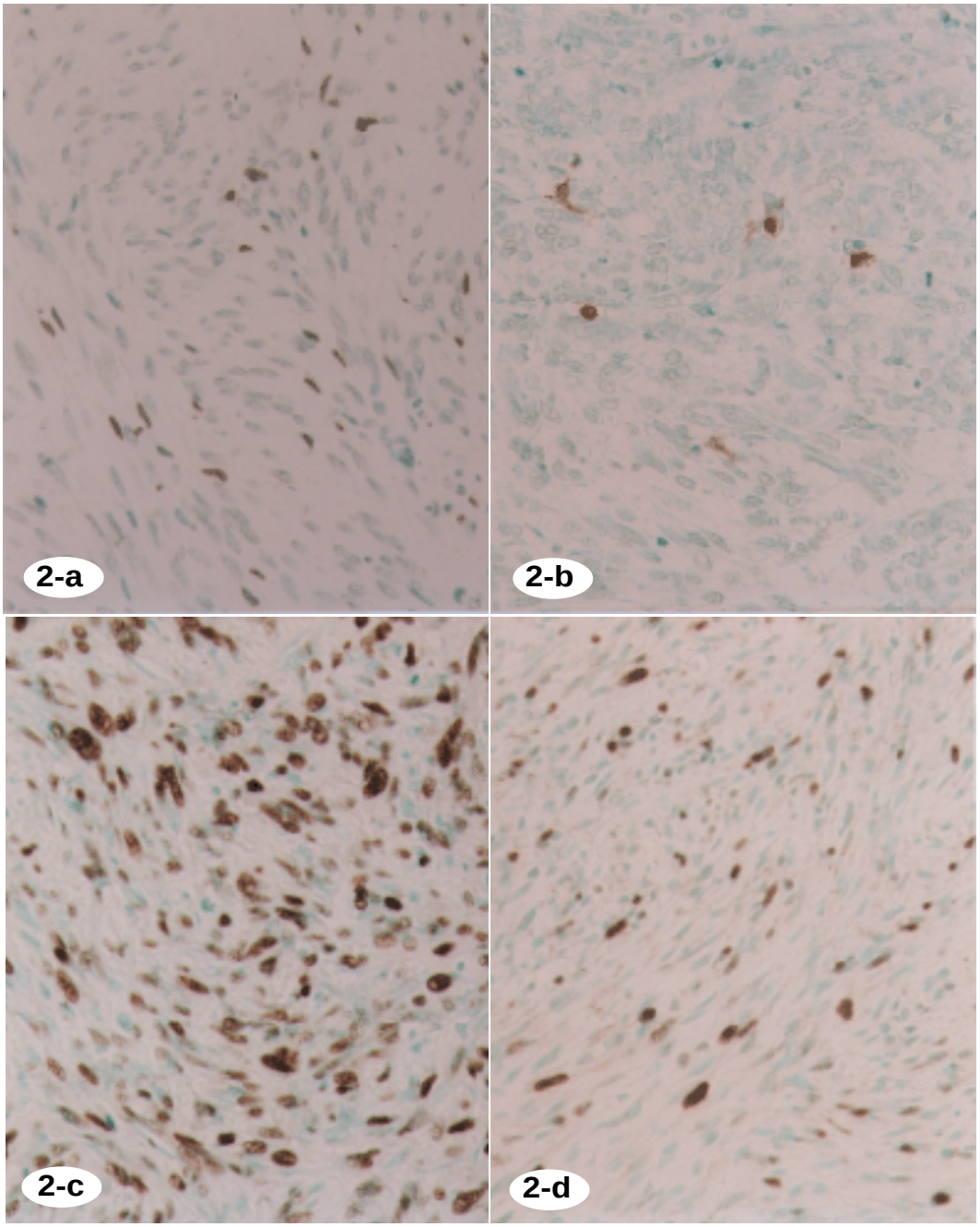

Figs. 2a and b. TUNEL for apoptotic cells. Positive for the tumor cell nuclei at a varying degree. **a:** AI = 5.1; third-recurrent tumor, 63/F. **b:** AI = 0.8; poorly-differentiated LMS, primary, 61/F.

Fig. 2c. Immunohistochemical stain of p53 protein. Strongly positive for the tumor cell nuclei. PI = 57, poorly-differentiated LMS, 74/F.

Fg. 2d. Immunohistochemical stain of Ki-67. Positive for the tumor cell nuclei at a various degree; 21.5%, poorly-differentiated LMS, 74/F.

Table 3. Comparative study on immunohistochemistry by the TUNEL method between 3 primary tumors and their recurrent/metastatic tumors

	Histological grading	Number of mitoses	AI	PI	Cell proliferation		
					Ki-67	PCNA	MCM2
Primary tumor [3]	Low	2– 9	0.4 ± 0.1	0.6 ± 0.5	0.5 ± 0.2	8.4 ± 2.4	3.8 ± 2.7
Recurrent/metastatic tumor* [9]	Intermediate-high	11–34	2.0 ± 0.7	12.3 ± 3.1	12.8 ± 3.5	22.1 ± 9.6	18.1 ± 7.9

Mean ± SD.
[ ], number of specimens.
* Recurrent period: 2–7 years.
AI, apoptotic index; MCM2, minimichrosome 2; PCNA, proliferating cell nuclear antigen; PI, p53 index; TUNEL, terminal deoxynuleotidyl transferase-mediated dUTP-biotin nick end labeling.

0.18 of PI, whereas 12 high-malignant LMSs were statistically higher at a rate of 1.1 ± 0.9 and 23.9 ± 15.0, respectively ($P < 0.05$). Three kinds of proliferative markers of Ki-67, PCNA and MCM2 proteins were examined to assess the proliferating activity of the tumor cells divided into 3 categories of malignancy. The nuclear positivities of Ki-67, PCNA and MCM2 increased statistically in the high-grade malignant LMSs at a rate of 16.7 ± 6.8 in Ki-67, 33.4 ± 6.8 in PCNA and 22.1 ± 10.7 in MCM2, more than those of the low-grade ones, respectively (2.0 ± 0.6 in Ki-67, 6.8 ± 1.2 in PCNA and 3.2 ± 0.8 in MCM2).

Relationship between TUNEL indices and p53 expression

TUNEL and p53 positivities were examined comparatively in primary LMSs (3 cases) and their recurrent/metastatic tumors (9 tumors) as shown in Table 3. TUNEL indices (AIs) of the recurrent/metastatic tumors were statistically higher (mean AI: 2.0 ± 0.7) in comparison with the primary site (0.4 ± 0.1). p53-positive cells increased significantly in cases of the recurrent/metastatic tumors (mean PI: 12.3 ± 3.1) than in those of the primary tumors (0.6 ± 0.5) ($P < 0.05$) in the same way.

Expression of apoptotic cells between p53-positive and -negative LMSs

Low-grade malignant LMS showed a rate of PI under 5 as shown in Table 2, and thus we figured that p53-positive tumors were rated over 5 PI. The p53-positive LMSs were found in 12 of 29 tumors as shown in Table 4 and revealed the AI at a mean of 2.2 ± 1.6, statistically higher than that (0.4 ± 0.1) of the p53-negative LMSs ($P < 0.05$). Histologically, the p53-positive group included 8 (66.7%) of 12 high-grade and 4 (50%) of 8 intermediate malignancies, whereas 9 (100%) low-grade and 4 (50%) of 8 intermediate malignant LMSs were included in the p53-negative group. The recurrence/metastasis-free

Table 4. AI between p53-negative and positive cases in 3 histological groups

	PI	Number of specimens	AI	Histological grading		
				Low	Intermediate	High
p53-negative	< 5	17	0.4 ± 0.1	9 [1]	4 [2]	4
p53-positive	5 ≤	12	1.5 ± 0.8	0	4 [3]	8 [3]

Mean ± SD.
[ ], number of recurrent/metastatic cases.
AI, apoptotic index; PI, p53 index.

survival rate was compared between the p53-positive and -negative groups (Fig. 3). The patients with p53-positive tumors was significantly worse than those with p53-negative tumors (log-rank test: $P = 0.047$).

Discussion

In this study, we evaluated the frequency of apoptotic cell death in an increasing malignant potential of LMS based on mitotic rate as well as expression of the proliferative markers of Ki-67/PCNA/MCM2 responsible of tumor growth. Secondly, the relationship between p53 overexpression and AIs was investigated to clarify the significance of apoptosis on tumor malignancy and prognostic factor. Our results showed a positive correlation between mitotic rate and increasing grade of histological malignancy on the one hand and the proliferative markers of Ki-67/PCNA/MCM2 on the other. These findings were exactly similar to those reported before by others (Drobijak et al., 1994; Yoo et al., 1997; Konomoto et al., 1998). MCM2 protein is part of the MCM family which is composed of 6 isoforms of MCM2 to MCM7. They are expressed in the G1/S phase of the cell cycle and bind to DNA strands for replication of DNA under the existence of DNA polymerase. The positivity for anti-MCM2 antibody ranged between those of Ki-67 and PCNA.

We observed p53 positivity in 12 (41.4%) of 29 LMSs, of which histological grading was high-malignant in 8 and intermediate malignant in 4. In the current study, p53-immunostaining was positive at a rate of 20 to 64% in various types of soft-tissue sarcomas (Yoo et al, 1997; Stefanou et al, 1998) and 21.6 to 43% of LMS of soft-tissue origin (O'Reilly et al., 1997; Konomoto et al., 1998). The mutated p53 gene loses its inherent transcriptional activity, resulting in enhancement of tumor cell proliferation (Endo et al., 1999). Yoo et al. (1997) suggested the role of the p53 mutation as the pathogenesis of soft-tissue sarcomas. Regarding the biological behavior or prognostic factor, however, the overexpression of p53 protein was estimated as being diverse from the one suggesting a positive correlation between p53 protein and prognosis (Drobijak et al., 1994; Toffoli et al., 1994; Yoo et al., 1997) to the others that indicated no relation between p53 expression and biological behavior (O'Reilly et al., 1997; Stefanou et al., 1998). We would like to emphasize here that the overexpression of p53 protein could be highly valuable as a prognostic factor taken from the findings of an increasing accumulation of p53 protein with an intimate relation to the grading of tumor malignancy.

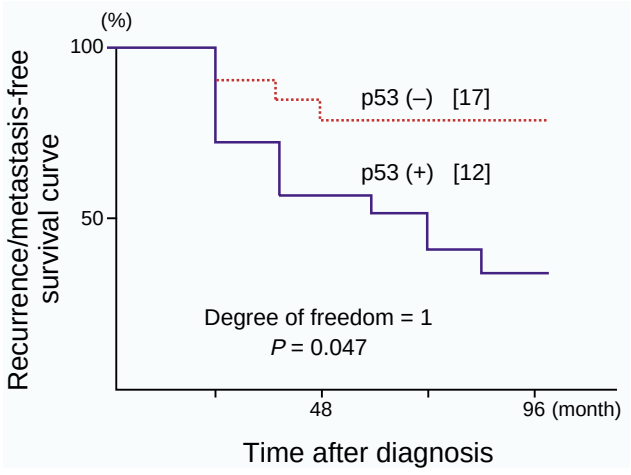

Fig. 3. Recurrence/metastasis-free survival curves of patients with p53-positive and -negative leiomyosarcoma (LMS). Patients with p53-positive tumors were significantly worse than those with p53-negative tumors (log-rank test: $P = 0.047$). [ ], number of specimens.

Detection and identification of apoptotic cells was successfully carried out on the paraffin-embedded sections by using the TUNEL method which was introduced and developed by Gavrieli et al. (1992) and Leoncini et al. (1993) and is based on detecting naturally occurring DNA strand breaks. TUNEL-positive, apoptotic cells were easily recognized as the cells with nuclear condensation, fragmentation separated by a clear halo around the cells in hematoxylin and eosin staining, in addition to the number of normal-looking cells as mentioned previ-

ously by others (Lowe et al., 1994; Aihara et al., 1994; Arai and Kino, 1995).

We compared AI and PI with histological grading resulting in a positive correlation of AI to PI which increased statistically from low-grade to high-grade malignancy. The AI value, furthermore, was statistically much higher in p53-positive cases of high-grade malignant LMS than in p53-negative cases. Thus, in cases of high-grade malignant LMS, especially recurrent/metastatic tumors, a more rapid growth of tumors caused by increased proliferative activity was probably due to a loss of regulation by p53. Apoptosis is regulated and controlled in its expression by various oncogens and suppresser genes (Kerr and Hormon, 1994). p53 gene is one of the major regulators of apoptosis which can easily allow apoptosis in association with MDM2 by regulating the cell cycle in the manner of growth arrest at G1/S or G2/S phase or apoptotic cell death, but the mutative p53 gene suppresses apoptosis by a loss of transcriptional activity to cyclin-dependent kinase (Aihara et al., 1994). Ikeda et al. (1998) demonstrated that naturally occurring apoptosis appeared to be induced predominantly via a p53-independent pathway from the observation of early and advanced gastric carcinoma. Kawauchi et al. (2000) suggested that apoptosis in cases of synovial sarcoma may be thought to be caused by a multiple apoptosis-regulating mechanism other than p53 protein. Our findings of a positive correlation between an increased amount of apoptotic cells and p53 positivity, however, is most likely to indicate that apoptotic status was induced via a p53-dependent pathway.

There has been a positive correlation reported between apoptotic cells and higher malignancies of non-Hodgkin's lymphomas (Leoncini et al., 1993), prostatic carcinomas (Aihara et al, 1994) and colorectal adenomas (Arai and Kino, 1995). Tatebe et al. (1996) also reported that apoptosis and cell proliferation were more frequent in metastatic foci than in primary lesions of colorectal carcinoma and suggested that apoptosis might reflect not only cell loss, but proliferative activity. A recent study on synovial sarcoma indicated that apoptosis may be an indicator of poor prognosis (Kawauchi et al., 2000). Our results support this hypothesis from the comparative examination of primary tumors and their recurrent/metastatic tumors. We used the materials of LMS originating in deep soft-tissue except for one from organ-specific LMS of the uterus and intestine which represented rather low mitotic activity. In fact, it is suggested that the susceptibility of undergoing apoptosis may reflect the histological differences between tumor types (Staunton et al., 1995). Therefore, the mechanism and role of apoptosis in LMS still remain to be elucidated.

Acknowledgments: This study was presented at the 33rd Annual Musculoskeletal Tumor Meeting of the Japanese Orthopaedic Association held on 14 July, 2000 in Kumamoto. The authors thank Mr. Hisayuki Matsumoto and Miss Yuki Torigai, both of whom are students at the College of Medical Care Technology, Tottori University.

References

1 Aihara M, Truong LD, Dunn JK, Wheeler TM, Scardino PT, Thompson TC. Frequency of apoptotic bodies positively correlates with Gleason grade in prostatic Cancer. Hum Pathol 1994; 25:797–801.
2 Arai T, Kino I. Role of apoptosis in modulation of the growth of human colorectal tubular and villous adenomas. J Pathol 1995;176:37–44.
3 Enzinger FM, Weiss SW. Soft tissue tumors. 2nd ed. St Lous: CV Mosby, 1988. Chapter 15. Leiomyosarcoma; p. 402–421.
4 Chen J, Wu X, Lin J, Levine AJ. mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol Cell Biol 1996; 16:2445–2452.
5 Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, et al. Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993;362:849–852.
6 Endo K, Sakatani T, Watanabe M, Yoshida H, Nannba E, Ito H. Wild-type p53 transfer resulted in cell cycle arrest, but not apoptosis of newly established human malignant fibrous histiocytoma cell line. Internat J Oncol 1999;15:935–942.
7 Drobijak M, Latres E, Pollack D, Karpeh M, Dudas M, Woodruff JM, et al. Prognositic implications of p53 nuclear overexpression and high

proliferation index of Ki-67 in adult soft-tissue sarcomas. J Natl Cancer Inst 1994;86:549–554.
8 Hashimoto H, Daimaru Y, Tsuneyoshi M, Enjoji M. Leiomyosarcoma of the external soft tissues. Cancer 1986;57:2077–2088.
9 Haupt Y, Barak Y, Oren M. Cell type-specific inhibition of p53-mediated apoptosis by mdm2. EMBO J 1996;15:1596–1606.
10 Gagvrielli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493–501.
11 Ikeda M, Shomori K, Endo K, Makino T, Matsuura T, Ito H. Frequent occurrence of apoptosis is an early event in the oncogenesis of human gastric carcinoma. Virchows Arch 1998;432:43–47.
12 Kawauchi S, Fukuda T, Oda Y, Saito T, Takeshita M, Yokoyama K, et al. Prognostic significance of apoptosis in synovial sarcoma: correlation with clinicopathologic parameters, cell proliferative activity, and expression of apoptosis-related proteins. Mod pathol 2000;13: 755–765.
13 Kasagi N, Gomyo Y, Shirai H, Tsujitani S, Ito H. Apoptotic cell death in human gastric carcinoma: analysis by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling. Jpn J Cancer Res 1994;85:939–945.
14 Konomoto T, Fukuda T, Hayashi K, Kumazawa J, Tsuneyoshi M. Leiomyosarcoma in soft tissue: examination of p53 status and cell proliferating factors in different locations. Hum Pathol 1998; 29:74–81.
15 Kerr JFK, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; 26:239–257.
16 Kerr JFR, Winterford CM, Hormon BV. Apoptosis: its significance in cancer and cancer therapy. Cancer 1994;73:2013–2026.
17 Leoncini L, Del Vacchio MT, Megha T, Barbini P, Galieni P, Pileri S, et al. Correlations between apoptotic and proliferative indices in malignant non-Hodgkin's lymphomas. Am J Pathol 1993; 142:755–763.
18 Lowe SW, Jacks T, Housman DE, Ruley HE. Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc Natl Acad Sci USA 1994;91:2026–2030.
19 Nakanishi H, Ohsawa M, Naka N, Uchida, Ochi T, Aozasa K. Immunohistochemical detection of bcl-2 and p53 proteins and apoptosis in soft tissue sarcoma: their correlations with prognosis. Oncology 1997;54:238–244.
20 Stefanou DG, Nonni AV, Agnantis NJ, Athnassiadou SE, Briassoulis E, Pavlidis N. p53/MDM-2 immunohistochemical expression correlated with proliferative activity in different types of human sarcomas: a ten-year follow-up study. Anticancer Res 1998;18:4673–4681.
21 O'Reilly PE, Raab SS, Niemann TH, Rodgers JR, Robinson RA. p53, proliferating cell nuclear antigen, and Ki-67 expression in extrauterine leiomyosarcoma. Mod Pathol 1997;10:91–97.
22 Staunton MJ, Gaffney EF. Tumor type is a determinant of susceptability to apoptosis. Am J Clin Pathol 1995;103:300–307.
23 Tatebe S, Ishida M, Kasagi N, Tsujitani S, Kaibara N, Ito H. Apoptosis occurs more frequently in metastatic foci than in primary lesions of human colorectal carcinomas: analysis by terminal-deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling. Int J Cancer 1996;65: 173–177.
24 Toffoli G, Doglioni C, Cernigoi C, Frustaci S, Perin T, Canal B, et al: p53 over-expression in human soft tissue sarcomas: relation to biological aggressiveness. Ann Oncol 1994;5:167–172.
25 Umansky SR. The genetic program of cell death. Hypothesis and some applications: transformation, carcinogenesis, aging. J Theor Biol 1982; 97:591–602.
26 Valenti MT, Azzarello G, Vinate O, Manconi R, Balducci E, Guidolin D, et al. Differentiation, proliferation and apoptosis levels in human leiomyoma and leiomyosarcoma. J Cancer Res Clin Oncol 1998;124:93–10
27 Wyllie AH. Cell death: a new classification separating apoptosis from necrosis. In: Bowen ID, Lockshin RA, eds. Cell death in biology and pathology. London: Chapman and Hall; 1981. p. 9–34.
28 Yoo J, Lee HK, Kang CS, Park WS, Lee JY, Shim SI. p53 gene mutation and p53 protein expression in human soft tissue sarcomas. Arch Pathol Lab Med 1997;121:395–399.
29 Yuki M, Yokota K, Okuyama S. Cell proliferation and cell death (apoptosis) as indices differentiating malignant from benign gastrointestinal myogenic tumors. Nippon Shokakibyo Gakkai Zasshi 1995;92:206–216 (in Japanese with English abstract).

Received April 12, 2001; accepted May 17, 2001

Corresponding author: Prof. Haruhiko Yoshida