the rate of kit mutation. A majority of gastric epithelioid GISTs have been reported to be the wild type for kit.[1,34] Likewise, EGIST might be a subtype with less frequent kit mutation, although the cell type (epithelioid vs. spindle) was not correlated with the frequency of kit mutation in EGIST.

PDGFRA is similar in structure to other receptor tyrosine kinase, such as c-kit and FLT3. Recent studies have described a gain-of-functional mutation of PDGFRA at the juxtamembrane domain (exon 12) and tyrosine kinase II domain (exon 18).[9,10] Heinrich et al have reported the PDGFRA mutation in 14 of 40 (35%) kit-wild GISTs.[9] Of the 14 cases, 10 had exon 18 mutations, including D842V (n = 8), Del DIMH 842-845 (n = 1), and Del HDSN 845-848P (n = 1), and 4 had exon 12 mutations, including V561D (n = 1) and the other types (n = 3). Hirota et al also reported the PDGFRA mutation in 5 of 8 KIT-wild GISTs, including 3 cases with D842V and 2 cases with V561D.[10] According to their data, D842V at exon 18 seems to be the most frequent mutation in the PDGFRA mutations of GIST. In the current study, two EGISTs had the PDGFRA mutation; one had V561D, a second hot spot in GIST, and the other had Del DIMH842-845, a minor type of mutation in GIST. This result suggests that the PDGFRA mutation may play an important role in the tumorigenesis of the small population of EGISTs.

However, there is still a question about the tumorigenetic mechanism of EGISTs lacking detectable c-kit and PDGFRA gene mutations. Rubin et al reported high-level c-kit phosphorylation and activation in GISTs with or without mutations.[26] In addition, Hirota et al showed that a small population of GISTs lacked both the kit and PDGFRA mutation.[10] It is likely that alternative oncogenic mechanisms other than c-kit and PDGFRA may be present in some populations of EGISTs. However, further details remain to be clarified.

In this study, 6 cases showed weak positivity for c-kit with consistent histologic and clinicopathologic characteristics of EGIST. Immunoreactivity for CD34 was strong in those cases, suggesting that weak immunoreactivity for c-kit is not due to concerns about the formalin fixation. As mentioned above, this subset might have molecular abnormalities other than kit activation. Of the 6 cases, 1 had the PDGFRA exon 12 mutation, which is compatible with the result of the recent immunoblot study showing that PDGFRA-mutant GIST tended to express a lower level of c-kit protein than kit-mutant GIST.[9]

In the current study, the c-kit gene mutations were not correlated with the prognosis of the patients with EGISTs. As for GISTs, several authors have discussed the importance of the c-kit gene mutation in predicting the prognosis. In the earlier literature, some groups suggested that the c-kit gene mutation might be an important prognostic factor.[7,23,31] However, most of the studies published from 1998 to 1999 have investigated only exon 11. However, more recent studies investigating the extracellular and kinase domains, as well as the juxtamembrane domain, reported no significant association between the presence of c-kit mutation and biologic behavior.[3,30] The clinicopathologic importance of the PDGFRA mutation is still unclear. In the current study, 2 cases of EGIST with the PDGFRA mutation had epithelioid morphology, a large tumor size (25 cm and 17 cm), low mitotic activity, and a low Ki-67 labeling index. One case with the exon 12 mutation (case no. 3) was malignant with short survival, whereas the other case with the exon 18 mutation (case no. 19) was free of recurrence for 4 years. Further study with a larger number of GISTs and EGISTs is expected to determine the correlation between the PDGFRA mutation and clinicopathologic factors or biologic behaviors.

In this study, a high mitotic rate (>5/50 HPF) and a high Ki-67 labeling index (>10%) were each significantly associated with an adverse outcome. However, Reith et al have suggested that more than 2 mitoses per 50 HPF may be useful in predicting the biologic behavior in EGISTs.[25] However, the shortness of the follow-up period in the Reith et al[25] series (median, 24 months) may lead a bias of their data. Although GISTs >5 cm in maximum diameter tend to be more aggressive,[5,19,24] this was not the case with EGISTs in our series. Furthermore, most of EGISTs were >5 cm in size. This may be explained by the anatomic site of EGISTs; alternatively, EGISTs appear to have enough space to grow and may only present clinical symptoms over a long term. Therefore, a grading system defined by a combination of mitotic rate and tumor size, which is commonly used in GISTs of the gastrointestinal tract, may not be applicable to EGISTs. Our results suggest that the tumor grade, defined by a combination of the mitotic rate (>5 HPF) and the MIB-1 labeling index (>10%), may be useful in predicting the aggressive biologic behavior in EGIST. Moreover, this grading system might be helpful in predicting the biologic behavior of GISTs of unknown origin (the digestive tract proper vs. soft tissue). However, we still cannot clarify the finding that EGISTs with a kit mutation were smaller in size than those without a mutation.

It is clinically important that EGIST have c-kit expression and c-kit gene mutations because c-kit is an important molecule, not only for the pathologic diagnosis but also as a therapeutic target. STI-571 (Gleevec) is a small molecule that selectively inhibits the tyrosine kinase activity of the abl (bcr-abl), platelet-derived growth factor receptor (PDGFR), and c-kit.[2] Although originally designed to target the bcr-abl of chronic myeloid leukemia, STI-571 can regulate the cell growth and lead to apoptosis of the GIST cell line.[32] Furthermore, a remarkable therapeutic effect on a patient with a metastatic GIST has been reported.[14] Recent phase I and II clinical trials of STI-571 in GISTs have demonstrated relatively good efficiency so far.[4,33] Our results suggest that the application of STI-571 could be a therapeutic strategy for EGISTs because EGISTs have kit abnormality at the molecular level. The effect of STI-571 for PDGFRA-mutant GISTs has not yet been established. However, PDGFRA may become a molecular thera-

peutic target of STI-571 in at least some populations of EGISTs and GISTs. We expect further clinical investigation.

In summary, we have demonstrated that EGISTs had c-kit mutations at the juxtamembrane domain and extracellular domain, as well as the PDGFRA mutation. Furthermore, STI-571 could be an effective therapy for EGISTs, as well as for GISTs, based on the similarity of their clinicopathologic and molecular characteristics.

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

Yukiko Takahashi[1], Yoshinao Oda[1], Ken-ichi Kawaguchi[1], Sadafumi Tamiya[1], Hidetaka Yamamoto[1], Sachiyo Suita[2] and Masazumi Tsuneyoshi[1]

[1]*Department of Anatomic Pathology and* [2]*Department of Pediatric Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

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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Keywords: rhabdomyosarcoma; children; cell-cycle-regulatory proteins

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]

Correspondence: Dr Y Oda, MD, Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
E-mail: oda@surgpath.med.kyushu-u.ac.jp
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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

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 registerated 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 2002[2] 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-μm) 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 μl 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 *Hpa*II site (CCGG) that is lost when the mutation occurs. *Hpa*II 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 *Hph*I (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. *Hph*I 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* ≥ 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

	Embryonal	*Alveolar*	*Pleomorphic*	*All types of RMS*
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.

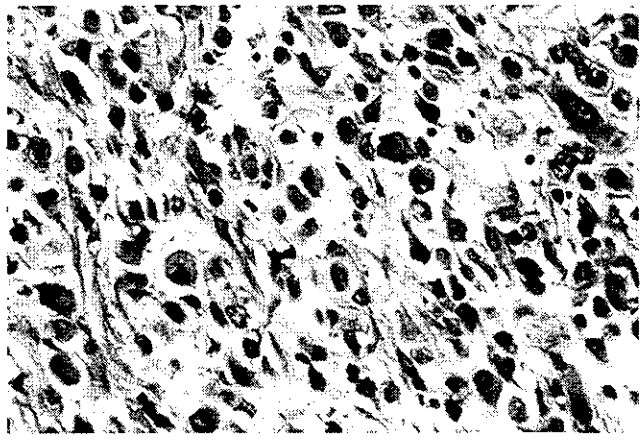

Figure 1 Embryonal type. The rounded tumor cells have hyperchromatic nuclei and some reveal an eosinophilic cytoplasm.

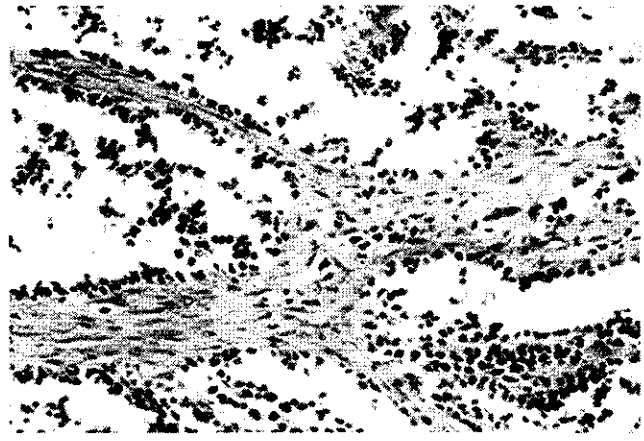

Figure 2 Alveolar type. The tumor is composed of a proliferation of rounded cells with thick collagenous septa.

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

	Embryonal (n = *36*)	*Alveolar* (n = *34*)	*Pleomorphic* (n = *2*)	*Overall* (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.

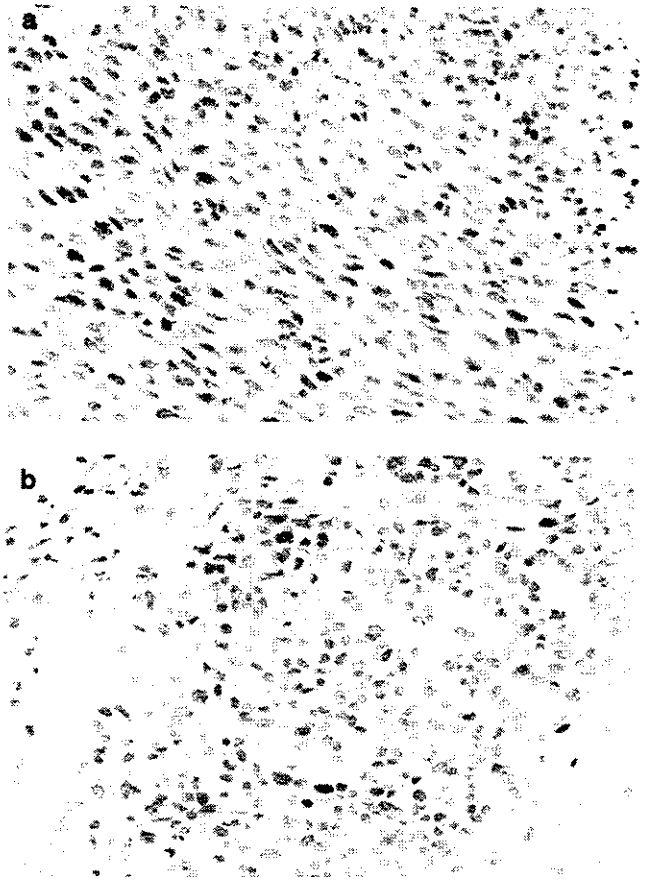

Figure 3 Immunohistochemical staining for pRB. (**a**) Tumor in the pelvic cavity of a 1-year-old female, embryonal type. The patient died of the disease 9 months after surgery (RMS5). A positive nuclear reaction in most tumor cells. (**b**) Tumor of the shoulder of a 13-year-old female, alveolar type.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

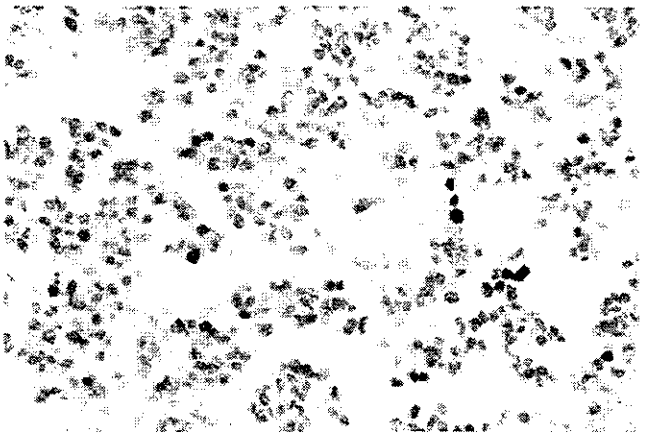

Figure 4 Immunohistochemical staining for cyclin D1 (LI = 6). Tumor of the finger of a 17-year-old female, alveolar type. The patient died of the disease 2 months after surgery (RMS9).

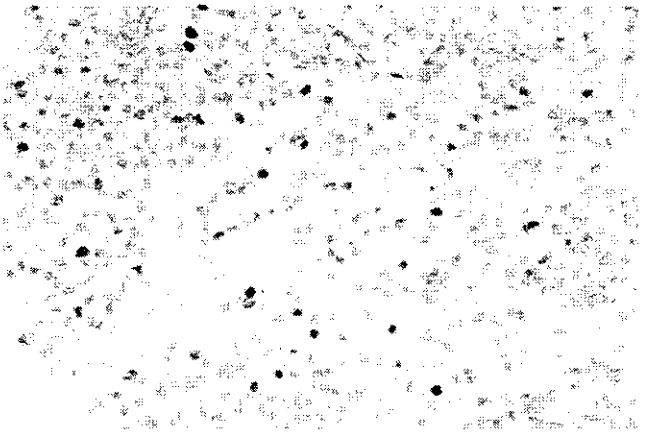

Figure 5 Immunohistochemical staining for E2F in alveolar type (LI = 14). Orbital tumor of 25-year-old male, alveolar type (RMS3).

($n = 25$,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 (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 overexpression 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.

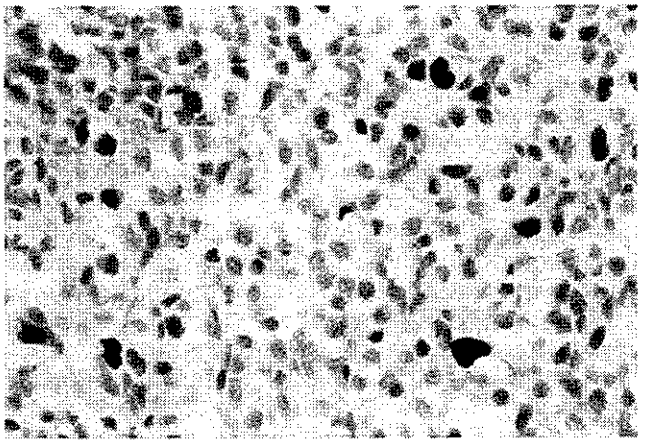

Figure 6 p53 immunoreactivity is observable in Case RMS51.

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.

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.

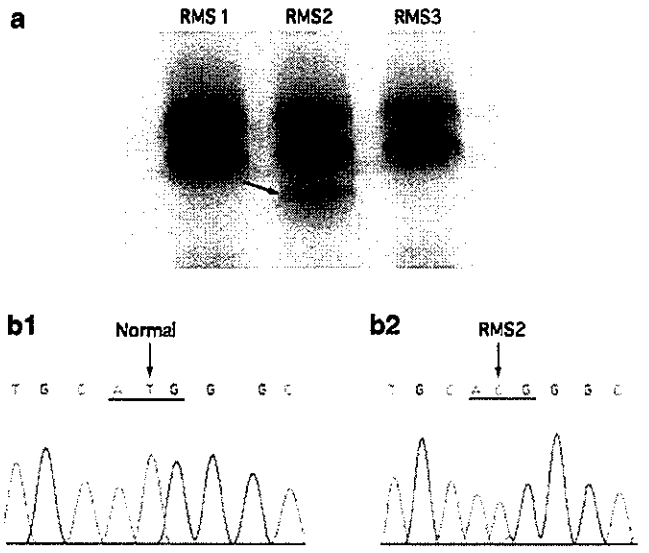

Figure 7 PCR–SSCP analysis at Exon 7. Abnormal band is evident in Case RMS2 (**a**). Direct DNA sequencing of Exon 7 in Case RMS2 (**b**). (Codon 243, ATG to ACG).

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 overepression 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-

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 age[7] 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 Würl *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.[6,20,21] 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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Alterations of the p16^{INK4a}/p14^{ARF} pathway in clear cell sarcoma

Tomonari Takahira,[1] Yoshinao Oda,[1,3] Sadafumi Tamiya,[1] Hidetaka Yamamoto,[1] Kenichi Kawaguchi,[1] Chikashi Kobayashi,[1] Yukihide Iwamoto[2] and Masazumi Tsuneyoshi[1,4]

Departments of [1]Anatomic Pathology (Second Department of Pathology), Pathological Sciences and [2]Orthopedic Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582

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Clear cell sarcoma (CCS) is a very rare soft tissue sarcoma with a poor prognosis. It has become apparent through immunohistochemical, ultrastructural, and microarray analyses that CCS is a soft tissue melanocytic neoplasm. Alterations in the *p16^{INK4a}/p14^{ARF}* gene are common in malignant melanoma, which is the prototypical melanocytic neoplasm. In the present study, we performed a clinicopathologic analysis and investigated p16 and cyclin D1 expression by immunohistochemistry in 14 cases. Furthermore, we investigated genetic changes of various tumor suppressor genes and an oncogene, including *p16^{INK4a}/p14^{ARF}*, *p53*, *β-catenin*, and *APC*, in 11 cases. The 5-year overall survival rate in all the patients was 33.3%. A high mitotic rate was a significant adverse prognostic factor (*P*=0.004). Decreased expression of p16 was observed in 4 (28.6%) of 14 cases. Overexpression of cyclin D1 was observed in 9 cases (64.3%). SSCP analysis followed by DNA direct sequencing revealed point mutations of the *p16^{INK4a}* gene in 2 of 11 cases (18.2%). In addition, one case with the *p14^{ARF}* mutation and 2 cases with the *p53* mutation were observed. None of the cases harbored mutation of the *β-catenin* or *APC* gene. Homozygous deletion of the *p16^{INK4a}/p14^{ARF}* gene was detected in one case. Methylation-specific PCR did not reveal hypermethylation of the *p16^{INK4a}/p14^{ARF}* promoter region in any of the cases. Three cases harbored genetic alterations of the *p16^{INK4a}/p14^{ARF}* gene (27.3%). All tumors with genetic alterations of the *p16^{INK4a}/p14^{ARF}* or *p53* gene showed a high mitotic rate or tumor necrosis. These alterations were considered to be influential in the poor prognosis of CCS patients. (Cancer Sci 2004; 95: 651–655)

Clear cell sarcoma (CCS) was first described by Enzinger in 1965.[1] Chung and Enzinger suggested the term "malignant melanoma of soft parts" in 1983 because of the presence of intracellular melanin.[2] Histopathologically, the tumor cells of CCS display a number of features indicative of a melanocytic line of differentiation, including the expression of S-100 and HMB-45 proteins, in addition to melanin synthesis.[3,4] Cytogenetic studies have distinguished CCS from melanoma by a unique translocation involving chromosomes 12 and 22.[5–7] The translocation results in rearrangements of the *EWS* gene at chromosome 22 and the *ATF1* gene at 12q13, and creation of a chimeric *EWS/ATF1* gene,[8–10] and its possible contribution to pathogenesis have been described. Other molecular genetic studies, except microsatellite instability[11] and microarray analysis,[12] in CCS patients are not well advanced.

The *p16^{INK4a}/p14^{ARF}* locus on the short arm of chromosome 9 is one of the most frequently altered sequences in human cancer. This locus encodes two distinct proteins, which are translated in different reading frames from alternatively spliced transcripts.[13] The α transcript (exons 1α, 2, and 3) encodes the p16 cyclin-dependent kinase (CDK) inhibitor, whereas the β transcript (exons 1β, 2, and 3) specifies the alternative product, p14. Alterations in the *p16^{INK4a}/p14^{ARF}* gene are common in malignant melanoma.[14,15] However, no one has reported the alterations of these genes in CCS of melanocytic origin.

In the present study, we first performed a clinicopathologic analysis in 14 cases of CCS. Further, mutational analysis screening for genetic changes of various tumor suppressor genes and an oncogene, including the *p16^{INK4a}/p14^{ARF}* locus, was performed in addition to immunohistochemical analysis.

Materials and Methods

Patients and DNA extraction. Fourteen patients with CCS were included in this study. All specimens were collected from soft tissue sarcomas registered in the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Japan between 1965 and 1998. Five cases had been examined in a previous electron microscopic analysis.[16] Materials were fixed in 10% formaldehyde and embedded in paraffin. Clinicopathological data of 14 patients are listed in Table 1. Clinical data in these cases were obtained from medical records. Survival data were available in 12 cases. The follow-up period ranged from 10 to 72 months (mean, 31 months). In order to assess the correlation between clinicopathologic parameters and the results of immunohistochemical and molecular analysis, we analyzed sex, age, anatomical site, tumor size, mitotic rate, tumor necrosis, and clinical stage according to the American Joint Committee on Cancer (AJCC). A high mitotic rate implied that there were more than 10 mitoses/10 high power fields (HPFs).

Immunohistochemical and molecular analyses were performed in 14 and 11 cases, respectively. Genomic DNA was isolated from these 11 cases by using standard proteinase K digestion and phenol/chloroform extraction, and was used for the following molecular analysis.

Immunohistochemistry. Immunohistochemistry was performed using anti-p16 mouse monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) and anti-cyclin D1 mouse mAb (Novocastra, Newcastle, UK; 1:25). The nuclear immunoreactivity of p16 protein was regarded as decreased when less than 10% of the tumor cells were stained for p16 protein, according to previously published criteria.[17] For evaluating the immunohistochemistry of cyclin D1, sections were considered to exhibit overexpression if more than 5% of the cells were positively stained.[18]

Mutational analysis by PCR-SSCP. Mutational analysis was performed for the *p16^{INK4a}/p14^{ARF}* (exons 1α, 1β, 2, 3), *p53* (exons 5–9), *APC* (mutation cluster region), and *β-catenin* (exon 3) genes. Primer sequences and PCR conditions were the same as those previously described.[19–24] Human genomic DNA (Clontech, Palo Alto, CA) was used as a positive control for each PCR and the subsequent reactions. SSCP was performed using a gel containing 12.5% acrylamide ("GenePhor," Amersham

[3]To whom correspondence should be addressed.
E-mail: oda@surgpath.med.kyushu-u.ac.jp
[4]To whom requests for reprints should be addressed.
E-mail: masazumi@surgpath.med.kyushu-u.ac.jp

Pharmacia Biotech, Uppsala, Sweden) and DNA fragment analyzer ("GenePhor," Amersham Pharmacia Biotech), and then the DNA bands were visualized by a DNA Silver Staining Kit ("GenePhor," Amersham Pharmacia Biotech). To increase the quantity of mutant DNA prior to sequencing, the extra bands that seemed to be aberrantly migrating were excised from the SSCP gel and re-amplified using the same primers. The sequence data were obtained using ABI Prism 310 Collection Software, and were analyzed using Sequencing Analysis and Sequence Navigator Software.

Differential PCR assay for p16^{INK4a}/p14^{ARF}. The differential PCR method for detecting homozygous deletions of exon 1α and 1β of *p16^{INK4a}/p14^{ARF}* was based on a modification of a reported method using the *β-actin* or *GAPDH* gene as an internal control.[19] Primers for this assay were the same as those for mutational analysis. PCR conditions were the same as those previously described.[25] Human genomic DNA (Clontech) was used as a normal control for each PCR and for the subsequent reactions. After amplification, 10 μl of the PCR products was electrophoresed through an 8% polyacrylamide gel, and the intensities of the DNA products were quantified using National Institutes of Health (NIH) Image software ver 1.56. Values of less than 25% for the target gene/internal control ratio were considered to represent a homozygous deletion.[19,25]

Methylation-specific PCR for the promoter region of *p16^{INK4a}/p14^{ARF}* genes. Bisulfite conversion was performed with 1 μg of genomic DNA and the reagents provided with Intergen's CpGenome DNA Modification Kit (Intergen, New York, NY). Methylation-specific PCR was performed to determine the DNA methylation status of CpG islands of the promoter region of the *p16^{INK4a}* and *p14^{ARF}* genes. The primer pairs used in this study were described previously.[20]

Statistical analysis. We estimated the influence of various factors on the overall survival rate using a log-rank test. We performed Fisher's exact test to assess the correlation among various factors. A *P* value of less than 0.05 was considered to be statistically significant.

Results

Clinical findings. The distribution of clinicopathologic characteristics of the 14 patients is outlined in Table 1. The mean age of the patients was 34.4 years (range, 10–63 years). There were 5 male (35.7%) and 9 female (64.3%) patients. In all cases, the tumor was located in the deep soft tissues of the extremities. The overall 5-year survival rate in this study was 33.3%. A high mitotic rate was observed in 5 of our 14 cases (35.7%, Fig. 1). High mitotic rate (≥10/10 HPFs) was a significant adverse prognostic factor (P=0.004). There was no correlation between other clinicopathologic parameters and patients' prognosis.

Mutation and deletion. The results of mutational analysis are summarized in Table 2. SSCP analysis followed by DNA direct sequencing revealed three sequence changes in exon 2 of the *p16^{INK4a}/p14^{ARF}* gene in 3 out of 11 cases (27.3%, Fig. 2). One of these 3 cases had a missense mutation of the *p16^{INK4a}* gene (case 1). The other one case had a missense mutation of the *p14^{ARF}* gene, in addition to a silent mutation of the *p16^{INK4a}* gene (case 3). The residual case showed the polymorphism frequently reported (codon 135; GGG>GGA),[26] and we confirmed it by analyzing the adjacent normal tissue. No cases had mutations of exon 1α or 3 of the *p16^{INK4a}* gene, or exon 1β of the *p14^{ARF}* gene. Homozygous deletion of the *p16^{INK4a}* gene was detected in one out of 11 cases (9.1%, case 8, Fig. 3). Case 8 also had homozygous deletion of the *p14^{ARF}* gene and showed decreased expression of p16 protein. Mutations of the *p53* gene was observed in 2 cases (18.2%), comprising one missense and one silent mutation. One case harbored three point mutations (case 3). None of the cases harbored mutation of exon 3 of the *β-catenin* gene or the *APC* gene. The presence of mutations of the *p53* gene adversely affected patients' overall survival rate (P=0.043).

Hypermethylation of the promoter region. No hypermethylation was detected in the promoter region of the *p16^{INK4}* or the *p14^{ARF}* genes in any of the 11 cases examined (Fig. 4).

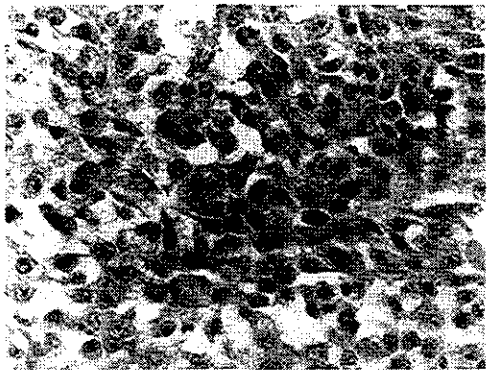

Fig. 1. A transition between clear cells and cells with a finely stippled eosinophilic cytoplasm. Mitotic figures are frequently seen (case 2, HE, original magnification ×200).

Table 1. Clinicopathologic features of 14 cases of CCS

Case	Age/Sex	Location	Size (cm)	Mitosis	Tumor necrosis	Stage	Prognosis
1	37/M	knee	5.5	4	(+)	III	Alive (60 mos)
2	26/M	buttock	7.5	24	(+)	III	DOD (19 mos)
3	21/F	buttock	8	14	(−)	III	DOD (10 mos)
4	47/M	finger	3	3	(−)	IIA	DOD (38 mos)
5	53/F	finger	3	7	(−)	IIA	DOD (23 mos)
6	63/F	thigh	5	9	(−)	IIA	N/A
7	12/F	lower leg	3	7	(−)	IIA	DOD (78 mos)
8	41/F	groin	8	40	(−)	III	N/A
9	30/M	knee	4	8	(−)	IIA	DOD (22 mos)
10	10/F	hand	5	2	(−)	IIA	DOD (13 mos)
11	26/F	foot	6	15	(−)	III	DOD (12 mos)
12	52/F	foot	4.5	4	(−)	IIA	Alive (72 mos)
13	33/M	knee	4.5	3	(−)	IIA	DOD (14 mos)
14	30/F	knee	2.2	10	(−)	IIA	DOD (12 mos)

Case numbers are identical throughout the manuscript.
N/A: not available.

Table 2. Results of immunohistochemical and molecular analysis

Case	Age/Sex	IHC[1]		Deletion		Mutation		
		p16	cyclin D1	$p16^{INK4}$	$p14^{ARF}$	$p16^{INK4}$	$p16^{ARF}$	p53
1	37/M	(–)[2]	(+)	(–)	(–)	codon 131, CGC (Arg) to CAC (His)	(–)	(–)
2	26/M	(+)[2]	(+)	(–)	(–)	(–)	(–)	codon 211, ACT (Thr) to ACA (Thr)
3	21/F	(–)	(+)	(–)	(–)	codon 88, GAG (Glu) to GAA (Glu)	codon 103, GGG (Gly) to AGG (Arg)	codon 246, ATG (Met) to GTG (Val) codon 249, AGG (Arg) to AAG (Lys) codon 259, GAC (Asp) to AAC (Asn)
4	47/M	(+)	(–)	(–)	(–)	(–)	(–)	(–)
5	53/F	(+)	(–)	(–)	(–)	(–)	(–)	(–)
6	63/F	(–)	(–)	N/A	N/A	N/A	N/A	N/A
7	12/F	(+)	(+)	(–)	(–)	codon 135, GGG (Gly) to GGA (Gly)[3]	(–)	(–)
8	41/F	(–)	(+)	(+)	(+)	(–)	(–)	(–)
9	30/M	(+)	(+)	(–)	(–)	(–)	(–)	(–)
10	10/F	(+)	(–)	(–)	(–)	(–)	(–)	(–)
11	26/F	(–)	(+)	N/A	N/A	N/A	N/A	N/A
12	52/F	(+)	(+)	(–)	(–)	(–)	(–)	(–)
13	33/M	(+)	(+)	(–)	(–)	(–)	(–)	(–)
14	30/F	(+)	(–)	N/A	N/A	N/A	N/A	N/A

1) Immunohistochemistry.
2) (–), decreased expression of p16 protein; (+), preserved expression of p16 protein.
3) Polymorphism.
N/A: not available.

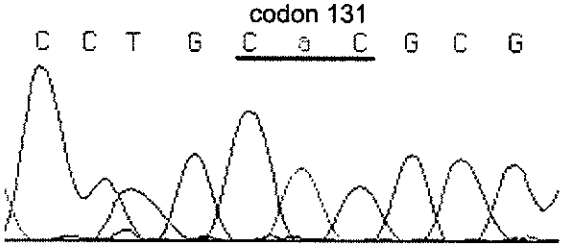

Fig. 2. Sequencing results for exon 2 of the $p16^{INK4a}$ gene. CAC (mutant) signals are observed at codon 131.

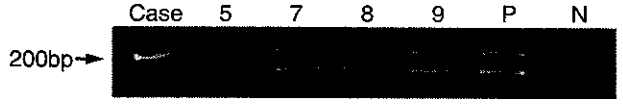

Fig. 3. Differential PCR for exon 1α of the $p16^{INK4a}$ homozygous deletion. Case 8 displays the absence of PCR products of the $p16^{INK4a}$ gene. P and N indicate positive and negative controls, respectively.

Immunohistochemistry. Decreased expression of p16 protein was recognized in 5 out of 14 cases (35.7%) (Fig. 5, A and B). All tumors with decreased expression of p16 protein measured 5 cm or more in diameter. Expression of p16 protein was significantly inversely correlated with the tumor size (P=0.021). Cases with alterations of the $p16^{INK4a}$ gene had a tendency to show decreased expression of p16, but the correlation was not significant (P=0.055). Nine cases (64.3%) showed overexpression of cyclin D1 (Fig. 6).

Discussion

CCS is a slow-growing malignant tumor with a poor prognosis

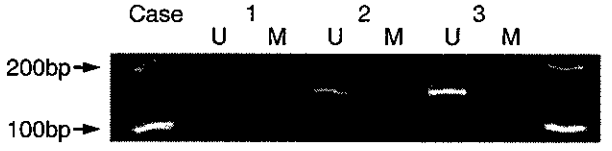

Fig. 4. Methylation status of the promoter region of the $p16^{INK4a}$ gene by methylation-specific PCR. PCR product with methylated primers was not recognized in any of the cases.

even in patients who received adequate treatments. The overall 5-year survival rate is less than 50%[10, 27, 28] in previous reports except one showing 67%.[29] The rate in the present study was 33.3%. It has been demonstrated that survival is inversely correlated with tumor size[28, 29] and tumor necrosis.[29] In the present study, only the high mitotic rate was a significant adverse prognostic factor. The mitotic rate varied from 2 to 40 mitotic figures per 10 HPFs. Most tumors with a high mitotic rate showed a transition between clear cells and cells with a finely stippled eosinophilic cytoplasm (Fig. 1). The mitotic rate tended to be higher in larger (5 cm or more in diameter) tumors, but the correlation between the rate and the tumor size was not statistically significant (P=0.10). Tumor necrosis was seen in only one case (case 1). This patient survived without evidence of disease for 5 years.

It has become apparent through immunohistochemical and ultrastructural studies that CCS is a soft tissue melanocytic neoplasm.[3, 4, 30] Recently, Segal *et al.* used cDNA microarrays to demonstrate that the gene expression profiles in CCS are similar to those of malignant melanoma, and genes encoding melanocyte differentiation antigens such as MITF are expressed.[12] Alterations in the $p16^{INK4a}$ gene are very common in melanoma cell lines, with the average alteration rate of approximately 70%.[14, 15, 31, 32] Some studies have revealed a relatively lower frequency of alteration in sporadic melanoma.[14, 15, 33, 34]

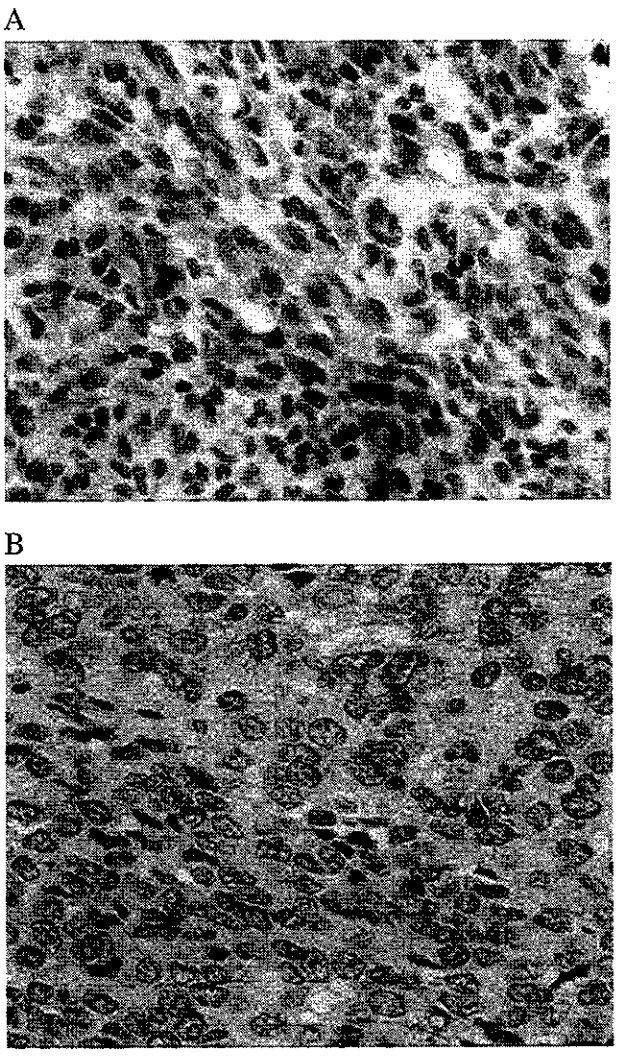

Fig. 5. Immunohistochemical staining of p16 protein. (A) Positive staining for p16 in case 9. (B) Tumor cells show decreased staining for p16 in case 8. This case showed homozygous deletion of $p16^{INK4a}$.

Mutations altering the glycogen synthase kinase 3β phosphorylation sites of β-catenin have been registered in 6 of 26 (23%) human melanoma cell lines,[35] but the mutational rate of the *β-catenin* gene in clinical samples is less than 5%.[36-38] We extensively analyzed genetic alterations of various tumor suppressor genes and an oncogene, including $p16^{INK4a}$ and *β-catenin*, which are frequently altered, at least in melanoma cell lines. Three of 11 cases (27.3%) harbored mutation or deletion of the $p16^{INK4a}$ gene, and the frequency of this alteration in CCS was similar to that in melanoma. One case (case 3) also had mutation of the $p14^{ARF}$ gene. Hypermethylation of the $p16^{INK4a}/p14^{ARF}$ gene promoter regions is associated with a loss of transcription, and has been demonstrated in various tumors. However, no hypermethylation of either gene was observed in the present study. Gonzalgo *et al.* showed that methylation-associated gene silencing does not represent a common mechanism for p16 inactivation in sporadic melanoma.[39] Our results suggested that hypermethylation of the promoter region of the $p16^{INK4a}$ gene is as rare an event for the inactivation of p16 in CCS as in melanoma. *p53* gene mutation was detected in 2 cases (18.2%). Although the number of the cases examined in this study was small, the presence of mutations of the *p53* gene adversely affected patients' overall survival rate (P=0.043). The relation of these genetic alterations with clinicopathological characteristics was investigated. Among 4 cases with alterations of the $p16^{INK4a}/p14^{ARF}$ or *p53* genes, 3 cases showed high mitotic rate, and one case showed a low mitotic rate and was a long-term survivor in spite of tumor necrosis (case 1). Based on these results, CCS with genetic alterations of the $p16^{INK4a}/p14^{ARF}$ or *p53* genes shows histological features of aggressiveness, such as high mitotic rate and tumor necrosis, and involves a poor prognosis. The impact of secondary alterations in the p53 pathway or *CDKN2A* on clinical behavior has been demonstrated for many soft tissue sarcomas with specific chromosomal translocations,[40] and our results confirmed that this is also the case for CCS with specific chromosomal translocation, t(12;22, q13;q12).

Transition from G1 to S phase of the cell cycle is mediated by interactions between the retinoblastoma gene product (pRb), p16 and cyclin D1. In the current study, decreased expression of p16 protein was recognized in 5 out of 14 cases (35.7%). Cases with alterations of the $p16^{INK4a}$ gene tended to show decreased expression of p16 (P=0.055). Increased cyclin D1 expression was observed in 9 out of 14 cases (62.3%). Four out of 5 cases with decreased p16 expression showed increased cyclin D1 expression. However, 5 cases with normal p16 expression also showed increased cyclin D1 expression. β-Catenin in the Wnt signaling pathway functions as a transcription factor when localized in the nucleus, and cyclin D1 is a target gene of β-catenin.[41,42] In our study, no mutation of the *β-catenin* or *APC* genes was detected. It was possible that other mechanisms were involved in overexpression of cyclin D1 rather than the Rb and Wnt pathway. Xie *et al.* demonstrated that the *SYT-SSX* fusion gene, which is specific for synovial sarcoma, interferes with ubiquitin-dependent degradation of cyclin D1.[43] The *EWS-ATF1* fusion gene of CCS may have similar mechanism of action.

In summary, we investigated the genetic alterations of some tumor suppressor genes and an oncogene, including the $p16^{INK4a}/p14^{ARF}$ gene, which are commonly mutated in malignant melanoma, in patients with CCS of melanocytic origin. Three out of 11 cases harbored mutations or deletion, and the frequency was similar to that of malignant melanoma. Among clinicopathological parameters, high mitotic rate was an adverse prognostic factor. All tumors with genetic alterations of the $p16^{INK4a}/p14^{ARF}$ or *p53* gene showed high mitotic rate or tu-

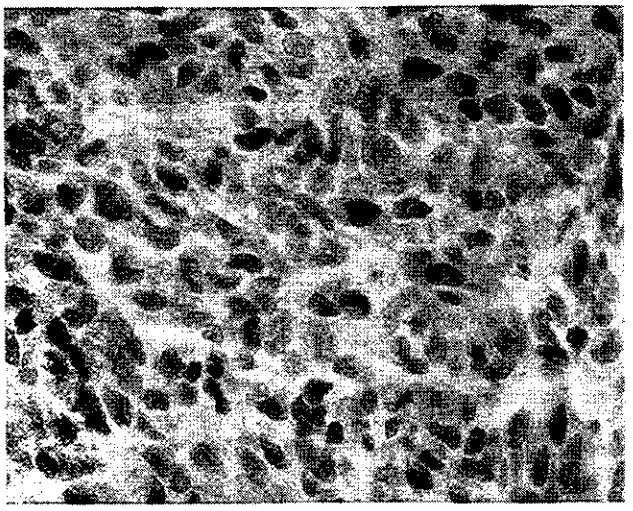

Fig. 6. Immunohistochemical staining of cyclin D1. More than 50% of the tumor cell nuclei were positively stained for cyclin D1 (case 9).

mor necrosis. These alterations were considered to be related to the poor prognosis of CCS patients.

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Histopathology 2004, 45, 29–38

Low-grade fibromyxoid sarcoma versus low-grade myxofibrosarcoma in the extremities and trunk. A comparison of clinicopathological and immunohistochemical features

Y Oda,[1] T Takahira,[1,2] K Kawaguchi,[1,2] H Yamamoto,[1] S Tamiya,[1] S Matsuda,[2] K Tanaka,[2] Y Iwamoto[2] & M Tsuneyoshi[1]

Departments of [1]Anatomic Pathology and [2]Orthopaedic Surgery, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

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Low-grade fibromyxoid sarcoma versus low-grade myxofibrosarcoma in the extremities and trunk. A comparison of clinicopathological and immunohistochemical features

Aims: Low-grade fibromyxoid sarcoma (LGFMS) is a distinctive variant of fibrosarcoma and has been reported to have metastatic potential despite its low-grade histological findings. Low-grade myxofibrosarcoma (MFS) is an important differential diagnosis of LGMFS, because it shows different biological behaviour. Of 75 MFSs in the extremities and trunk, we defined 22 grade 1 tumours as low-grade MFS according to the French Federation of Cancer Centres grading system and compared the clinicopathological factors and immunohistochemical expression of cell cycle regulators with those of 11 LGFMSs.

Methods and results: The two entities could be distinguished on histological grounds. Low-grade MFS was characterized by the presence of prominent elongated, curvilinear capillaries and pseudolipoblasts, accompanied by an abundant myxoid matrix. It had no extensive solid areas. LGFMS was composed of bland spindle cells arranged in a whorled pattern with alternating myxoid and fibrous stroma. Curvilinear capillaries were not prominent and cytological atypia was absent. No tumour necrosis was observed in any of the 11 LGFMSs, whereas only one case showed tumour necrosis in less than 50% of the tumour in 22 low-grade MFSs. The patients with low-grade MFS were significantly older than those with LGFMS (low-grade MFS average, 60.1 years; LGFMS average, 31.5 years; $P < 0.0001$) and low-grade MFS occurred more frequently in a superficial location (low-grade MFS 14/20; LGFMS 2/11; $P = 0.0077$). As for cell cycle regulator expression, the MIB-1 labelling index (LI) (14.76 on average) and cyclin E LI (11.55 on average) in low-grade MFS were significantly higher than those (MIB-1 LI, 4.68 on average; cyclin E LI, 3.38 on average) of LGFMS, while p21 LI (25.53 on average) and p27 LI (42.68 on average) in low-grade MFS were significantly lower than those (p21 LI, 42.74 on average; p27 LI, 57.28 on average) of LGFMS.

Conclusions: We conclude that low-grade MFS and LGFMS are distinctly different clinicopathological entities and the assessment of the immunohistochemical expression of MIB-1, cyclin E, p21 and p27 as well as conventional clinicopathological features may be helpful to distinguish low-grade MFS from LGFMS.

Keywords: cell cycle regulators, immunohistochemistry, low-grade fibromyxoid sarcoma, myxofibrosarcoma, myxoid malignant fibrous histiocytoma

Abbreviations: AJCC, American Joint Committee on Cancer; FNCLCC, French Federation of Cancer Centres; LGFMS, low-grade fibromyxoid sarcoma; LI, labelling index; MFH, malignant fibrous histiocytoma; MFS, low-grade myxofibrosarcoma

Address for correspondence: Yoshinao Oda MD, PhD, Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. e-mail: oda@surgpath.med.kyushu-u.ac.jp

Introduction

Low-grade fibromyxoid sarcoma (LGFMS) was initially described by Evans in 1987[1] and characterized as a bland fibromyxoid neoplasm arising in deep soft tissue with occasional metastatic potential.[1–3] Recently, some authors have regarded this entity as a fibromyxoid type of fibrosarcoma.[4,5] The term myxofibrosarcoma (MFS) was first used by Angervall *et al.*[6] to describe a lesion of fibroblastic origin with a myxoid matrix. Nowadays, MFS is considered to be synonymous with myxoid malignant fibrous histiocytoma (MFH). The morphological distinction of LGFMS from low-grade MFS is sometimes difficult and problematic.[7] However, it is clinically important to differentiate these two entities because they have different biological behaviour. In this study, we classified MFS according to a reproducible French Federation of Cancer Centres (FNCLCC) grading system[8] and defined grade 1 tumours as low-grade MFS and compared their clinicopathological features with LGFMS.

Many tumour suppressor genes and oncogenes directly participate in or regulate signal transduction pathways linking extracellular stimuli to cell cycle progression in cancer cells. Alteration of certain cell cycle regulators has been implicated in tumour progression of certain soft tissue sarcomas.[9–12] The investigations have been relatively limited in high-grade soft tissue sarcomas and there has been no study of low-grade fibroblastic sarcomas.

In the current study, we compared the clinicopathological features of and the expression of cell cycle regulators including p53, MDM2, p16, p21, p27, cyclin A, cyclin D1 and cyclin E in LGFMS and low-grade MFS.

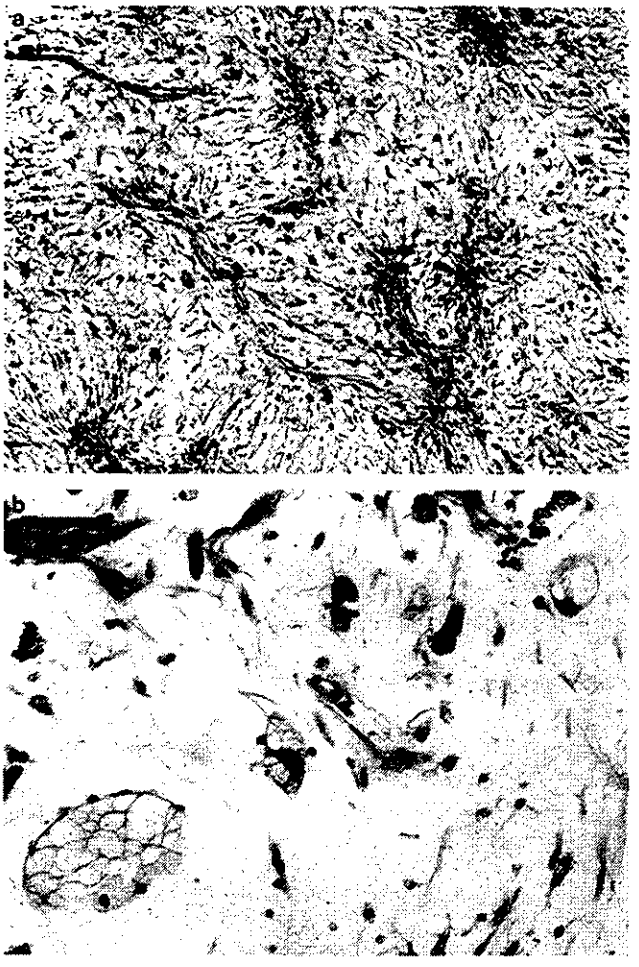

Figure 1. a, Typical histological features of low-grade myxofibrosarcoma (MFS). Pleomorphic tumour cells are scattered throughout abundant myxoid matrix, accompanied by curvilinear vessels along which the tumour cells are anchored. b, Pseudolipoblasts in low-grade MFS. The vacuoles are more irregular than lipoblasts.

Materials and methods

DIAGNOSTIC CRITERIA AND CASES

Myxofibrosarcoma (MFS/myxoid MFH) was defined as a spectrum of malignant fibroblastic lesions in which at least 50% of the entire tumour displays a highly vascularized and myxoid stroma with distinctive curvilinear vessels, according to the World Health Organization classification[13] (Figure 1a). Frequently, vacuolated neoplastic fibroblastic cells, so-called pseudolipoblasts, are noted (Figure 1b). LGFMS is composed of bland spindle-shaped cells with alternating myxoid and fibrous stroma (Figure 2a). Tumour cells are generally arranged in a whorled pattern and a fine network of curvilinear capillaries is not prominent, in contrast to MFS (Figure 2b). Cytological atypia of tumour cells is absent or minimal in LGFMS (Figure 2c). Hyalinizing spindle cell tumour with giant rosettes was included in LGFMS in this study, because it is considered to be a variant of LGFMS[14,15] (Figure 3). In each case, haematoxylin and eosin-stained slides of the tumours were carefully reviewed and the number of evaluated histological slides ranged from 1 to 31, with a mean of five slides per case in LGFMS, and from one to six, with a mean of three slides per case in low-grade MFS.

Seventy-five cases of MFS of the extremities and trunk, and 11 cases of LGFMS were retrieved from our soft tumour file dating from 1971 to 2000. Among 75 MFSs, 22 tumours were grade 1 according to the FNCLCC grading system[8] and defined as low-grade MFS. Each case was also evaluated according to the new American Joint Committee on Cancer (AJCC) staging system.[16]

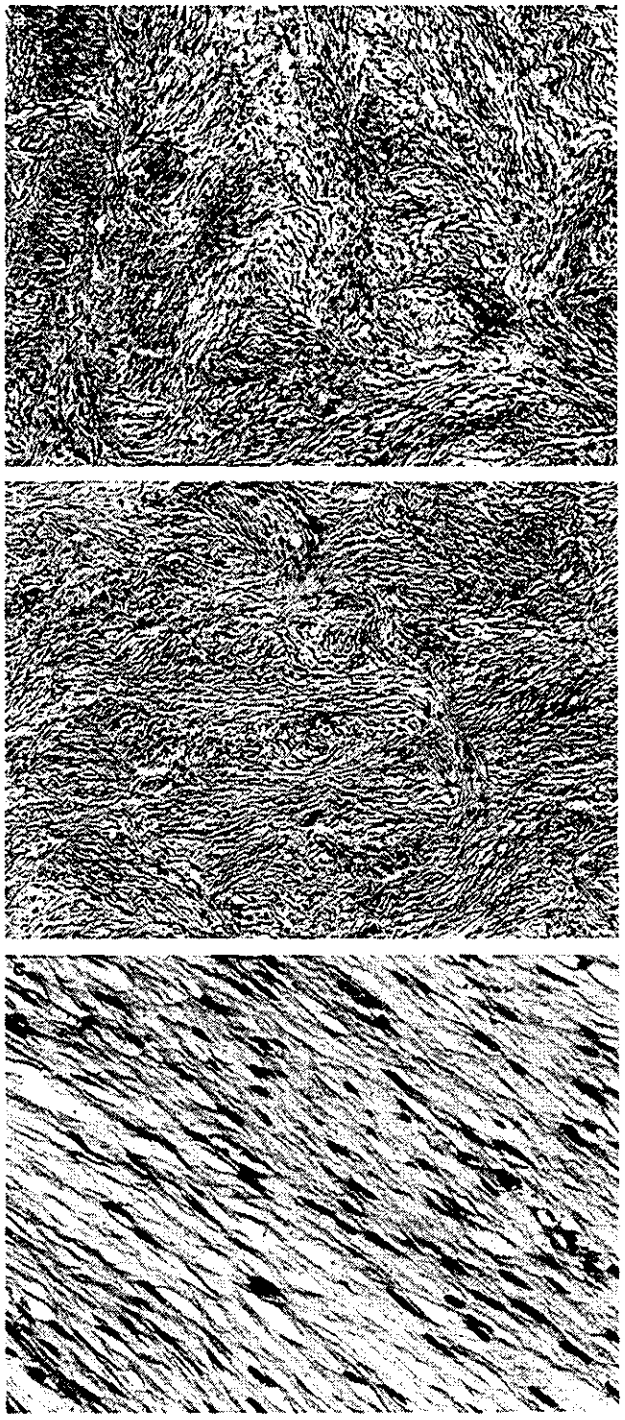

Figure 2. a, Typical morphological features of low-grade fibromyxoid sarcoma (LGFMS). Alternating fibrous and myxoid areas are prominent. b, Spindle-shaped cells are arranged in a whorled pattern. c, Tumour cells show minimum atypia.

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Representative formalin-fixed paraffin-embedded materials for immunohistochemistry were available in nine

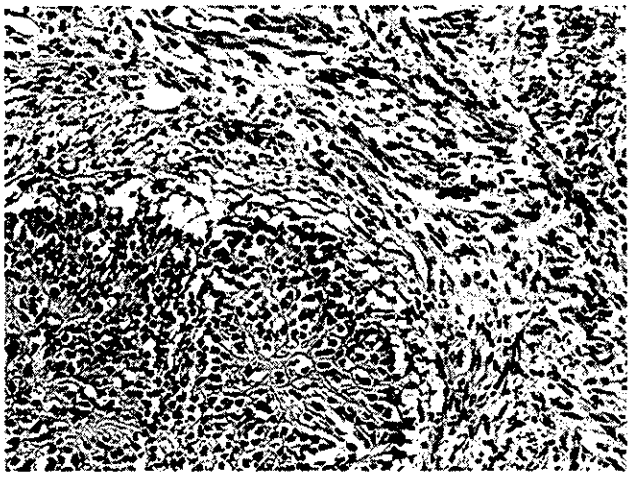

Figure 3. Low-grade fibromyxoid sarcoma (LGFMS) with giant rosettes. Large collagen rosettes (bottom left) surrounded by epithelioid fibroblasts are the characteristic features of LGFMS.

cases of LGFMS and 13 cases of low-grade MFS. For immunohistochemical study, the following monoclonal antibodies were used: p53 (1 : 100, Pab 1801; Oncogene Research Products, Cambridge, MA, USA), MDM2 (1 : 40, IF2; Oncogene Research Products), MIB-1 (1 : 100; Immunotech, Marseille, France), p21WAF1 (1 : 100; Oncogene Research Products), p27 (1 : 20, 1B4; Novocastra, Newcastle, UK), p16 (1 : 50, F-12; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), cyclin A (1 : 50, 6E6; Novocastra), cyclin D1 (1 : 25; Novocastra), and cyclin E (1 : 100; Oncogene Research Products). Histological sections (4 μm thick) of 10% formalin-fixed paraffin-embedded materials were cut, mounted on glass slides coated by 3-aminopropyltriethoxysilane, and 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) for 20 min or 10 min (p53 and MDM2) 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).

For p53 and MDM2, staining of >10% of the nuclei was needed for a case to be regarded as positive. As for p16 expression, if stained nuclei were present in all areas of the tumour, it was considered 'normal'. If there was absence of nuclear staining in a portion of a tumour, or in an entire tumour section, even though admixed non-neoplastic cells showed nuclear reactivity, the lesion was considered 'abnormal'.[17] The MIB-1, cyclin D1, cyclin A, cyclin E, p21 and p27 labelling

