Altered expression and molecular abnormalities of cell-cycle-regulatory proteins in rhabdomyosarcoma

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Rhabdomyosarcoma is the most commonly occurring soft-tissue sarcoma in children. Some reports have discussed the altered expression and molecular abnormalities of cell-cycle-regulatory proteins in rhabdomyosarcoma; however, variable frequencies of occurrence have been noted. In the current study, among 72 cases of rhabdomyosarcoma, the authors evaluated for the expression of p53, MDM2, p16, p21/WAF1, p27, cyclin D1, cyclin E, pRb and E2F-1 protein immunohistochemically and assessed for proliferative activities using MIB-1. We also analyzed the mutation of the *p53* gene in 45 cases, the amplification of the *MDM2* gene in 18 cases and the mutation of the *H-ras* gene in 29 cases, using formalin-fixed paraffin-embedded materials. Furthermore, we assessed the correlation between clinicopathologic factors and the results of both immunohistochemical and molecular analyses. Alveolar type affected older patients, and it had a significantly higher mitotic rate compared with the embryonal type ($P = 0.0226$). p53 overexpression was detected in 22 (30.6%) of 72 cases, and 10 (22.2%) of 45 cases had *p53* gene abnormalities. As for MDM2, its overexpression was found in nine (12.5%) of 72 cases, and three (16.7%) of 18 cases showed *MDM2* amplification. A statistically significant association was observed between immunoreaction for MDM2 and p53 overexpression ($P = 0.0002$), and p53 and MDM2 overexpression was significantly correlated with high MIB-1 labeling indices. E2F-1 labeling indices showed a significantly higher score in alveolar type compared with that seen in embryonal type ($P = 0.0334$), but MIB-1 did not. In conclusion, our study suggests that p53 overexpression may be related to tumor progression because tumors with p53 overexpression have a high proliferative activity in the current study. Alveolar type had a significantly higher both mitotic rate and E2F-1 labeling indices when compared with the embryonal type. The current study is the first report of the correlation of E2F-1 with alveolar rhabdomyosarcoma.

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Rhabdomyosarcoma is the most commonly occurring soft-tissue sarcoma in children.¹ Based on histopathologic features, rhabdomyosarcomas can be categorized into the following types: embryonal, alveolar and pleomorphic subtypes.² It has been reported that both the biological behavior and the clinical behavior are different depending on the above subtypes of rhabdomyosarcoma, and even within the same subtype, differences with regard to malignant behavior have been noted.³

There have been several reports concerning the altered expression and the molecular abnormalities of the cell-cycle-regulatory proteins in rhabdomyosarcoma, as has also been reported for other soft-tissue tumors, and as a result, variable frequencies of occurrence have been noted.^{4–16} Altered cell-cycle regulation may underlie the development and/or progression of human malignancies. Many of the tumor-suppressor genes and oncogenes directly participate in or regulate the signal transduction pathways. The genes and their proteins, acting at the G1-S checkpoint, represent one of the more frequent targets in molecular tumorigenesis.

Mutation of the *p53* gene is the most common alteration found in human malignant tumors and the alterations of *p53* have been suggested as playing a role in tumor progression.^{4,6,8,17} The *MDM2* gene products bind to p53 protein, forming a complex

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that can inhibit the p53 growth-suppressive function by blocking the transactivation domain of p53.^{18,19} On the other hand, the coexpression of p53 and MDM2 protein has been demonstrated as being an adverse prognostic factor in several types of soft-tissue sarcomas.^{8,20,21}

Although several kinds of sarcoma have been analyzed, there have been few investigations utilizing a series of childhood malignant solid tumors, including rhabdomyosarcoma, especially where the focus has been on clinical specimens.^{4,5,7,10,12,14–16} The number of samples studied has been very small so far, and the data concerning the occurrence of disturbance in the cell-cycle-regulatory proteins involving the p53 and the Rb pathway have been limited, with great variations in data, such as the rate of p53 mutations ranging from 0 to 45%.^{4–8,17} Furthermore, there has not been enough evaluation of the correlation between the alterations of these proteins and the clinicopathological findings in rhabdomyosarcoma.²²

In addition, the *ras* gene family has been reported to be associated with human neoplasms, and frequent *ras* mutations have been reported in a number of human cancers. As for sarcomas, *H-ras* codon 12 mutations have been reported in rhabdomyosarcoma and in malignant fibrous histiocytoma.²³

The aim of this study was to define the frequency of altered expression and molecular abnormalities of cell-cycle regulatory proteins involving the p53 and the Rb pathway in a rather large series of rhabdomyosarcoma using clinical specimens, and to investigate their possible association with clinicopathologic features and proliferative activities.

Materials and methods

Case Materials

In all, 72 formalin-fixed, paraffin-embedded rhabdomyosarcoma specimens registered in the Department of Anatomic Pathology between 1970 and 2001 were available for immunohistochemical studies. The diagnosis of all cases was based on light microscopic examination with hematoxylin–eosin (HE) staining according to the WHO classification in 2002² and, where necessary, immunoperoxidase procedures using the streptavidin–biotin peroxidase (SAB) method were carried out. All the samples were from different patients, and among them nine cases had already been treated by chemotherapy or radiotherapy. Survival data were available for 64 cases. Follow-up ranged from 1 to 257 months (mean, 46.0 months). On the other hand, from among these 72 cases, 45 cases, in which the DNA was of good quality, were used for further molecular analyses.

We assessed the correlation between clinicopathologic factors (sex, age, anatomical site, histologic

subtype and mitotic rate) and the results of both immunohistochemical and molecular analysis. The mitotic count was also estimated by counting mitotic figures in 10 high-power fields (HPFs).

Immunohistochemistry

The expression of cell-cycle-related proteins was investigated by immunohistochemical studies. The primary antibodies used in this study are summarized in Table 1. Histologic sections (4- μ m) of 10% formalin-fixed, paraffin-embedded materials were cut, mounted on glass slides coated by 3-aminopropyltriethoxysilane and then air-dried overnight at room temperature. The sections were deparaffinized in xylene and rehydrated in ethanol, and endogenous peroxidase was blocked by methanol containing 3% hydrogen peroxidase for 15 min. For staining with the above antibodies, sections were pretreated with citrate buffer (0.01 mol/l citric acid: pH 6.0) four times, each for 5 min at 100°C in a microwave oven. The sections were incubated with primary antibodies at 4°C overnight, followed by staining with a streptavidin–biotin peroxidase kit (Nichirei, Tokyo, Japan). The sections were then finally reacted in a 3,3'-diaminobenzidine, peroxytrichloride substrate solution and counterstained with methyl green.

In the cases of p53 and MDM2, staining of more than 10% of the nuclei was needed for positive cases. In the cases of p16 and Rb, the immunoreactivities were classified into three categories, defined as follows: minus sign, 0% of the nuclei were reactive; heterogeneous staining, 1–49% of the nuclei were reactive; and plus sign, more than 50% of the nuclei were reactive. Samples showing either minus or heterogeneous staining were considered abnormal. The p21, p27, cyclin D1, cyclin E, MIB-1 and E2F-1 labeling indices (LI) were determined by counting the positively stained nuclei in at least 500 tumor cells. The scoring was performed by a single

Table 1 Monoclonal antibodies used in the study

Antigen	Dilution	Source
Ki-67	1:100	Immunotech, Marseille, France
p53	1:100	Oncogene Science, New York, NY, USA
MDM2	1:40	Oncogene Research Products, Cambridge, MA, USA
p21/WAF1	1:100	Oncogene Research Products
p27	1:40	Novocastra Laboratories, Newcastle upon Tyne, UK, UK
p16	1:50	Santa Cruz, CA, USA
cyclin D1	1:25	Novocastra Laboratories
cyclin E	1:100	Calbiochem, Cambridge, MA, USA
pRb	1:800	Pharmingen Laboratories, San Diego, CA, USA
E2F-1	1:100	Santa Cruz, CA, USA

pathologist who had no prior knowledge of the clinical characteristics.

Polymerase Chain Reaction-Single-Strand Conformation Polymorphism (PCR-SSCP) and DNA Sequencing for *p53*

Genomic DNA was purified from paraffin-embedded tissues of 45 rhabdomyosarcomas, using standard proteinase K digestion and phenol/chloroform extraction.

The Polymerase Chain Reaction-Single-Strand Conformation Polymorphism (PCR-SSCP) method in this study was used as the method of Oda *et al.*²⁴ Mutations of the *p53* gene were examined from exons 5–9. After the purification of aberrantly migrating bands of *p53* in the SSCP gel, direct sequencing was carried out by the dideoxy chain termination methods using a Perkin Elmer ABI Prism 310 sequence analyzer (Applied Biosystems, Foster City, CA, USA). The primers used for direct sequences were the sense and antisense primers used for PCR-SSCP in *p53*.

Differential PCR for *MDM2*

The differential PCR method used to detect the amplification of *MDM2* in this study was a modification of the method of Reid *et al.*^{24,25} As for a positive control, DNA of the SA-1 cell line (American Type Cell Collection, Rockville, MD, USA), which is known to show seven-fold amplification of the *MDM2* gene by the radio-labeled differential PCR method, was used. After amplification, 15 μ l of each reaction was analyzed by electrophoresis on a 3% agarose gel, stained with ethidium bromide, and the intensities of the DNA products were quantified using a National Institutes of Health (NIH) Image Ver 1.56. The level of *MDM2* amplification was determined by comparing the ratio of the intensities of the *MDM2* and *PAH* PCR products for each of the samples with positive SA-1 cells (seven-fold). Samples showing more than two-fold amplification were judged as positive.⁹

Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) for *H-ras*

We used the PCR–RFLP procedure to detect *H-ras* mutations at codons 12 and 13 with strategy primers as reported.²⁶ Codon 12 mutations were detected thanks to a naturally occurring *HpaII* site (CCGG) that is lost when the mutation occurs. *HpaII* digests the 71-bp-amplified fragment into two fragments (41- and 30-bp), thus revealing the presence of the normal allele, while the mutant allele remains within the undigested 71-bp fragment. This method allows the detection of a mutation of the second and third positions of codon 11 and the first and second

positions of codon 12. To detect codon 13 mutation, nested primers were used to create a new restriction site for *HphI* (GGTGA) by changing a T for an A in the second position of codon 14, thereby allowing the detection of any mutation of codon 13 of the *H-ras* gene. *HphI* digests the same 71-bp-amplified fragment into two fragments (58- and 13-bp), thus revealing the presence of the normal allele, while the mutant allele remains within the undigested 71-bp fragment. The DNA bands were analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide and then photographed.

Statistical Analysis

Fisher's exact test was used to evaluate the association between two dichotomous variables for *p53*, *MDM2*, p16 and pRb. The clinicopathologic parameters investigated in this study were classified as follows: age (<10 vs $\geq$ 10), location (orbital or genitourinary (restricted nonbladder/prostate) vs others), mitotic rate (<15/10 HPFs vs $\geq$ 15/10 HPFs) and histological subtype (embryonal vs alveolar). As regards the histological subtypes of this tumor, two cases with pleomorphic type were excluded from the statistical analysis because the number of samples was too small to analyze. The difference in the labeling indices of p21, p27, cyclin D1, cyclin E, MIB-1 and E2F-1 between the two groups was estimated by the Mann–Whitney test. A *P* value of less than 0.05 was considered statistically significant.

Results

Clinicopathologic data for the patients with rhabdomyosarcoma are summarized in Table 2. There were 41 male and 31 female patients, ranging in age from 15 days to 38 years (mean, 11.9 years). Histologically, 36 tumors were categorized as embryonal type (Figure 1), of which two as botryoid type, four as spindle cell type and one as anaplastic type. In all, 34 tumors were categorized as alveolar type (Figure 2) and two as pleomorphic type. Alveolar type (mean age, 15.8 years) occurred in older individuals than embryonal type (mean age, 8.2 years). Although rhabdomyosarcomas occurred at various sites, the most common site was the head and neck (23 cases), followed by the genitourinary area (17 cases), the extremities (12 cases), the trunk (12 cases), the retroperitoneum (three cases), the peritoneum (two cases), the pelvis (two cases), and the lung (one case). Embryonal type frequently occurred in the genitourinary area (13/36; 36.1%), whereas alveolar type frequently occurred at the extremities (10/34; 29.4%). As for two cases of pleomorphic type, one 10-year-old female having a tumor in the oral cavity died of the disease 14 months after surgery, and another 16-year-old female having a tumor in the chest wall also died of the disease 7 months after

Table 2 Clinicopathological data for rhabdomyosarcoma

	<i>Embryonal</i>	<i>Alveolar</i>	<i>Pleomorphic</i>	<i>All types of RMS</i>
Number	36 (50.0%)	34 (47.2%)	2 (2.8%)	72
Sex (m:f)	22:14	19:15	0:2	41:31
Ratio	1.6:1.0	1.3:1.0		1.3:1.0
Age (mean)	8.1 years	15.2 years	13.0 years	11.9 years
Range; median	15 day–38 years; 4 years	2 mo–36 years; 15 years	10 years–16 years; 13 years	15 day–13 years; 10 years
Location (no.)				
Head/neck	10	12	1	23
Parameningeal area	1	0	0	1
Orbit	2	2	0	4
Extremities	2	10	0	12
Genitourinary area	13	4	0	17
Bladder, prostate	8	0	0	8
Other	11	8	1	20
Mitotic rate (no.)				
≥ 15	32	22	1	55
< 15	4	12	1	17
Follow-up (no.)				
DOD	17	21	2	40
AWD	17	7	0	24

mo = month.

Figure 1 Embryonal type. The rounded tumor cells have hyperchromatic nuclei and some reveal an eosinophilic cytoplasm.

Figure 2 Alveolar type. The tumor is composed of a proliferation of rounded cells with thick collagenous septa.

surgery. The mitotic rate ranged from 0 to 83 per 10 HPFs (mean, 10.3 per 10 HPFs). A total of 52 tumors (72.2%) had less than nine mitoses per 10 HPFs, whereas 11 (15.3%) had between 10 and 19 mitoses per 10 HPFs. Nine tumors (12.5%) had more than 20 mitoses per 10 HPFs, of which seven cases were alveolar type. The mitotic rate was recorded as being high in 17 cases (23.6%), including one case of pleomorphic type, with more than 15 mitoses per 10 HPFs. The alveolar type had significantly more numerous mitoses compared with the embryonal type (mean value for alveolar type, 16.1; mean value for embryonal type, 4.5; $P = 0.0226$).

Immunoreactivity for All Cell-cycle-regulatory Proteins

The results of immunohistochemical analysis are summarized in Table 3. PRb immunoreactivity showed normal expression in 19 samples (26.4%) (Figure 3a), while altered patterns were observed in 53 samples (73.6%) (Figure 3b). Altered staining patterns included heterogeneous expression with some cells (1–49%) positive and others negative within the same samples. As for p16, 24 (33.3%) samples were considered normal, while 48 (66.6%) demonstrated altered patterns, either heterogeneous

Table 3 Immunohistochemical analysis of histological types of rhabdomyosarcoma

	<i>Embryonal</i> (n = 36)	<i>Alveolar</i> (n = 34)	<i>Pleomorphic</i> (n = 2)	<i>Overall</i> (n = 72)
p53+	14 (38.8%)	7 (20.6%)	1 (50.0%)	22 (30.6%)
MDM2+	7 (19.4%)	2 (5.8%)	0 (0%)	9 (12.5%)
p16+	21 (58.3%)	26 (76.5%)	1 (50.0%)	48 (66.7%)
RB+	30 (83.3%)	22 (64.7%)	1 (50.0%)	53 (73.6%)
*p21-LI (range)	7.5 (0–68.0)	3.7 (0–20.0)	4.5 (2.0–7.0)	5.6 (0–68.0)
*p27-LI (range)	5.6 (0–48.0)	5.6 (0–64.0)	2.0 (0–4.0)	5.5 (0–64.0)
*cyclin D1-LI (range)	4.7 (0–40.0)	4.9 (0–40.0)	0.5 (0–1.0)	4.7 (0–40.0)
*cyclin E-LI (range)	1.1 (0–18.0)	1.1 (0–18.0)	1.1 (0–18.0)	1.1 (0–18.0)
*E2F 1-LI (range)	5.6 (0–30.0)	10.3 (0–38.1)	5.3 (0–10.6)	7.7 (0–38.1)
*MIB1-LI (range)	13.3 (4.1–40.3)	10.4 (1.7–33.9)	11.5 (6.5–16.5)	11.8 (1.7–40.3)

*Mean value.

Figure 3 Immunohistochemical staining for pRB. (a) Tumor in the pelvic cavity of a 1-year-old female, embryonal type. The patient died of the disease 9 months after surgery (RMS5). A positive nuclear reaction in most tumor cells. (b) Tumor of the shoulder of a 13-year-old female, alveolar type. The patient remains alive 153 months after surgery (RMS51). Heterogeneous expression can be seen in some cells.

(n = 25, 34.7%) or negative (n = 23, 31.9%). The mean LI and SD for p21, p27, cyclin D1, cyclin E, MIB-1 and E2F-1 were 5.64 and 11.86 (range, 0–68.0), 5.49 and 11.22 (range, 0–64.0), 4.68 and 8.89 (range, 0–40.0), 2.31 and 5.11 (range, 0–28.0), 11.80 and 8.46

Figure 4 Immunohistochemical staining for cyclin D1 (LI=6). Tumor of the finger of a 17-year-old female, alveolar type. The patient died of the disease 2 months after surgery (RMS9).

Figure 5 Immunohistochemical staining for E2F in alveolar type (LI = 14). Orbital tumor of 25-year-old male, alveolar type (RMS3).

(range, 1.7–40.3) and 7.65 and 9.47 (range, 0–38.1), respectively (Figures 4 and 5).

Of the 72 rhabdomyosarcoma cases analyzed, nuclear accumulation of p53 was detected in 22 cases (30.5%) (Figure 6), while overexpression of MDM2 was found in nine cases (12.5%).

There were some relationships between the expressions of cell-cycle-related proteins and clinicopathological parameters. Altered pRb levels ($P=0.0297$) and a high cyclin D1 LI ($P=0.048$) were significantly correlated with those tumor sites that were related to poor prognosis. Cyclin E (embryonal type: 1.11 vs alveolar type: 3.68, $P=0.037$) and E2F-LI (embryonal type: 5.58 vs alveolar type: 10.33, $P=0.0334$) showed a high score in the alveolar type compared with that noted in the embryonal type. A statistically significant association was observed between p53 overexpres-

Figure 6 p53 immunoreactivity is observable in Case RMS51.

Table 4 Correlation between immunoreactivity or labeling index of cell cycle regulators and labeling index of MIB 1 and E2F 1 in rhabdomyosarcoma

	MIB1-LI Mean	P value	E2F 1-LI Mean	P value
p53				
–(n = 49)	10.5	0.0350*	7.7	0.4957
+(n = 21)	15.2		8.4	
MDM2				
–(n = 61)	11	0.0360*	7.8	0.8384
+(n = 9)	18.2		8.5	
p16				
–(n = 17)	13.6	0.3801	7.3	0.8313
+(n = 53)	11.4		8.1	
RB				
–(n = 18)	14.7	0.1167	9.4	0.3331
+(n = 52)	10.9		7.4	
p21LI				
L (n = 54)	11.6	0.5705	6.5	0.0185*
H (n = 16)	13		12.7	
p27-LI				
L (n = 52)	10.4	0.0094*	7.5	0.5173
H (n = 18)	16.4		9.1	
cyclin D1-LI				
L (n = 52)	12	0.9185	6.3	0.0123*
H (n = 18)	11.7		12.6	
cyclin E-LI				
L (n = 61)	11.9	0.988	6.8	0.0085*
H (n = 9)	11.9		15.5	

*Statistically significant; L: low LI; H: high LI.

sion and altered p16 staining ($P=0.0292$), but no association was found with clinicopathologic parameters. A statistically significant association was also observed between MDM2 and both p21 ($P=0.0246$) and p27 expression ($P=0.0070$), and between cyclin E and both p21 ($P=0.0246$) and cyclin D1 expression ($P=0.0427$).

Table 4 presents the relationships between the MIB-1 and E2F LI and the expressions of different proteins. We used the mean values as the cutoff values for each of p21, p27, cyclin D and cyclin E LI (mean, 5.6, 5.5, 4.7, 5.1, respectively). The MIB-1 LI was significantly correlated with the immunohistochemical expression of p53 ($P=0.0350$), MDM2 ($P=0.0360$) and high p27 LI ($P=0.0448$), whereas the E2F-1 LI was significantly correlated with the immunohistochemical expression of high p21 LI ($P=0.0185$), high cyclin D1 LI ($P=0.0123$) and high cyclin E LI ($P=0.0085$).

P53, MDM2, p21, p27, p16, pRb, cyclin D1 and cyclin E all showed no association with the patients' prognoses.

p53 Mutations in Exons 5–9

In all, 10 of the 45 cases (22.2%) had *p53* gene alterations detected by molecular biological analysis (Figure 7). There were 11 mutational sites, two being silent mutations (Cases 17 and 72), and nine being missense mutations (Table 5). One tumor, which was a single case showing anaplasia (Case 31), contained two mutations at codons 247 and 249 and also demonstrated nuclear accumulation of p53 protein. As for the type of base change, there were 10 sites of transition and one site of transversion. No positive correlation was observed between *p53* mutation status and the patients' prognoses or any of the other clinicopathologic parameters. In all 22 of the 72 cases (30.6%) demonstrated nuclear accumulation of p53 protein in more than 10% of the tumor cells. Among them, four cases contained five *p53* mutations comprising one silent mutation and four missense mutations. There was no significant correlation between p53 immunoreactivity and *p53* mutation status.

MDM2 Amplification

Differential PCR for *MDM2* was available in 18 cases. Three cases (16.7%) showed *MDM2* amplification. Nine out of the 72 cases (12.5%) showed immunoreaction for MDM2. A statistically significant association was observed between immunoreaction for MDM2 and p53 overexpression ($P=0.0002$). Eight cases showed the coexpression of both p53 and MDM2 protein, but this did not affect the prognosis of the patients. There was no correlation between *MDM2* amplification and either p53 or MDM2 overexpression. In addition, MDM2 showed no association with the patients' prognoses

or with any of the other clinicopathologic parameters, including histological type and age.

H-ras Mutation Status

Overall, *H-ras* mutations at Codon 12 or 13 were not detected in any of these rhabdomyosarcoma clinical specimens.

Discussion

Rhabdomyosarcoma is the most commonly occurring soft-tissue sarcoma in children, accounting for 5–8% of all malignancies.¹ Based on histopathologic features, rhabdomyosarcomas can be categorized into the following types: embryonal including botryoid and spindle-cell subtypes, alveolar, and also pleomorphic subtype, although this type is rare.² It has been reported that the alveolar subtype has more aggressive features

than the embryonal subtype, with both the biological and clinical behavior differing among the subtypes of RMS; even within the same subtype, differences with regard to malignant behavior have been noted. It has also been reported that tumors occurring in the orbit, paratestis or head and neck show a better prognosis than those at other sites. The reasons for these findings are still unknown. In the previous reports of rhabdomyosarcoma, data concerning alteration of the cell-cycle-regulatory proteins involving the p53 and the Rb pathways have been limited. In this study, we tried to define both the frequency of altered expression and the molecular abnormalities of cell-cycle-regulatory proteins involving the p53/Rb pathway in a rather large series of rhabdomyosarcoma clinical specimens.

The expression of p53 protein in rhabdomyosarcoma has been recognized in 19–75% of cases.^{6,8,9–12} Ayan *et al* commented that the data demonstrated an apparent correlation between p53 overexpression and event-free survival, but not overall survival. Lai *et al* also reported that p53 overexpression was related to both tumor differentiation and the degree of malignancy, and so p53 positivity may indicate a poor prognosis. In our series, of the 72 cases of rhabdomyosarcoma analyzed, nuclear accumulation of p53 was detected in 22 cases (30.5%). In line with their report that there was no correlation between p53 overexpression and age, sex or histopathologic subtype,^{10,11} our data also demonstrated that p53 showed no association with the patients' prognoses or with any of the other clinicopathologic parameters. In the present study, the MIB-1 LI was significantly correlated with the immunohistochemical expression of p53 ($P=0.0350$). No positive correlation was observed between p53 overexpression and the patients' prognoses, however, p53 overexpression was associated with a high MIB-LI, which is known as a tumor proliferation marker. Therefore, p53 overexpression would appear to be related to tumor aggressiveness in rhabdomyosarcoma.

The wild-type p53 protein is a transcriptional activator of target genes, functioning as a G1 cell-

Figure 7 PCR-SSCP analysis at Exon 7. Abnormal band is evident in Case RMS2 (a). Direct DNA sequencing of Exon 7 in Case RMS2 (b). (Codon 243, ATG to ACG).

Table 5 p53 mutations in 45 cases of rhabdomyosarcoma

Case	Age/sex	Location	Histology	Exon	Codon	Base change	Amino-acid	p53 IHC	Follow-up
RMS2	25/F	Orbit	Alveolar	7	243	ATG→ACG	Met→Thr	+	NA
RMS7	5/F	Forearm	Alveolar	8	291	AAG→CAG	Cys→Gln	–	NA
RMS17	2/F	Back	Embryonal	7	240	AGT→AGC	Ser→Ser	+	64 mo/DOD
RMS21	2/F	Buttock	Embryonal	7	245	GGC→TGC	Gly→Cys	–	61 mo/DOD
RMS31	1/M	Cheek	Embryonal	7	247	AAC→GAC	Asn→Asp	+	40 mo/AWD
					249	AGG→GGG	Arg→Gly		
RMS39	16/F	Forearm	Alveolar	6	204	GAG→GGG	Glu→Gly	+	24 mo/DOD
RMS43	5/M	Axilla	Alveolar	8	295	CCT→CAT	Pro→His	–	NA
RMS54	16/F	Chest wall	Pleomorphic	6	209	AGA→ACA	Arg→Thr	–	7 mo/DOD
RMS55	14/M	Paratestis	Embryonal	6	223	CCT→CGT	Pro→Arg	–	319 mo/AWD
RMS72	19/F	Lung	Embryonal	6	207	GAT→GAC	Asp→Asp	+	NA

mo = month.

cycle checkpoint. In previous molecular studies of *p53* mutation in rhabdomyosarcoma, the materials studied comprised both cell lines and clinical tumor samples, and the number of samples studied was very small, leading to results that varied greatly. In case of the materials restricted to only clinical tumor samples, the results of the previous reports of *p53* gene abnormalities in rhabdomyosarcoma were low, ranging from 5 to 33%.^{4–7,13,14} Some investigators have reported that most patients with *p53* mutations were of a younger age⁷ and had tumors of the embryonal subtype. In the current study, 10 of the 45 cases (22.2%) had *p53* gene abnormalities detected by molecular biological analysis, including four cases of alveolar type. Although a number of mutations of *p53* were detected in rhabdomyosarcoma cell lines of both embryonal and alveolar type origin, to the best of our knowledge, this is the first report of such many alveolar rhabdomyosarcoma patients with *p53* mutations from tumor specimens. No positive correlation was observed between *p53* mutation status and the patients' prognoses or any of the other clinicopathologic parameters, including age and histological type.

MDM2 has been shown to function as a negative regulator of *p53*. It binds to *p53* protein, thereby inactivating its transcriptional activity.^{18,19} In the previous limited reports, MDM2 immunoreactivity and the frequency of *MDM2* gene amplification in rhabdomyosarcoma have been found to be low. Taylor *et al* demonstrated that of the 20 rhabdomyosarcoma cases analyzed, two cases (10%) showed *MDM2* amplification. In the report of Würfl *et al*, positive staining was observed in 36% (4/11) of rhabdomyosarcoma cases and all the positive cases were Grade 3 tumor samples. Leuschner *et al* reported that MDM2 was expressed at low levels in pediatric rhabdomyosarcoma cases, and 10% of the cases (15/150) showed positive staining in more than 5% of the tumor cells. In addition, they did not have any cases showing both strong *p53* and MDM2 expression. In our study, cases showing staining in more than 10% of the nuclei were regarded as positive cases, overexpression of MDM2 was found in nine out of 72 cases (13%), and of the 18 rhabdomyosarcoma analyzed, three cases (16.7%) showed *MDM2* amplification. There was no correlation between *MDM2* amplification and MDM2 overexpression. Xia *et al* showed that *MDM2* amplification events occur frequently in alveolar type, but only rarely in embryonal type.²⁷ In our study, of the three cases with *MDM2* amplification, one case was alveolar type while two were embryonal type. MDM2 showed no association with the patients' prognoses or with any of the other clinicopathologic parameters, including histological type and age. Some reports have demonstrated that the coexpression of both *p53* and MDM2 protein is an adverse prognostic factor in soft-tissue sarcomas.^{8,20,21} In our study, eight cases showed the coexpression of both *p53* and MDM2 protein, but

this did not affect the prognosis of the patients. The MIB-1 LI was significantly correlated with the immunohistochemical expression of MDM2 ($P=0.0360$). A similar relationship between *p53* expression and MIB-1 LI was observed, indicating that MDM2-mediated inactivation of *p53* may occur in some cases. Therefore, MDM2 overexpression as well as *p53* is related to tumor aggressiveness in rhabdomyosarcoma.

Several proteins that inhibit these kinase activities have been identified. All the components involved in restriction point control are targeted in oncogenesis, both the positive controllers (cyclins and CDKs) as oncoproteins as well as the negative controllers (the CKIs p16, p21 and p27) as potential tumor suppressor proteins.²⁸ The correlation between the alterations of these proteins in rhabdomyosarcoma has not been adequately evaluated.²²

Gao *et al*¹⁵ is showed that the loss of p16 protein expression in rhabdomyosarcoma was 38.3% (18/47 cases), and this was more frequently observed in alveolar type than in embryonal type. In our study, the altered expression was identified in 66.6% of cases (48/72), that is, 76.5% of the alveolar type cases and 58.3% of the embryonal type cases. A statistically significant association was observed between *p53* overexpression and altered p16 staining, but no association with clinicopathologic parameters was seen. A statistically significant association was also observed between MDM2 and both p21 and p27 expression, and between cyclin E and both p21 and cyclin D, however, each of MDM2, p21, p27, cyclin D1, cyclin E and pRb showed no association with the patients' prognoses or any of the other clinicopathologic parameters.

The Rb/E2F pathway is essential in the passage of cells through the G1 phase of the cell cycle. Some studies have shown that the levels of expression of the *Rb* gene product vary in high-grade primary sarcomas and that lower levels of Rb protein are correlated with poor prognosis, thereby indicating that Rb levels may be an important prognostic variable in these tumors.^{16,29–33} In the present study, the tumor cells showed a variability in pRb staining, but we were unable to clarify any prognostic significance to the alterations in Rb protein expression found in rhabdomyosarcoma.

E2F-1 is the ultimate transcription factor along the Rb/E2F pathway. E2F-1 could act as an oncogene causing uncontrolled cellular proliferation, by counteracting the negative effects of cyclin-cdk inhibitors. pRb expression was inversely correlated with MIB-1 proliferative activity in tumor cells.^{34,35} There was a positive correlation between the MIB-1 and E2F-1 indices,^{36–38} and an increase in these two proliferative indices could be interpreted as consistent with an inverse correlation with pRb function. There were some reports about correlation between E2F-1 and clinicopathological prognostic factors. Zhang *et al*³⁶ reported that increased E2F-1 index correlated with the known prognostic factors of

breast cancer, such as histological grade, stage and metastatic status. Carllotti *et al*³⁹ showed that E2F-1 LI was significantly lower in patients with choroid plexus carcinoma in children after chemotherapy than before. In the present study, E2F-1 LI showed a significantly higher score in alveolar type compared with embryonal type ($P = 0.0334$), and the alveolar type is known as one of the poor prognostic factors. As free E2F-1 mediates G1-S progression by forming complexes with other proteins, our results indicated that E2F-1 may be related to tumor progression in some cases of rhabdomyosarcoma, especially the alveolar type.

In conclusion, this is the first report of assessment of E2F-1 LI in rhabdomyosarcoma. The result is that E2F-1 LI showed a high score in the alveolar type, indicating that E2F-1 may be related to tumor progression in some cases of rhabdomyosarcoma.

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