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Apoptotic Cell Death and p53 Expression in Leiomyosarcoma of Soft-Tissue Origin

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Leiomyosarcoma (LMS) of soft-tissue origin was studied on the expression and the biological significance of apoptosis in relation to p53 oncoprotein. Immunohistochemical analysis was performed initially on the paraffin-embbeded sections taken from 29 surgical tissues (20 cases) including 9 recurrent/metastatic tumors. The results are as follows: A positive correlation was observed between the p53 indices (PIs) and the proliferative markers designated by Ki-67, PCNA and MCM2, both of which increased significantly in the high-grade malignant LMS much more than in the low-grade one ($P < 0.001$). Apoptotic cells were detected by the TUNEL method and evaluated as the apoptotic index (AI). A high AI-level was shown in the high-grade malignant LMS, especially in cases of the recurrent/metastatic sites in comparison with the tumors of the primary site ($P < 0.05$). The AI was statistically higher in the p53-positive cases of high-grade malignant LMS than in the p53-negative cases of low-grade malignant LMS. In conclusion, apoptotic activity paralleled the overexpression of p53 protein along with an increasing grade of malignancy and may be related intimately to the increased malignant potential, especially to recurrence/metastasis.

Key words: apoptosis; immunohistochemistry; leiomyosarcoma; p53 protein

Leiomyosarcoma (LMS) is now known as one of the most frequent malignant tumors occurring in the soft-tissue of adults. It has been divided into 3 categories according to location by Enzinger and Weiss (1988), because of its clinical and biological behavior. Hashimoto et al. (1986) emphasized a prognostic difference between superficial and deep forms of LMS, an adverse prognosis of the deep form as compared to the superficial form. Histologically, conventional LMS is classified currently into well, moderately and poorly/anaplastic differentiated types depending on the degree of differentiation, but it is very difficult to evaluate the malignancy in predicting metastasis despite the multiple factors of size, cellularity, atypia and necrosis which are known as prognostic factors. Even at the level of mitotic activity, a serious decision can hardly be made on the biological behavior of LMS (Enzinger and Weiss, 1988).

Apoptosis is an active process of cell death occurring in both normal and neoplastic conditions and is characterized as the orderly participation of biological, morphological and molecular genetic events (Kerr et al., 1972, 1994; Wyllie, 1981; Umansky, 1982). It is induced by a variety of agents including oncogenic proteins, mainly p53 protein, the bcl-2 gene family and Fas/Fas legand among the process and regulation of apoptosis (Kerr et al., 1994). Recent studies have disclosed 2 pathways for the expression of apoptosis (Clarke and Purdie, 1993;

Abbreviations: AI, apoptotic index; LMS, leiomyosarcoma; MCM2, minimichrosome 2; PCNA, proliferating cell nuclear antigen; PI, p53 index; TUNEL, terminal deoxynuleotidyl transferase-mediated dUTP-biotin nick end labeling

Endo et al., 1999): p53 gene-dependent (Haupt et al., 1966; Chen et al., 1996) and -independent pathways, the latter of which can be induced by growth factors, hormones, chemotherapeutic agents and radiation (Clarke and Purdie, 1993; Endo et al., 1999). In considering malignant neoplasma, apoptosis may play an important role in the regulation of tumor growth in intimate relation to the p53 gene regulating the cell cycle in conjunction with cell proliferation.

The relationship between p53 gene status and apoptosis, however, has rarely been reported on soft-tissue sarcoma (Staunton and Gaffney, 1995; Yuki et al., 1995; Nakanishi et al., 1997; Valenti et al., 1998). We examined the relationship between the p53 protein and apoptosis in 29 surgical specimens from 20 cases of LMS of soft-tissue origin which were classified histologically from low-grade to high-grade malignancies according to the mitotic rate and proliferative cell markers. In addition, we discussed the significance and evaluation of apoptotic activity as a biological and prognostic factor of LMS.

Materials and Methods

This study was performed on 29 paraffin-embedded tumor tissues from 20 cases of LMS of soft-tissue origin, all of which were removed surgically. The histological diagnosis of these cases were determined in addition to immunohistochemical findings of vimentin, alpha-smooth muscle actin and HHF 35, and classified into 3 groups according to tumor cell differentiation from poorly to well differentiated types. Mitotic activity is an another aid in evaluating tumor malignancy and thus, the number of mitoses was counted in 10 high-power fields selected randomly as a mitotic rate: under 10 as low-grade, 10 to 20 as intermediate and over 20 as high-grade malignancies.

The clinical data for these cases were collected from medical records. Each case in this study was analyzed according to the tumor nodes metastases classification developed by the Union Internationale Contra le Cancer to evaluate the relation of apoptotic activity in LMS to their biological behavior.

Immunohistochemistry

An immunohistochemical study was performed by the streptavidin-biotin-complex method using the following monoclonal antibodies: anti-p53 nuclear protein (DO-7, 1:50, Dako, Glostrup, Denmark), anti-Ki-67 (MIB-1, 1;50, Immunotech, Marceille, France), anti-proliferating cell nuclear antigen (PCNA) (PC10, Nichirei, Tokyo, Japan) and anti-minimichrosome maintenance 2 protein (MCM2). MCM proteins are composed of 6 isoforms ranging from MCM2 to MCM7. They are expressed in the G1/S phase of the cell cycle and bind to DNA strands for a replication of DNA under the presence of DNA polymerase. Monoclonal antibody (2H10 clone) against MCM2 protein was produced by the immunization of oligopeptide of N-terminal from MCM2 protein with 892 amino acids and was reported as a proliferative marker available to examine the grading of malignancy*. The deparaffinized sections were pretreated with citrate buffer (0.01 mol/L citric acid: pH 6.0) at 92˚C for 30 min in a microwave oven (Azumaya MI-77, Osaka, Japan) for all antibodies except anti-PCNA antibody and then incubated at 4˚C overnight with the primary antibodies. The positive cells were counted and evaluated as the ratio of immunoreactive cells to the total number of 1,000 tumor cells.

TUNEL

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) was employed for the detection of apoptotic cells using an ApopTag Plus in situ apoptosis detection kit (Oncor, Gaithersburg, MD) which was followed by the method originally developed by Kerr et al. (1972). Briefly, the sections were incubated with 20 μg/mL proteinase K (Behringer Mannheim/Yamanouchi, Tokyo) for 15 min at 37˚C after blocking endogenous peroxidase in methanol for 30 min at room temperature. Thereafter, the sections were layered and incubated on terminal deoxynucleotidyl transferase with digoxigenin-11-dUTP in a

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moist chamber for 90 min at 37˚C. After stopping the reaction, anti-digoxigenin-peroxidase was applied to the reaction mixture for 30 min at room temperature. The positive reaction was estimated with 3,3'-diaminobenzidine reaction at room temperature. The positive cells were counted and evaluated as the ratio of TUNEL-positive cells to a total number of more than 1,000 tumor cells.

Table 1. Clinical data of the cases used in this study

Age range (mean)*	35–76 (65.6)
Sex ratio (male:female)	7:13
Primary site	
Retroperitoneum and abdomen	11
Upper extremity	1
Lower extremity	4
Body	1
Uterus	1
Recurrence/metastasis	5

*year.

Results

The clinicopathologic data are shown in Table 1. The patients were comprised of 7 males and 13 females. The age distribution was from 35 to 76 years old (mean age, 65.6 years). The primary sites of the tumors involved 12 from the retroperitoneum and abdomen, 4 from the lower extremities, 1 from the upper extremity and 1 from the trunk in addition to 1 from the uterus. Recurrent/metastatic cases were included in 4 cases (10 specimens). Twenty nine specimens from 20 cases of LMS of soft-tissue origin were histologically evaluated into 3 categories of malignancy based on tumor cell differentiation and the mitotic index: 9 low-grade, 8 intermediate and 12 highly malignant LMSs (Table 2). The cases of low-grade malignancy appeared very clear in cell differentiation showing less than 10 mitotic figures per 10 high-power fields selected randomly. The cases of intermediate malignancy were well to moderate in cell differentiation associated with 10 to 20 mitotic figures, and in high-grade malignancy, the tumor cells were poorly differentiated assuming pleomorphic giant cells abundantly. The mitotic figures were easily encountered at a ratio of over 20 per 10 high-power fields (Figs. 1a–d).

TUNEL labeling for apoptotic cells

Apoptotic cells were determined by the TUNEL method and evaluated as the apoptotic index (AI). Apoptotic cells were distinguished as cells with condensed and fragmented nuclei separated by a clear halo in hematoxylin and eosin stain (Fig. 1d). TUNEL-positivity was observed at a varying degree to the cell nuclei with condensed and fragmented nuclei and occasionally to tumor cells which looked normal (Figs. 2a and b). Table 2 shows the AI, positivities of p53 and the proliferative markers (Ki-67, PCNA and MCM2) of LMS categorized into 3 groups according to the mitotic rate. The immunohistochemical findings of p53 as well as Ki-67, PCNA and MCM2 are shown in Figs. 2c and d. Nine low-grade malignant LMSs showed 0.4 ± 0.2 of AI (mean ± SD) and 0.4 ±

Table 2. Comparative studies on LMS categorized in 3 groups according to the mitotic rate

Malignancy	Number of specimens	Cell differentiation	AI	PI	Cell proliferation		
					Ki-67	PCNA	MCM2
Low-grade (< 10)	9	Well	0.4 ± 0.2	0.4 ± 1.8	2.0 ± 0.6	6.8 ± 1.2	3.2 ± 0.8
Intermediate (10–20)	8	Moderately	1.2 ± 0.5	4.6 ± 2.7	5.0 ± 3.2	16.9 ± 2.6	9.7 ± 2.1
High-grade (20 <)	12	Poorly	1.3 ± 0.9	23.7 ±15.0	16.7 ± 5.0	33.4 ± 6.8	22.1 ±10.7

Mean ± SD.
( ), mitotic rate/10 high-power field.
AI, apoptotic index; LMS, leiomyosarcoma; MCM2, minimichrosome 2; PCNA, proliferating cell nuclear antigen; PI, p53 index; TUNEL, terminal deoxynuleotidyl transferase-mediated dUTP-biotin nick end labeling.

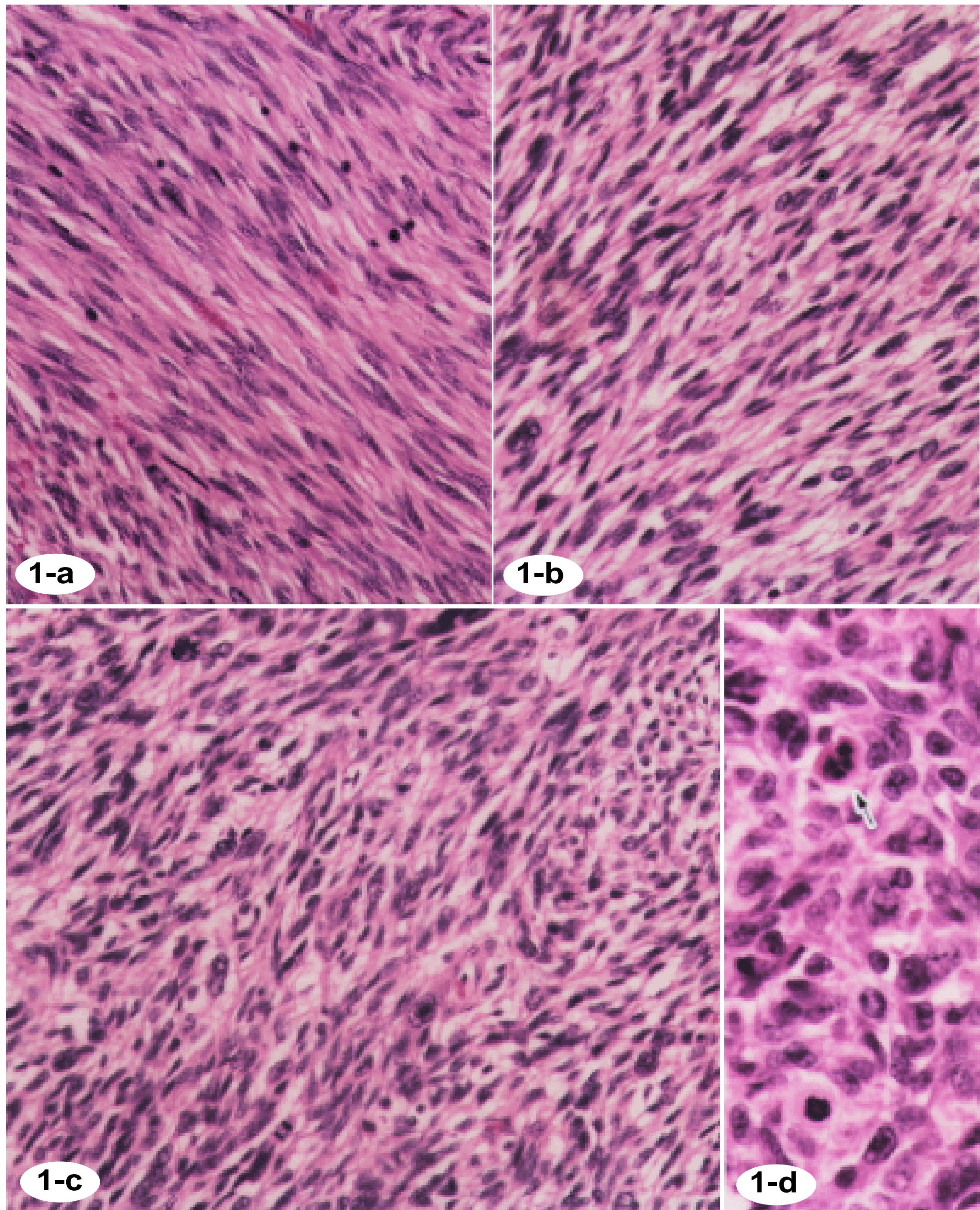

Fig. 1. Histological findings of LMS. Hematoxylin and eosin stain, × 200.

a: Well-differentiated LMS; mitotic cells, 9/10 HPF, low-grade malignancy, 71/F.
b: Moderately-differentiated LMS; mitotic cells, 10/10 HPF, intermediate malignancy, 76/F.
c: Poorly-differentiated LMS; mitotic cells, 34/10 HPF, high-grade malignancy, 63/F.
d: Arrow indicates an apoptotic cell.