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Genetic and molecular alterations in rhabdomyosarcoma: mRNA overexpression of MCL1 and MAP2K4 genes

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Summary. Rhabdomyosarcoma, the most common soft tissue sarcoma in childhood, belongs to the small round cell tumor family and is classified according to its histopathological features as embryonal, alveolar and pleomorphic. In this study we propose to explore genetic alterations involved in rhabdomyosarcoma tumorigenesis and assess the level of mRNA gene expression of controlling survival signalling pathways. For genetic and molecular analysis, array-based comparative genomic hybridization, combined with Real Time PCR using the comparative method, was performed on 14 primary well-characterized human primary rhabdomyosarcomas. Multiple changes affecting chromosome arms were detected in all cases, including gain or loss of specific regions harbouring cancer progression-associated genes. Evaluation of mRNA levels showed in the majority of cases overexpression of MCL1 and MAP2K4 genes, both involved in cell viability regulation. Our findings on rhabdomyosarcoma samples showed multiple copy number alterations in chromosome regions implicated in malignancy progression and indicated a strong expression of MAP2K4 and MCL1 genes, both involved in different biological functions of complicated signalling pathways.

Key words: CGH-microarray, Gene expression, Real-Time PCR, Rhabdomyosarcoma

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Introduction

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in childhood and likely is a result of an imbalance in proliferation and differentiation of precursor cells during the skeletal myogenesis program (Merlino and Helman, 1999; Charge and Rudnicki, 2004).

According to histopathological features, RMS belongs to the small round cell tumor family and is classified as embryonal (ERMS), its most frequent form; alveolar (ARMS) the most aggressive and pleomorphic (PRMS) representing a rare variant that occurs in adults (Fletcher et al., 2002).

Unlike ERMS and PRMS, ARMS is cytogenetically well-characterized by recurrent translocation (65%) between gene FKHR located at 13q14 and gene PAX3 at 2q35, or less commonly (15%) between FKHR and PAX7 located at 1p36. Some reports suggest that FKHR-PAX3 occurring during fetal development contributes to oncogenesis through abnormal control of growth, apoptosis and differentiation (Barr, 2001). Tumors with FKHR-PAX7 fusion transcript show a predilection for younger patients, appear in the extremities and have a better prognosis.

Although the second genetic events are not specific, full transformation to RMS might require additional mutations leading to downstream activation of the growth factor signalling pathway (Helman and Meltzer, 2003).

In vitro studies by microarray analysis identified a cluster of new genes related to the aberrant differentiation program of human RMS cells, suggesting a potential role as myogenic or repressor markers of differentiation (Astolfi et al., 2001). Other studies demonstrated that some genes, such as insulin-growth

factor receptor 2 (IGF2), seem to be strongly linked to ARMS growth, activating myogenic transcription programs (Khan et al., 1999) and inducing abnormalities in signal transduction pathways (Kalebic et al., 1998). ERMS has a complex karyotype and is characterized by loss of heterozygosity on the 11p15.5 chromosome region (Gallego and Sanchez de Toledo Codina, 2007), extra copies of chromosomes 2, 8, 12 and 13, consistent with amplification of MYCN(2p24), MDM2, GLI, CDK4(12q13-15), FKHR(13q14) and rearrangements of chromosome 1 (Anderson et al.,1999; Pandita et al., 1999). However, our previous studies on a series of human RMS samples demonstrated that genes located on 12q13-15 were distributed with similar frequency between ARMS and ERMS with anaplastic histological features (Ragazzini et al., 2004). Furthermore, conventional comparative genomic hybridization (CGH) of primary RMS tumors have confirmed quantitative alterations of large chromosomal regions, including 2q, 8p, 17p and have seen new alterations of novel loci, such as 15q25-26 and 5q32ter (Gordon et al., 2003). A recent study by CGH array revealed a high aneuploidy level with double minutes and additional structural aberrations in all RMS histological sub-types, consistent with multiple amplification or deletion events involving several gene families (Goldstein et al., 2006).

In the present study, carried out on a series of primary RMS from patients with poor prognosis in terms of disease-free survival including metastases and local recurrences, the evaluation of mRNA expression of the genes located on the chromosome regions most frequently altered, 17p11.2 and 1q21, revealed overexpression of MAP kinase kinase 4 (MAP2K4) and Myeloid cell leukaemia 1 (MCL1) in the majority of the cases studied. MAP2K4 is located on chromosome 17p11.2 and directly phosphorylates and activates c-Jun-NH2 terminal kinase (JNK) in response to cellular stress. MCL1, located at 1q21 region is an anti-apoptotic Bcl-2 family member. In addition, the increased DNA copy number at 17p locus results in an amplification of non-muscle myosin, heavy chain 10 (MYH10), involved in tight junction and in the regulation of actin cytoskeleton.

Materials and methods

Primary tumor tissues and patients

Tumor samples from 14 RMS, including 5 ARMS, 5 ERMS and 4 PRMS referred at the Rizzoli Orthopaedic Institute were considered. Fresh non-necrotic tissue (≥ 90% tumor cells) was obtained from biopsy or excision before chemo- and/or radiotherapy. Diagnosis was based on hematoxylin-eosin stained samples according to histopathological criteria (Weiss and Goldblum, 2001). For differential diagnosis, cytogenetic analysis and immunohistochemistry with specific antibodies for striated muscle cell differentiation were performed.

4 out of 5 ARMS presented the specific translocation t(2;13).

Clinical and pathological characteristics of patients are reported in Table 1.

11/14 patients had metastases during follow-up and 3 locally relapsed. 8 patients died of disease.

Oligonucleotide CGH Microarrays

Array-based comparative genomic hybridization (CGH Microarray) analysis was performed on 4 cases of ARMS.

Isolation of DNA was performed using QIAamp DNA Micro Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. For each CGH array, genomic DNA from the experimental sample was random primer-amplified using components from BioPrime, Array CGH labeling kit (Invitrogen, Milan) as previously described (Morandi et al. 2006). Reaction consisted of 300 ng/mL random oligodeoxy-ribonucleotide primers, 20 U of Klenow enzyme, 200

Table 1. Clinical table of the patients.

Tumor	Sex	Age	Site	M/LR	Follow-up months	Outcome
ARMS 1	F	2	thigh	M	39	AWD
ARMS 2	F	6	foot	LR	42	NED
ARMS 3	M	23	armpit	M	11	DOD
ARMS 4	M	16	arm	M	26	DOD
ARMS 5	F	29	pelvic girdle	M	31	DOD
PRMS 1	M	70	shoulder	M	12	NED
PRMS 2	M	23	gluteus	M	7	DOD
PRMS 3	F	62	trunk	M	6	DOD
PRMS 4	F	2	leg	M	12	AWD
ERMS 1	M	17	arm	LR	6	NED
ERMS 2	F	13	thigh	M	12	DOD
ERMS 3	F	22	leg	M	113	DOD
ERMS 4	M	27	pelvic girdle	M	9	DOD
ERMS 5	M	0	arm	LR	18	NED

ARMS: alveolar rhabdomyosarcoma; PRMS: pleomorfic rhabdomyosarcoma; ERMS: embryonal rhabdomyosarcoma; M: metastatic tumor; LR: local recurrence; DOD: died of disease; AWD: alive with disease; NED: no evidence of disease.

mM dNTPs and total DNA extracted from the microdissected specimen. Total DNA from each sample and random primers were denatured at 95ºC for 5 minutes and then immediately cooled in ice. The remaining reagents were then added, and the reaction was incubated at 37°C for 2 hours. Amplified DNA was further purified by MiniElute spin columns (Qiagen GmbH, Hilden Germany) following the Qiagen protocol.

1 µl of amplified DNAs was further analyzed for quantity and length by Agilent 2100 Bioanalyzer. A second round of the same linear amplification system was used to label amplified DNA using Cy3-dCTP for reference DNA and Cy5-dCTP (Perkin Elmer) for test-DNA following the protocol of Barrett MT et al. (Barret et al., 2004). Labeling reactions were performed in a volume of 50 ml with a modified dNTP pool containing 120 mM each of dATP, dGTP, dTTP, 60 mM of dCTP and Cy5-dCTP or Cy3-dCTP. Labeled targets were subsequently purified by MiniElute spin columns (Qiagen) and eluted in ultra pure water (Invitrogen). Experimental and reference targets for each hybridization were pooled and mixed with 100 mg of human Cot-1 DNA (Invitrogen)/ 50 ml of 1x hybridization control targets (SP310, Operon Technologies, Alameda CA)/ 50 mg of yeast tRNA (Invitrogen) in a final volume of 250 ml and mixed together with 250 ml of 2x hybridization solution (Agilent). Before hybridization to the array, the 500 ml hybridization mixtures were denatured at 100°C for 1.5 min and incubated at 37°C for 30 min. To remove any precipitate, the mixture was centrifuged at 14,000 x *g* for 5 min and transferred to a new tube, leaving a small residual volume (<5ml). The sample was applied to the 44K Agilent Oligo array by using an Agilent hybridization chamber, and hybridization was carried out for 17 hours at 65°C in a rotating oven (Robbins Scientific, Mountain View, CA) at 4 rpm. Arrays were then disassembled in 6x SSPE/0.00025% of N-Laurylsarcosine, and washed once for 1 min with 6x SSPE/0.005% of N-Laurylsarcosine, and once for 1 min with 0.06x SSPE/0.005% N-Laurylsarcosine, both at room temperature. Slides were immersed in the Stabilization solution (Agilent Cod. 5185-5979), dried and scanned using an Agilent 2565A scanner with 5 mm resolution.

Reference DNA for CGH hybridization was derived from a pool of wild type female DNA extracted from lymphocytes of 10 different healthy women.

Image and data analysis

Microarray images were analyzed using the Feature Extraction Software (Agilent version 7.5) following the protocol described from Barrett et al (Barrett et al., 2004). In brief, default settings were employed, using signals from negative control features for background subtraction. Several filtering procedures were applied, eliminating: 1) features that did not cross the threshold of calculated background for both channels; 2) outlier features for each experiment; 3) features not included between -0.1 and 0.1 values obtained in the female/male CGH experiments. Discovery of gains and losses was made using CGH-Explorer (http://www.ifi.uio.no/bioinf/Papers/CGH/) software for analysis of copy errors (ACE) (Lingjaerde et al. 2005). Single genes were partitioned into subsequences, or "runs", of positive or negative copy numbers (after calculating the running mean of data, with five neighbors). Each run was characterized by its length and height (average value), and the collection of (length, height) pairs was compared to distribution of (length and height) pairs in a theoretical null population of normal copy number ratios, available from the male/female experiment. This method computes an estimate of positive false discovery rate (pFDR) and provides a table of different pDFR-

Table 2. a. PRIMERS for DNA analysis.

MCL1	
Forward:	ATGCACCTGTGTTCCTCGCT
Reverse:	GTGCTCATTGCTGCCTCAAC
MYH10	
Forward:	CAGCGTGACATTTTTTTCTCCTT
Reverse:	CCTGGTTATGATAATGGACTCCACT
MAP2K4	
Forward:	TCCAATTCCTGGGTTCAAGTG
Reverse:	GCTGGGTGTGGTGGCATATAC

Table 2. b. TaqMan Gene Expression Assays®.

GAPDH	Assay ID: Hs99999905_m1
MCL1	Assay ID: Hs00172036_m1
MYH10	Assay ID: Hs00292551_m1
MAP2K4	Assay ID: Hs00387426_m1

Table 3. Most frequent genetic and molecular alterations in ARMS.

a.

CGH Array gains	CGH Array losses
1q21	2q33
6p21	7q11
12q24	9q12
14q32	19q13
17p13-11	23q13

b.

Chromosomic regions	Genes
1q21	MCL1-BNPL
2q32	STAT1,MYO1B, NEDL2, PREI3
6p21	Histone gene family
7q11	ZNF gene family
9q12	ANKRD20A,MGC21881
12q24	ARK5, RFX4, SELPGL, UBE3B
14q32	MTA1, IGHG4
17p13-11	EIF4A1, NDEL1,MYH10,MAP2K4,GAS7
19p12	TIZ, SMG1
23(X)q13	OGT,CXCR3,PHKA1,PGK1,ZCCHC5,ITM2A,DACH2

values and corresponding numbers of significant genes. pDFR-value was chosen in order to select the maximum number of mutated genes compared to the minimum number of false positive mutations found by male/female hybridization control. A data table was obtained by this algorithm with converted values (wild type = 0, gain = + 1, loss = -1).

Real Time DNA quantification

Analysis of DNA copy number, at loci 1q21, 2q33, 12q24, 17p13, 17p11.2 and 19q13 was extended to all 14 RMS samples. Tumor DNA was extracted from fresh tissue according to phenol–chloroform method (Sambrook et al., 1989). PCR reactions included 2 µl of the extracted DNA (20ng), primers (400 nmols each) (Table 2a) and the 12.5 µl Sybr Green Master Mix (Applied Biosystems, CA, US), as well as water added to a final volume of 25 µl. Analysis was performed using an ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA) under the following cycling conditions: an initial denaturation step at 95°C for 10 min, followed by 40 cycles of amplification (95°C for 5 s, 60°C for 60 s). All assays were performed in duplicate.

For calculation of gene copy number we used the $2^{-\Delta\Delta CT}$ comparative method (Holzmann et al., 2004). Copy number of target genes was normalized to a housekeeping ß-actin gene (TaqMan Assays-Applied Biosystems, Foster City, CA) and DNA from normal tissue was used as calibrator.

RNA extraction and reverse transcription

Total RNA was extracted from all RMS frozen samples (~150 mg) using TRIzol Reagent (Invitrogen, Carlsbad CA) according to 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. 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 the manufacturer's instructions.

Real-Time (RT) PCR

Quantitative RT-PCR was performed using an ABI 7900 sequence detection system (Applied Biosystems, Foster City, CA). Expression of target genes MYH10, MAP2K4 and MCL1 was quantified using TaqMan Expression Assays (Applied Biosystems, Foster City, CA) (Table 2b) 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, genes were amplified by 45 cycles (95°C for 10 min, 95°C for 5 s, 60°C for 1 min). For calculation of gene expression we used the $2^{-\Delta\Delta CT}$ comparative method. Expression of target genes was normalized to a housekeeping GAPDH gene (TaqMan Expression Assays - Applied Biosystems, Foster City, CA) and normal striated muscle was used as calibrator.

Results

CGH array and real time PCR validation

Using array based CGH-analysis, multiple copy number changes were detected in 4 ARMS samples

Table 4. Relative DNA copy number analysed by Real Time PCR.

	MCL1 (ch1q21) $2^{-\Delta\Delta CT}$ ± SD	MYH10 (ch17p13) $2^{-\Delta\Delta CT}$ ± SD	MAP2K4 (ch17p11.2) $2^{-\Delta\Delta CT}$ ± SD	SELPLG (ch12q24) $2^{-\Delta\Delta CT}$ ± SD	PIK4CB (ch1q21) $2^{-\Delta\Delta CT}$ ± SD	PREI3 (ch2q33) $2^{-\Delta\Delta CT}$ ± SD	BIRC8 (ch19q13) $2^{-\Delta\Delta CT}$ ± SD
ARMS 1	1.02±0.09	0.99±0.09	1±0.08	0,97±0,17	0,66±0,43	0.38±0.22	0.94±1.09
ARMS 2	0.69±0.09	1.06±0.15	1.12±0.20	0,57±0,08	0,62±0,15	0.63±0.00	0.49±0.6
ARMS 3	1.67±0.17	0.73±0.03	2.67±0.21	0,58±1,22	0,87±0,04	0.27±0.69	0.76±0
ARMS 4	1.78±0.25	1.9±0.28	1.96±0.24	2,33±0,25	1,49±0,25	0.38±0.1	0.49±1.0
ARMS 5	0.64±0.23	0.72±0.07	1.77±0.07	0,51±0,49	0.55±0.08	0.06±0.6	0.04±0.3
PRMS 1	0.85±0.06	0.64±0.05	2.33±0.2	0,81±0,07	0,86±0,11	0.84±0.16	0.27±0.7
PRMS 2	0.99±0.21	0.8±0.27	0.9±0.15	0,37±0,23	0,84±0,44	0.53±0.36	2.04±0.05
PRMS 3	0.39±0.14	0.48±0.09	0.75±0.17	0,6±0,13	0,38±0,08	0.45±0.5	0.44±0
PRMS 4	0.64±0.80	0.57±0.18	1.98±0.83	0,94±0,78	0,46±0,15	0.40±0	0.07±0
ERMS 1	0.52±0.40	1.64±0.01	0.88±0.10	0,17±0,01	0,53±0,07	0.03±1.02	0.28±1.0
ERMS 2	0.38±0.58	0.41±0.19	1.19±0.59	0,48±0,16	0,33±0,53	2.28±0.33	1.37±0
ERMS 3	0.65±0.09	1.37±0.20	1.06±0.48	0,57±0,05	0,7±0,04	4.59±1.4	5.11±0.3
ERMS 4	0.4±0.46	1.12±0.31	1.88±0.33	0,54±0,05	0,28±0,30	0.38±0.8	0.36±1.06
ERMS 5	0.35±0.40	0.43±0.18	1.66±0.43	0,5±0,39	0,37±0,19	0.08±0.7	0.39±0.1

$2^{-\Delta\Delta CT}$ value (DNA copy number): > 1.5 was scored as gain and < 0.5 was scored as loss.

(Table 3a).

These included gains in 1q21, 6p21, 12q24, 14q32 and 17p13-11 and losses in 2q33, 7q11, 9q12, 19q13 and 23q13, likely leading to activation or inactivation of genes involved in malignancy progression, such as transcription factors (RFX4), signal transducers (STAT1), anti-apoptotic (MCL1-BNIPL), proliferating (ARK5-NEDL1-MAP2K4-CXCR3) and pro-metastatic (MTA1) genes (Table 3b).

To confirm CGH data, we assessed gene level at loci 1q21 (MCL1-PIK4CB), 2q33 (PREI3), 12q24 (SELPLG), 17p13(MYH10), 17p11.2(MAP2K4) and 19q13 (BIRC8) by comparative Real Time PCR method. DNA copy number > 1.5 ($2^{-\Delta\Delta CT}$) was scored as gain, < 0.5 ($2^{-\Delta\Delta CT}$) as loss. Our results showed 75% concordance with CGH array data except for BIRC8 gene which had 100% concordance.

Then, we analysed the copy number alterations by Real Time PCR in all tumor samples selected. Gains of gene sequences were found in 1/14 for SELPLG and PIKACB, in 2/14 for MCL1, MYH10, BIRC8 and PREI3, and in 7/14 for MAP2K4.

DNA losses were present in 4/14 for MCL1, in 3/14 for MYH10 and SELPLG, in 5/14 for PIKACB, in 9/14 for BIRC8 and PREI3 (Table 4). Variability of gene copy number changes confirmed lack of specific genetic events in RMS.

RNA expression

Evaluation of RNA expression of 14 RMS by Real-Time PCR was performed according to $2^{-\Delta\Delta CT}$ comparative method (Winer et al., 1999). Each gene was considered more expressed when the value was >1±SD, and less expressed when the value was < 1±SD. Value 1 corresponds to fluorescence emission in each amplification reaction for target and reference in normal striated muscle.

MCL1 was found to be more expressed in 9/14 cases *(*ARMS 3,4,5; PRMS 2,4; ERMS 1,2,3,5*)* with mRNA values ranging from 1.25 to 34.5. Genes located on chromosome 17p presented high mRNA expression in 10 out of 14 tumor samples, with value variability ranging from 1.78 to 39.94 for MYH10 and from 1.16 to 192.6 for MAP2K4.

MYH10 was overexpressed in ARMS 1,3,5; PRMS 1,2,4; ERMS 1,3,4,5*,* while MAP2K4 expression was found to be increased in all ARMS samples, in PRMS 1,4 and ERMS 1,3,5 (Table 5).

Table 5. mRNA expression analysed by Real Time PCR.

	MCL1 (ch1q21) $2^{-\Delta\Delta CT}$± SD	MYH10(ch17p13) $2^{-\Delta\Delta CT}$± SD	MAP2K4 (ch17p11.2) $2^{-\Delta\Delta CT}$± SD
ARMS 1	0.13±0	9.68±0.09	192.67±0
ARMS 2	0.16±0.3	0.47±0.06	1.91±0.04
ARMS 3	2.2±0.2	14.52±1.03	13.64±0.13
ARMS 4	2.36±0	0.41±0.1	1.67±3.32
ARMS 5	2.96±0.2	1.78±1.13	1.54±1.25
PRMS 1	1.04±0.04	10.81±0.2	2.18±0.82
PRMS 2	1.25±2	25.72±1.15	0.39±0
PRMS 3	0.03±0	0.19±0.7	0.35±0.2
PRMS 4	2.08±0.8	7.44±0.07	3.9±2.3
ERMS 1	2.42±0.9	3.32±0	22.94±3
ERMS 2	34.53±0	0.13±0.4	0.75±0.14
ERMS 3	3.29±0.7	39.94±1.27	1.16±0.5
ERMS 4	1.02±0.3	16.97±0.57	0.71±0.24
ERMS 5	2.74±0	31.56±0	3.06±0.1

Each gene was considered more expressed when the $2^{-\Delta\Delta CT}$ value was > 1 ±SD.

Discussion

Array-based CGH is a powerful tool for simultaneous detection of gene alterations, allowing identification of novel candidate genes in carcinogenesis (Holzmann et al., 2004). However, Real-time PCR is a less expensive and more accurate quantitative method useful for assessing exact copy number changes (Winer et al., 1999).

In our RMS series, array-based CGH analysis combined with quantitative Real-Time PCR detected remarkable copy number changes in 1q21, 17p, 12q24, 2q33 19q13 chromosome regions, harbouring genes involved in cell proliferation, malignancy progression, apoptosis and cell viability. In particular, by Real Time PCR we found amplifications in 17p and 1q21 regions including genes involved in signal-transduction and apoptosis, such as MAP2K4, and MCL1. MAP2K4 is a member of the mitogen-activated protein kinase (MAPK) cascade, characterized as a core signal transduction pathway (Ichijo, 1999). Cell lines lacking MAP2K4 exhibited defective activation of JNK and AP-1 dependent transcription activity in response to specific stimuli (Cuenda, 2000).

However, the role of MAP2K4 and of its downstream targets in tumor progression is still under investigation.

One of the most confirmed hypotheses is that members of both MAPK and Bcl-2 gene families contribute with other gene products to cell viability regulation (Townsend et al., 1998). In this study, MAP2K4 was found amplified in 7 out of 14 RMS, while in no cases were DNA losses evident. Although its genetic inactivation has been implicated in pancreatic and prostatic carcinogenesis in a role of tumor suppressor gene (Xin et al., 2004), other studies on many different tumor specimens found a very low frequence of MAP2K4 genetic alterations, suggesting that the possible loss of heterozigosity at TP53 locus might not extend into the MAP2K4 gene (Chae et al., 2002). On the other hand, we assessed increased expression of MAP2K4 in the majority of RMS samples. It is known that MAP2K4 acts upstream of c-JUN NH2-terminal kinase (JNK), phosphorylating and activating it. Expression of the activated form of JNK may influence apoptotic (Koyama et al., 2006) or survival signalling

pathways (Davis, 2000; Lee and McCubrey, 2002). *In vitro* studies demonstrated that the complexity of MAPK pathways suggests a pro-oncogenic activity of MAP2K4 /c-Jun NH2-terminal kinase depending on the presence of cell type-specific factors, activity of tumor proliferating or suppressor pathways (Lee et al., 2003; Wang et al., 2004).

Furthermore, a clinical study showed a correlation between MAP2K4 overexpression and short relapse-free and overall survival in patients with gastric adenocarcinoma (Wu et al., 2000).

Similarly, amplification of chromosome 17p led to overexpression of a gene involved in muscle contraction, MYH10, which encodes the protein "Mammalian non-muscle myosin II" involved in cytokinesis, cell migration, and cell shape change (Kim et al., 2005).

MCL1 protein plays an important role in the development of various malignancies and is expressed in a wide variety of tissues and neoplastic cells (Shighemasa et al., 2002; Krajewski et al., 2005). It has been demonstrated that its increased expression can enhance survival of hemopoietic cells in response to apoptotic stimuli (Zhou et al., 1997).

In addition to its anti-apoptotic effect, MCL1 has also been proven to be involved in the regulation of cell-cycle progression (Fujise et al., 2000).

In our series, only 2 cases had gains at 1q21 region, but 9 out of 14 RMS had increased mRNA values.

It is known that MCL1 is regulated both at transcriptional and post-transcriptional level by activation of MAPK family members. Ras/MEK/MAPK pathway influences MCL1 transcription such as PI3-kinase seems to be necessary to stimulate MCL1 protein translation (Schubert and Duronio, 2001). MCL1 mRNA levels have been found to be increased in human hepatocellular carcinoma and to be related to apoptosis resistance (Fleischer et al., 2006). Accordingly, a recent study observed strong MCL1 expression in sarcoma cell lines of different histological origin, associated with inhibition of apoptosis probably due to down-regulation of cell-damage-induced mitochondrial cytochrome *c* release (Wang and Studzinski, 1997).

The hypothesis that MCL1 may be a chemoresistant target was confirmed by pro-apoptotic effects of a combination of MCL1 antisense oligonucleotides with low-dose cyclophosphamide, promising new treatment strategy in human sarcoma therapy (Thallinger et al., 2004).

In conclusion, our data on highly aggressive RMS from patients with poor prognosis indicated a possibile involvement of specific chromosome regions in progression of clinical malignancy. The alterated expression of MAP2K4 and MCL1 genes both involved in different biological functions of complicated signaling pathways, appeared to be associated with disease relapse rather than with histological or cytogenetic features.
Further studies are required to understand the biological significance of their altered regulation in RMS cells.

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References

Anderson J., Gordon A., Pritchard-Jones K. and Shipley J. (1999).Gene chromosomes and rhabdomyosarcoma. Genes Chromosomes Cancer 26, 275-285.

Astolfi A., De Giovanni C., Landuzzi L., Nicoletti G., Ricci G., Croci S., Scopece L., Nanni P. and Lollini P. (2001). Identification of new genes related to the myogenic differentiation arrest of human rhabdomyosarcoma cells. Gene 274, 139-149.

Barr F.G. (2001). Gene fusion involving PAX and FOS family members in alveolar rhabdomyosarcoma. Oncogene 20, 5736-5746.

Barrett M.T., Scheffer A., Ben-Dor A., Sampas N., Lipson D., Kincaid R., Tsang P., Curry B., Baird K., Meltzer PS., Yakhini Z., Bruhn L. and Laderman S. (2004). Comparative genomic hybridization using oligonucleotide microarrays and total genomic DNA. PNAS 101, 1765-1777.

Chae K.S., Ryu B.K., Lee M-G., Byun D.S. and Chi S.G. (2002). Expression and mutation analyses of *MKK4*, a candidate tumour suppressor gene encoded by chromosome 17p, in human gastric adenocarcinoma. Eur. J. Cancer 38, 2048-2057.

Charge S.B. and Rudnicki M.A. (2004). Cellular and molecular regulation of muscle regeneration. Physiol. Rev. 84, 209-238.

Cuenda A. (2000). Mitogen-activated protein kinase kinase 4 (MKK4). Int. J. Biochem. Cell Biol. 32, 581-587.

Davis R.J. (2000). Signal Transduction by the JNK Group of MAP Kinases Cell 103, 239-252.

Fleischer B., Schulze-Bergkamen H., Schuchmann M., Weber A., Biesterfeld S., Muller M., Krammer P.H. and Galle P.R. (2006). Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. Int. J. Oncol. 28, 25-32.

Fletcher C.D.M., Unni K.K. and Martens F. (2002). World Health Organization: Classification of tumors pathology and genetics of tumours of Soft tissue and Bone. IARC press. Lyon (France). pp 146-154.

Fujise K., Zhang D., Liu J. and Yeh E.T. (2000). Regulation of apoptosis and cell cycle progression by MCL1. Differential role of proliferating cell nuclear antigen. J. Biol. Chem. 275, 39458-39465.

Gallego M. and Sanchez de Toledo Codina J. (2007). Molecular biology of rhabdomyosarcoma. Clin. Transl. Oncol. 9, 415-419.

Goldstein M., Meller I., Issakov J. And Orr-Urtreger A. (2006). Novel genes implicated in embryonal, alveolar and pleomorphic rhabdomyosarcoma: a cytogenetic and molecular analysis of primary tumors. Neoplasia 8, 332-343.

Gordon A., McManus A., Anderson J., Fischer C., Abe S., Nojima T., Pritchard-Jones K. and Shipley J. (2003). Chromosomal imbalances in pleomorphic rhabdomosarcomas and identification of the alveolar rhabdomyosarcoma-associated PAX3-FOXO1A fusion gene in one case. Cancer Genet. Cytogenet. 140, 73-77.

Helman L.J. and Meltzer P. (2003). Mechanisms of sarcoma

development. Nature Rev. Cancer 3, 685-694.

Holzmann K., Kohlhammer H., Schwaenen C., Wessendorf S., Kestler H.A., Schowoerer A., Rau B., Radlwimmer B., Dohner H., Lichter P., Gresss T. and Bentz M. (2004). Genomic DNA-chip hybridization reveals a higher incidence of genomic amplifications in pancreatic cancer than conventional comparative genomic hybridization and leads to the identification of novel candidate genes. Cancer Res. 64, 4428-4433.

Ichijo H. (1999). From receptors to stress-activated MAP kinases. Oncogene 18, 6087-6093.

Kalebic T., Blakesley V., Slade C., Plasschaert S., Leroith D. and Helman L.J. (1998). Expression of a kinase-deficient IGF-I-R suppresses tumorigenicity of rhabdomyosarcoma cells constitutively expressing a wild type IGF-I-R. Int. J. Cancer 76; 223-227.

Kim K.Y., Kovacs M., Kawamoto S., Seller J.R. and Adelstein R.S. (2005). Disease-associated mutations and alternative splicing alter the enzymatic and motile activity of nonmuscle myosins II-B and II-C. J. Biol. Chem. 280, 22769-75.

Khan J., Bittner M.L., Saal L.H., Teichmann U., Azorsa D.O., Gooden G.C., Pavan W.J. Trent J.M. and Meltzer P.S. (1999). cDNA microarrays detect activation of a myogenic transcription program by the PAX3-FKHR fusion oncogene. PNAS 96, 13264-13269.

Koyama T., Mikami T., Imakiire A., Yamamoto K., Toyota H. and Mizuguchi J. (2006). Apoptosis induced by chemotherapeutic agents involves c-Jun N- terminal kinase activation in sarcoma cell lines. J. Othop. Res. 24,1153-1162.

Krajewski S., Bodrug S., Krajewska M., Shabaik A., Gascoyne R., Beren K. and Reed J.C. (1995). Immunohistochemical analysis of Mcl-1 protein in human tissues. Differential regulation of Mcl-1 and Bcl-2 protein production suggests a unique role for Mcl-1 in control of programmed cell death in vivo. Am. J. Pathol. 146, 1309-1319.

Lee H.Y., Srinivas H., Xia D., Lu Y., Superty R., LaPushin R., Gomez-Manzano C., Gal A.M., Walsh G.L., Force T., Ueki K., Mills G.B. and Kurie J.M. (2003). Evidence that phosphatidylinositol 3-kinase- and mitogen-activated protein kinase kinase-4/c-JunNH2-terminal kinase-dependent pathways cooperate to maintain lung cancer cell survival. J. Biol. Chem. 278, 23630-23638.

Lee J.Y. Jr and McCubrey J.A. (2002). The Raf/MEK/ERK signal transduction cascade as a target fot chemotherapeutic intervention in leukemia. Leukemia 16, 486-507.

Lingjaerde O.C., Baumbusch L.O., Liestol K., Glad I.K. and Borresen-Dale A.L. (2005). CGH-Explorer: a program for analysis of array-CGH. Bioinformatics 21, 821-822.

Merlino G. and Helman L.J. (1999). Rhabdomyosarcoma- working out in pathways. Oncogene 18, 5340-5348.

Morandi L., Marucci G., Foschini M.P., Cattani M.G., Pession A., Riva C. and Eusebi V. (2006). Genetic similarities and differences between lobular in situ neoplasia (LN) and invasive lobular carcinoma of the breast. Virchows Arch. 449, 14-23.

Pandita A., Zielenska M., Thorner P., Bayani J., Godbout R., Greenberg M. and Squire J.A. (1999). Application of comparative genomic hybridization , spectral karyotyping and microarray analysis in the identification of sub-type specific patterns of genomic changes in rhabdomyosarcoma. Neoplasia 1, 262-275.

Ragazzini P., Gamberi G., Pazzaglia L., Serra M., Magagnoli G., Ponticelli F., Ferrari C., Ghinelli C., Alberghini M., Bretoni F., Picci P. and Benassi M.S. (2004). Amplification of CDK4, MDM2,SAS, and GLI genes in leiomyosarcoma, alveolar and embryonal rhabdomyosarcoma. Histol. Histopathol. 19, 401-411.

Sambrook J., Fritsch E.F., Maniatis T. and Harbor C.S. (1989). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press. New York.

Schubert K.M. and Duronio V. (2001). Distinct roles for extarcellular - signal-regulated protein kinase (ERK) mitogen-activated protein kinase and phosphotidylinositol 3-kinase in the regulation of MCL1 synthesis. Biochem. J. 356,473-480.

Shighemasa K., Katoh O., Shiroyama Y., Mihara S., Mukai K., Nagai N. and Ohama K. (2002). Increased MCL-1 expression is associated with poor prognosis in ovarian carcinomas. Jpn. J. Cancer Res. 93, 542-50.

Thallinger C., Wolschek M.F., Maierhofer H., Skwara H., Pehamberger H., Monia B.P., Jansen B., Wacheck V. and Selzer E. (2004). MCL1 is a novel therapeutic target for human sarcoma: synergistic inhibition of human sarcoma xenotransplants by a combination of MCL-1 antisense oligonucleotides with low dose cyclophosphamide. Clin, Cancer Res, 10, 4185-4241.

Townsend K.J., Trusty J.L., Traupman M,A., Eastman A. and Graig R.W. (1998). Expression of antiapoptotic MCL1 gene product is regulated by mitogene activated protein kinases-mediated pathway triggered through microtubule disruption and protein kinases C. Oncogene 17, 1223-1234.

Wang L., Pan Y. and Dai J.L. (2004). Evidence of MKK4 pro-oncogenic activity in breast and pancreatic tumors. Oncogene 23, 5978-5985.

Wang X. and Studzinski G.P. (1997). Antiapoptotic action of 1,25-dihydroxyvitamin D3 is associated with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome c. Exp. Cell Res. 235, 210-217.

Weiss S.W. and Goldblum J.R. (2001). Malignant soft tissue tumors of incertain type. In: Enzinger and Weiss's eds, Soft tissue tumors (Fourth edition). St Louis. Mosby. 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 expresion in cardiac myocytes in vitro. Anal. Biochem. 270, 41-49.

Wu C.W., Li A.F, Chi C.W., Huang C.L., Shen K.H., Liu W.Y. and Lin W. (2000). Human gastric cancer kinase profile and prognostic significance of MKK4 kinase. Am. J. Pathol. 156, 2007-2015.

Xin W., Yun K.J., Ricci F., Zahurak M., Qiu W., Su G.H., Yeo C.J., Hruban R.H., Kern S.E. and Iacobuzio-Donahue C.A. (2004). MAP2K4/MKK4 expression in pancreatic cancer: genetic validation of immunohistochemistry and relationship to disease course. Clin. Cancer Res. 10, 8516-8520.

Zhou P., Qian L., Kozopas K.M. and Craig R.W. (1997). Mcl-1, a Bcl-2 family member, delays the death of hematopoietic cells under a variety of apoptosis-inducing conditions. Blood 89, 630-643.

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