparable to those of ARMS. Moreover, a cytogenetic study on ARMS and ERMS with and without anaplastic features (Bridge et al., 2002) showed that ARMS and anaplastic ERMS exhibited

Table 8. Cases with both protein high expression and gene alterations.

TYPE OF SARCOMA	CDK4			MDM2			GLI		
	protein high expr.	gene ampl.	gene overrep.	protein high expr.	gene ampl.	gene overrep.	protein high expr.	gene ampl.	gene overrep.
LMS (29 cases)	11	2	4	3	1	2	10	0	0
ARMS (9 cases)	3	1	1	2	1	1	3	1	0
Anaplastic ERMS (7 cases)	5	1	2	1	0	1	2	0	1
Classic ERMS (6 cases)	2	0	0	1	0	0	0	0	0

LMS: leiomyosarcoma; ARMS: alveolar rhabdomyosarcoma; ERMS: embryonal rhabdomyosarcoma; expr.: expression; ampl.: amplification (≥ 10 copies); overrep.: overrepresentation (from 3 to 10 copies).

genetic alteration more frequently than classic ERMS.

In the present study, amplification and overrepresentation of one or more of the genes here considered (CDK4, MDM2, GLI and SAS) was found in 45% of LMS and 54% of RMS, suggesting that these events may be of importance for the pathogenesis of these neoplasms.

In particular, among RMS, PCR analysis of 9 ARMS and 13 ERMS revealed that gene alteration was distributed with comparable frequencies between two histologic subtypes. Moreover, alteration of CDK4, MDM2, GLI and SAS was detected highly predominant in the ERMS characterized by a focal or diffuse anaplasia, compared with classic ERMS. Previous CGH studies (Weber-Hall et al., 1996; Pandita et al., 1999; Bridge et al., 2000), have shown that genomic amplification was frequently present in ARMS and ERMS with anaplasia, whereas it was a rare event in classic ERMS. Our results demonstrate and support the hypothesis that amplification and overrepresentation of these genes are frequent not only in ARMS, but also in anaplastic ERMS, which makes these alterations an important differentiating feature between the anaplastic ERMS and the classic ERMS subtypes.

Among the genes considered in this study, CDK4 and SAS were the most frequently alterated, both in LMS and in RMS. Almost all LMS (21%) and RMS (36%) with CDK4 gene amplification or overrepresentation also showed high expression of cdk4 protein by immunohistochemistry, indicating that increased levels of this protein mostly derived from an increased transcription of CDK4 consequent to gene alteration. These findings are in agreement with other studies on different tumors, which revealed increased levels of cdk4 protein in association with the amplification of this gene (An et al., 1999; Wunder et al., 1999). On the other hand, high expression of cdk4 protein in absence of CDK4 gene amplification was observed in 5 LMS and 5 RMS. This may result from a higher transcriptional activity of the gene or from post-transcriptional modifications that increase the half-life of the protein (An et al., 1999). Alteration of CDK4 gene and consequent high expression of cdk4 protein could be of pathogenetic significance for neoplastic growth, since CDK4 belongs to the family of cyclin-dependent kinases that, associated with cyclins, play a crucial role in the regulation of cell cycle progression in eukaryotic cells (Sherr, 1993) .

SAS amplification was detected in 24% of LMS and in 36% of RMS with a higher frequency in anaplastic ERMS (57%) compared with classic ERMS (33%). Less is known about the physiological functions of SAS. The SAS gene product belongs to the transmembrane 4 protein superfamily (TM4SF), whose members have been found to be involved in signal transduction and growth control (Jankowski et al., 1994) . Therefore, SAS amplification could result in disturbances of cellular growth control.

The analysis of MDM2 alteration revealed the presence of increased copy number of this gene in 14% of LMS and 23% of RMS, without involving classic ERMS. In agreement with other studies (Oliner et al., 1992; Wunder et al., 1999), the incidence of MDM2 alteration resulted lower than that of CDK4 and SAS.

Although MDM2 gene may play an important role in tumorigenesis, through the interaction with p53 function (Momand et al., 1992), the relatively low frequency of MDM2 alteration in LMS and RMS suggests that it is not the primary target of gene alteration at 12q13-15 in these tumors. Moreover physical mapping studies have demonstrated that MDM2 locus is separated by over 1 Mb from the loci of CDK4 and SAS, suggesting that, at this chromosomal region, MDM2 amplification is probably independent from that of other genes (Nilbert et al., 1995; Berner et al., 1996; Elkahloun et al., 1996; Wunder et al., 1999).

High expression of mdm2 protein was detected in most LMS and RMS carrying MDM2 gene amplification or overrepresentation, suggesting its link to gene alteration, in agreement with other published data (Oliner et al., 1992; Cordon-Cardo et al., 1994; Nakayama et al., 1995). Amplification of GLI gene was not detectable in the series of LMS analyzed in this study. Only RMS exhibited GLI alteration in 14% of cases, in particular the amplification occurred in ARMS (11%) and overrepresentation in anaplastic ERMS (29%). It is noteworthy that, in LMS, increased level of gli protein was present in 10 cases in absence of a concomitant alteration of the gene, suggesting that the elevated expression of this protein may be indicative of the aggressiveness of these high grade LMS.

This is in line with other studies describing LMS and childhood sarcomas (Roberts et al., 1989; Stein et al., 1999) that found the GLI gene frequently amplified in RMS (Forus et al., 1993; Roberts et al., 1989), especially the alveolar subtype (Gordon et al., 2000), but it was within the norm in LMS (Kinzler et al., 1988). In our study high expression of gli protein was seen in some RMS carrying gene amplification and overrepresentation.

In summary, quantitative real-time PCR and immunohistochemistry analyses of LMS and RMS specimens have shown that all the genes considered, CDK4, MDM2, GLI and SAS were frequently alterated and/or highly expressed in LMS and RMS, indicating that genes located at 12q13-15 may be important for tumorigenesis of these neoplasms. Furthermore, the observed more frequent alteration of genes in ARMS and anaplastic ERMS compared with classic ERMS, indicates that the presence of alterations could be considered an important differentiating feature between ARMS and ERMS subtypes. No significant association was found between 12q13-15 gene alterations and clinicopathologic feature (data not shown). However, additional studies are required to identify the potential prognostic significance of increased copy number of 12q13-15 genes and the relantionship between protein and gene alterations during malignant transformation of

these tumors.

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