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Prognostic Impact of P53 Status, *TLS-CHOP* Fusion Transcript Structure, and Histological Grade in Myxoid Liposarcoma: A Molecular and Clinicopathologic Study of 82 Cases[1]

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ABSTRACT

Purpose: **A specific *TLS-CHOP* fusion gene resulting from the t(12;16) is present in at least 95% of myxoid liposarcomas (MLS). Three common forms of the *TLS-CHOP* fusion have been described, differing by the presence or absence of *TLS* exons 6–8 in the fusion product. Type 5-2 (also known as type II) consists of *TLS* exons 1–5 fused to *CHOP* exon 2; type 7-2 (also known as type I) also includes *TLS* exons 6 and 7 in the fusion, whereas type 8-2 (also known as type III) fuses *TLS* exons 1–8 to *CHOP* exon 2. We sought to determine the impact of *TLS-CHOP* fusion transcript structure on clinical outcome in a group of well-characterized MLS cases. We also analyzed P53 status, because this parameter has been found to have a significant prognostic impact in other sarcomas with chromosomal translocations.**

Methods: **We analyzed *TLS-CHOP* fusion transcripts by reverse-transcription PCR using RNA extracted from frozen tissue in 82 MLS confirmed previously to harbor a *CHOP* rearrangement either by Southern blotting or by cytogenetic detection of the t(12;16). Parameters analyzed included age, location, size, percentage of round cell (RC) component, areas of increased cellularity, necrosis, and surgical margins. In 71 (87%) cases, adequate tumor tissue was available for immunohistochemical analysis of P53 status, using DO7 antibody. The Kaplan-Meier method, log-rank, and Cox regression tests were used for survival analyses.**

Results: **Most MLS were >10 cm (73%), arising in the thigh (70%), and localized at presentation (89%). RC component was <5% in 47 (57%) cases and ≥5% in 35 (43%). The *TLS-CHOP* fusion transcript was type 5-2 in 55 (67%), type 7-2 in 16 cases (20%), and type 8-2 in 8 (10%). One tumor had a unique variant fusion, between exon 6 *TLS* and exon 2 *CHOP*. Two other cases (2%) showed an *EWS-CHOP* fusion transcript. Overexpression of P53 (defined as ≥10% nuclear staining) was detected in 12 (17%) cases. High histological grade (defined as ≥5% RC; $P < 0.01$), presence of necrosis (≥5% of tumor mass; $P < 0.05$), and overexpression of P53 ($P < 0.001$) correlated with reduced metastatic disease-free survival in localized tumors. The presence of negative surgical margins ($P < 0.01$) and extremity location ($P = 0.02$) were found to be significant in predicting local recurrence in the entire group as well as localized cases by univariate and multivariate analysis. Although there was no significant correlation between *TLS-CHOP* transcript type and histological grade or disease-specific survival, an association was found between the P53 status and type 5-2 fusion ($P < 0.01$).**

Conclusion: **In contrast to some other translocation-associated sarcomas, the molecular variability of *TLS-CHOP* fusion transcript structure does not appear to have a significant impact on clinical outcome in MLS. Instead, high histological grade (≥5% RC), presence of necrosis, and P53 overexpression are predictors of unfavorable outcome in localized MLS.**

INTRODUCTION

MLS,[3] the most common subtype of liposarcoma, occurs predominantly in the extremities of adults and has a tendency either to recur locally or to metastasize to unusual soft tissue locations (1–3). A proportion of cases show histological progression to RC morphology, a feature significantly associated with a poor prognosis (2). The karyotypic hallmark of MLS and RC liposarcomas is the t(12;16)(q13;p11), which is specific for this tumor type and is present cytogenetically in >95% of cases (4). The translocation leads to the fusion of the *CHOP* and *TLS*

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[3] The abbreviations are: MLS, myxoid liposarcomas; RC, round cell; CHOP, CCAAT/enhancer-binding protein homologous protein; MSKCC, Memorial Sloan-Kettering Cancer Center; UNMC, University of Nebraska Medical Center; RT-PCR, reverse transcription-PCR; LR, long range; LRFS, local recurrence-free survival; MDFS, metastatic disease-free survival; DSS, disease-specific survival; WDLS, well-differentiated liposarcoma.

Table 1 *TLS-CHOP* fusion transcript structure, pathological findings, and patient demographic information

LS no.	PCR	Age	Sex	Site[a]	Size	%RC	At dx	LRdx	Mdx	LFU	Status
1	(5-2)	35	M	RP/l-abd	12	10	1	8	10	48	DOD
2	(5-2)	31	F	Thigh	5.5	0	1			49	NED
3	(7-2)	33	M	Thigh	14	20	M		0	12	AWD
4	(5-2)	68	M	Thigh	17	<5	M	9	0	44	DOD
5	(5-2)	72	M	Thigh	6	0	1	11		11	NED
6	ews	33	F	Thigh	11.5	30	M		0	51	DOD
7	(5-2)	68	M	Thigh	10.2	40	1			23	NED
8	(5-2)	36	M	RP/l-abd	18	<5	1	35		68	AWD
9	(5-2)	44	M	Thigh	32	40	1		30	32	AWD
10	(5-2)	31	F	Pelvis	10	0	1	22		99	AWD
11	(5-2)	37	M	Thigh	17	0	1	26		76	NED
12	(5-2)	50	F	Thigh	14	30	1			100	NED
13	(5-2)	33	M	Thigh	14	<5	1	19		20	AWD
14	(5-2)	63	F	Thigh	15	35	1		33	35	AWD
15	(5-2)	51	M	Thigh	16	20	1		3	23	DOD
16	(5-2)	35	M	Thigh	7.5	0	1	193		221	NED
17	(7-2)	79	F	Thigh	15.5	0	1			1	NED
18	(5-2)	43	M	Thigh	16.5	<5	1			51	NED
19	(5-2)	42	F	Buttock	6.2	>50	1	100	95	107	DOD
20	(5-2)	46	F	Thigh	11.4	0	1			55	NED
21	(6-2)*	35	M	Thigh	12	0	1			83	NED
22	(5-2)	44	M	Buttock	20	<5	1			50	NED
23	(5-2)	45	M	Leg	20	0	1			91	NED
24	(8-2)	29	M	Thigh	11	0	1			42	NED
25	(5-2)	37	M	Thigh	18	0	1			61	NED
26	(5-2)	42	M	Groin	15.5	0	1	16	25	44	DOD
27	(7-2)	31	M	Buttock	23	10	1			2	NED
28	(8-2)	27	M	Thigh	7	10	1		14	72	DOD
29	(5-2)	56	F	Thigh	24	10	1		11	36	DOD
30	(7-2)	42	F	Thigh	14.5	<5	1			30	NED
31	(5-2)	54	F	Thigh	17	20	1		5	90	AWD
32	(5-2)*	69	F	Groin	25	20	1			16	DWD
33	(8-2)	48	M	Thigh	18	10	1			25	NED
34	(5-2)	65	M	Thigh	12	0	M	12	0	20	DOD
35	(7-2)	35	M	Thigh	21	10	1		45	51	AWD
36	(8-2)	46	M	Thigh	12	20	1			14	NED
37	(8-2)	33	M	Axilla	11.5	0	1	49		57	NED
38	(5-2)	71	M	Buttock	8	0	1	49		65	NED
39	(8-2)	60	M	Groin	14.5	0	1	42	67	120	DOD
40	(5-2)	25	F	Axilla	30	>50	M		0	8	DOD
41	(5-2)	35	M	Thigh	15	>50	1			50	NED
42	(5-2)	61	F	Thigh	16	25	M		0	23	AWD
43	(5-2)	55	M	Foot	9.5	<5	1		11	18	DOD
44	(5-2)	62	M	Buttock	14	0	M	36	0	37	DOD
45	(8-2)	59	M	Pelvis	16.2	<5	1	6		40	DWD
46	(5-2)	37	M	Thigh	14	0	1		51	139	AWD
47	(8-2)	49	M	Thigh	8	0	1			1	NED
48	(5-2)	59	F	Upper arm	10	<5	1	35		123	AWD
49	(5-2)	13	M	Thigh	5.2	0	1			1	NED
50	(7-2)	48	F	Thigh	15	0	1			75	NED
51	(5-2)	38	M	Buttock	23	0	1	16	25	34	DOD
52	(5-2)	37	M	Thigh	15	0	1			30	NED
53	(5-2)	42	F	Thigh	14	0	1			43	NED
54	(5-2)	56	M	Thigh	24	5	1			72	NED
55	(7-2)	65	F	Shoulder	6	0	1	108		156	NED
56	(5-2)	61	F	Calf	7	0	1		42	180	NED
57	(7-2)	37	M	Thigh	20	>50	M		0	13	DOD
58	(5-2)	52	F	Thigh	15	20	1		32	50	DOD
59	(7-2)	38	F	Thigh	10	30	1	96	84	98	DOD
60	(5-2)	59	F	Thigh	15	5	1	42	59	61	DOD
61	(5-2)	35	F	Thigh	10	10	1		288	288	AWD
62	(5-2)	40	M	Thigh	4	0	1		22	56	AWD
63	(5-2)	23	F	Calf	6	0	1			37	NED
64	(5-2)	74	F	Thigh	11	0	1			12	NED
65	(5-2)	50	M	Thigh	9	<5	1			53	NED
66	(5-2)	57	M	Thigh	8	35	1			156	NED

[a] RP/l-abd, retroperitoneum-lower abdomen.
[b] DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease.

Table 1 Continued

LS no.	PCR	Age	Sex	Site[a]	Size	%RC	At dx	LRdx	Mdx	LFU	Status
67	(5-2)	53	M	Thigh	28	>50	1		9	11	DOD
68	(5-2)	28	F	Thigh	15	0	1	76		135	NED
69	(5-2)	31	M	Thigh	17	0	1			24	NED
70	(7-2)	39	F	Thigh	32	5	M		0	24	DOD
71	(7-2)	42	F	Thigh	5	<5	1			34	NED
72	(7-2)	61	M	Scapula	7.5	25	1			N/A	N/A
73	(5-2)	42	M	Calf	8	0	1			13	NED
74	(7-2)	56	M	Thigh	5.5	0	1			7	NED
75	(5-2)	45	M	Thigh	12.5	20	1			3	NED
76	(5-2)	65	F	Groin	12	25	1			52	NED
77	(7-2)	41	M	Calf	23	20	1			N/A	N/A
78	(7-2)	37	F	Thigh	3.5	0	1			N/A	N/A
79	(7-2)	42	M	Thigh	13	5	1			15	NED
80	(5-2)	34	M	Thigh	15	0	1			12	NED
81	(5-2)	40	F	Thigh	14	0	1			52	NED
82	ews	33	F	Thigh	19	20	1			37	NED

[a] RP/l-abd, retroperitoneum-lower abdomen. At dx, stage at diagnosis (1, localized; M, metastatic).
[b] DOD, dead of disease; NED, no evidence of disease; AWD, alive with disease.

genes at 12q13 and 16p11, respectively, and the generation of a TLS-CHOP hybrid protein (5–7). The *TLS* gene is also known as *FUS* (6). In rare cases of MLS, a variant chromosomal translocation has been described, t(12;22)(q13;q12), resulting in an *EWS-CHOP* fusion gene (3, 8, 9).

The *CHOP* gene is a member of CHOP family of leucine zipper transcription factors, implicated in adipocyte differentiation and growth arrest (10–12). The *CHOP* gene is normally expressed at very low levels in most cells, including adipocytes; however, it is markedly activated by perturbations that induce cellular stress. In MLS, the hybrid *TLS-CHOP* gene encodes a protein that consists of the 5′ portion of *TLS* fused to the entire coding region of *CHOP*. The TLS-CHOP protein is thought to function primarily as an aberrant transcriptional regulator that interferes with adipocyte differentiation, favoring proliferation over terminal differentiation.

TLS-CHOP fusion transcripts occur as different recurrent structural variants reflecting the inclusion of different portions of *TLS*, caused by different genomic breakpoints (13). Of the possible *TLS* genomic breakpoints, only breaks in *TLS* introns 5, 7, and 8 give rise to in-frame fusion transcripts joining *TLS* exons 5, 7, and 8, respectively, to exon 2 of *CHOP*. Thus, three major recurrent fusion transcript types have been reported in cases of MLS: type 7-2 (also known as type I); type 5-2 (also known as type II); and type 8-2 (also known as type III). Exons 1–5 of *TLS* are thus invariably present in all *TLS-CHOP* fusions.

On the basis of experience with other translocation-associated sarcomas, such as Ewing sarcoma, alveolar rhabdomyosarcoma, or synovial sarcoma, in which the genetic variability of the fusion structure impacts on survival (see "Discussion"), we sought to evaluate the potential impact of *TLS-CHOP* fusion transcript structure on clinical outcome in MLS. We also analyzed P53 status, because P53 alteration has been found to be a strong negative prognostic factor in other sarcomas with chromosomal translocations, for instance Ewing sarcoma and synovial sarcoma (14, 15). The prognostic value of *TLS-CHOP* fusion transcript structure and P53 status was also compared with established conventional clinical and pathological prognostic factors in MLS.

PATIENTS AND METHODS

Study Group. This study included a total of 82 patients with MLS from three institutions, including MSKCC (52 patients); Cleveland Clinic Foundation (19 patients), and the UNMC (11 patients). The retrospective collection of frozen tumor samples included patients treated at the above institutions over a 17-year period (1983–2000). All tumors had a typical histological appearance of MLS. Among the 82 patients, there were 50 (61%) males and 32 (39%) females. Mean age at diagnosis was 45.8 years, and ages ranged from 13 to 79 years of age. The tumors were frequently located within the lower extremity [67 (82%)], with a strong predilection for the thigh [57 (70%)]. The remaining tumors were located in the upper extremity, 1 (1%) case; trunk, 10 (12%) cases; and retroperitoneum/pelvis, 4 (5%) cases. Most of the tumors were large, measuring ≥10 cm in 60 (73%) cases. Twenty (25%) tumors were ≥5 cm but <10 cm; only 2 (2%) cases were <5 cm. The majority of tumors were localized at presentation (89%), with only 9 (11%) patients presenting with distant metastases at the time of diagnosis (Table 1).

In all cases, microscopic slides from the en-bloc resection specimen of the primary tumor were available for pathological review, and our standard practice was to submit one block/per cm^3 of tumor for histological examination in each case. The tumors were scored for the percentage of RC component or transitional areas (increased cellularity; Ref. 16). The percentage of RC component was estimated by scanning all individual sections, using the entire tumor volume as a denominator. The cases were divided into low and high histological grades using, in parallel, two previously described cutoff points, first with ≥5% (17) RC component and the second with ≥25% RC component (2), to assign low and high grades. The transitional areas were defined as areas of increased cellularity where the tumor cells were not closely packed and which retained a small amount of intercellular myxoid stroma and a discernible plexiform vascular pattern. In cases with <5% RC component, the degree of increased cellularity was also assessed, because previous reports suggested a possible prognostic role (16). There-

fore, these cases were divided into two groups: (*a*) pure MLS cases, lacking RC component or increased cellularity; and (*b*) MLS with areas of increased cellularity (≥5%). The degree of necrosis was also assessed, arbitrarily using ≥ 5% microscopic areas of necrosis as a threshold for positive cases.

All primary tumors were managed by surgical resection with a curative intent. In 46 (56%) cases, the tumors were entirely resected with negative microscopic margins; in 14 (17%) cases, the inked surgical margins were free but close (<1 mm); in the remaining 22 (27%) cases, there were positive microscopic margins. Adjuvant chemotherapy and/or radiotherapy were given based on evaluation of prognostic factors in each individual case and were thus not randomized. Therefore, adjuvant therapy was not included in the analyses performed.

Molecular Analysis. Tumor samples for molecular analysis were snap-frozen in liquid nitrogen and stored at −70°C. The tumor tissues available for molecular analysis were obtained from primary tumor sites in 58 (71%) cases, from a local recurrence in 14 (17%) cases, or from a distant metastatic site in 10 (12%) cases. All cases included for analysis were previously confirmed to harbor *CHOP* rearrangement by Southern blotting (87% of cases) at MSKCC or by conventional cytogenetic demonstration (at UNMC) of a t(12;16)/t(12;22) (13% of cases), using standard protocols as described previously (18). Forty cases were included in a previous study of *CHOP* and *TLS* genomic rearrangements in MLS (19).

RT-PCR for *TLS-CHOP* and *EWS-CHOP* Fusion Transcripts. Extraction of total RNA was based on the guanidinium isothiocyanate-phenol chloroform method using the RNA Wiz reagent (Ambion, Inc., Austin, TX). Analysis by RT-PCR for *TLS-CHOP* and if indicated, for *EWS-CHOP* transcripts, was performed on all 82 cases at one of the centers (MSKCC). The adequacy of the extracted RNA was assessed by RT-PCR, using primers for *phosphoglycerate kinase* gene transcripts. Negative controls that lacked either tumor RNA or reverse transcriptase were used routinely. Three μg of total RNA were subjected to RT-PCR using Qiagen 1 Step RT-PCR kit (Qiagen, Inc., Valencia, CA), using a forward primer within exon 5 of *TLS* (5′-CAG CCA GCA GCC TAG CTA TG-3′) or in exon 7 of *EWS* (5′-CTG GAT CCT ACA GCC AAG CTC CAA G-3′) and a reverse primer in exon 3 of *CHOP* (5′-TGT CCC GAA GGA GAA AGG CAA TG-3′). The Qiagen 1 Step RT-PCR conditions included: (1) the reverse transcription step: 50°C for 30 min; followed by (2) 95°C for 15 min; (3) the PCR cycles of 95°C for 45 s, 64°C for 45 s, 72°C for 1 min (35 cycles); and (4) 72°C for 7 min. The RT-PCR products were identified by agarose gel electrophoresis. The expected sizes were 526 bp for type 7-2, 250 bp for type 5-2, and 625 bp for type 8-2 *TLS-CHOP* fusion transcripts. All non-type 5-2 transcripts and randomly selected type 5-2 transcripts were confirmed by direct automated sequencing.

Long-Range DNA PCR for *TLS-CHOP* and *CHOP-TLS* Genomic Junction Fragments. To independently confirm the RT-PCR results, LR PCR was performed in 61 (74%) cases with adequate/available DNA. DNA was isolated from snap-frozen tissue using a standard organic extraction protocol. One μg of DNA was amplified using the Expand System LR-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN), per package instructions. Three sets of primers were used: one set each for the detection of either *TLS-CHOP* or *CHOP-TLS* genomic junction fragments in cases with type 5-2 (5-2) transcripts, and one set for the detection of *TLS-CHOP* genomic junction fragments in both type 7-2 (7-2) and type 8-2 (8-2) cases. The primers for *TLS-CHOP* in cases with type 5-2 transcripts were: LR TLS-5 forward (5′-GTG GAG GTG GAG GTG GAG-3′), designed close to the 3′ end of exon 5 of *TLS* (nucleotides 584–601, GenBank accession no. S62138) and LR CHOP-2 reverse (5′-CAT CTG CTT TCA GGT GTG GTG-3′), ~30 bp from the 5′-end of exon 2 *CHOP* (nucleotides 908–928; GenBank accession no. S62138). The expected product size range was 60–3250 bp, and the annealing temperature used was 60°C. The primers for reciprocal *CHOP-TLS* junction fragment used in cases with the type 5-2 transcript were: LR CHOP-1 forward (5′-GCA GCG ACA GAG CCA AAA TCA GAG C-3′) and LR TLS-6 reverse (5′-TCC ACC ACT CTG GTC TTG ATT GC-3′; position 662–684 in *TLS*). The expected product size range was 850-3220 bp with an annealing temperature of 62°C (20). The primer set used to amplify *TLS-CHOP* genomic junction fragments in cases with type 7-2 (7-2 fusion) or type 8-2 (8-2 fusion) transcript was: LR TLS-7 forward (5′-CCG TGG TGG CTT CAA TAA A-3′), positioned at the 3′-end of exon 7 (position 852–870) *TLS* and the same LR CHOP-2 reverse primer, in *CHOP* exon 2, with an annealing temperature of 58°C. The expected size range for products was 949-4046 bp (for the type 8-2 fusion) and 80–3504 bp (for the type 7-2 fusion).

Immunohistochemical Analysis. In 71 (87%) cases, paraffin-embedded representative tumor tissue was available for P53 immunohistochemical analysis. The single tumor tissue paraffin block used for the immunohistochemical analysis was selected to contain the most cellular area or the RC component from each case. A mouse monoclonal antibody, clone DO7 (DAKO, Carpinteria, CA; 1:500; 0.2 μg/ml) was used for detection of a P53 epitope located between amino acids 19 and 26 of wild-type and mutant human P53 proteins. Deparaffinized sections were treated with 3% H_2O_2 to block endogenous peroxidase activity. Sections were subsequently immersed in boiling 0.01% citric acid (pH 6.0) in a microwave oven for 15 min to enhance antigen retrieval, allowed to cool, and incubated with 10% normal horse serum (mouse monoclonal antibodies) to block nonspecific tissue immunoreactivities. Primary antibodies were then incubated overnight at 4°C. Biotinylated horse anti-mouse IgG antibodies (Vector Laboratories, Burlingame, CA; 1:500 dilution) were applied for 1 h, followed by avidin-biotin-peroxidase complexes that were applied for 30 min (Vector Laboratories; 1:25 dilution). Diaminobenzidine was used as the final chromogen, and hematoxylin was used as the nuclear counterstain. Nuclear immunoreactivities were scored on a continuous scale with values that ranged from undetectable levels or 0% to homogeneous staining or 100%. The staining profile in tumor cells was compared with the negative internal tissues, as well as with both negative and positive control samples. On the basis of other reports (15, 21) a cutoff point of ≥10% nuclear staining was used to designate positive cases. This type of aberrant P53 staining in sarcomas correlates well with the presence of *P53* point mutations.

Statistical Analysis. All time-to-event end points were modeled using Kaplan-Meier survival plots and analyzed by the

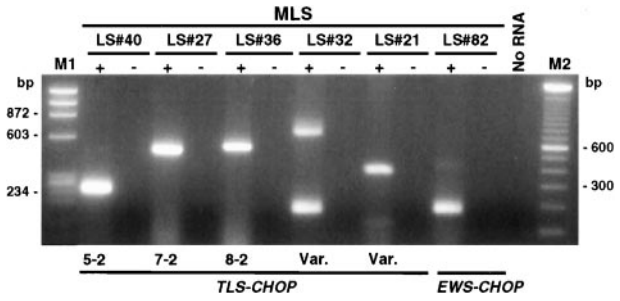

Fig. 1 Detection of *TLS-CHOP* or *EWS-CHOP* transcripts by RT-PCR. *M1*, size marker (PhiX174RF DNA/*Hae*III); *LS#40*, type 5-2 *TLS-CHOP* fusion (250 bp), +/− reverse transcriptase; *LS#27*, type 7-2 *TLS-CHOP* fusion (526 bp), +/− reverse transcriptase; *LS#36*, type 8-2 *TLS-CHOP* fusion (625 bp), +/− reverse transcriptase; *LS#32*, (5-2 variant) *TLS-CHOP* fusion (169 bp and 782 bp, see text), +/− reverse transcriptase; *LS#21*, (6-2 variant) *TLS-CHOP* fusion (382 bp), +/− reverse transcriptase; *LS#82*, *EWS-CHOP* fusion (179 bp), +/− RT; *M2*, size marker (100-bp DNA ladder). *Var.*, variant.

log-rank test. Significant factors by log-rank test were analyzed for independent prognostic importance by the Cox proportional hazards regression using a stepwise procedure. Because of the modest number of cases in this study, only factors found to have $P < 0.2$ by univariate analysis were entered into the Cox model. Fisher's exact tests were used to assess associations between factors. The statistical analysis was performed for all patients with available follow-up and for the subgroup of patients presenting with localized disease.

The clinical end points analyzed were time to local recurrence (LRFS), to distant metastasis (MDFS), and to death from disease (DSS). The factors analyzed included: age, size, location, histological grade (≥5% RC; ≥25% RC; and ≥5% increased cellularity in the subgroup of patients with <5% RC), necrosis (≥5% of the tumor mass), surgical margins, and *TLS-CHOP* fusion transcript type, and status of P53 protein overexpression.

RESULTS

Histological Features and Grade. All cases had a typical morphological appearance of MLS. The high-grade areas were restricted to the RC component. None of the 82 cases showed areas of dedifferentiation or nonlipogenic spindle cell proliferation. Furthermore, no histological features overlapping with other types of liposarcoma, such as well-differentiated or pleomorphic types, were noted.

Using ≥5% RC as the cutoff point, there were 47 (57%) low-grade and 35 (43%) high-grade cases. When ≥25% RC cutoff was used, there were 67 (82%) low-grade and 15 (18%) high-grade cases. Of the 47 cases with <5% RC, 27 (57%) showed areas of increased cellularity. Thirty-nine (48%) cases showed microscopic evidence of tumor necrosis in ≥5%.

RT-PCR Results. The most common *TLS-CHOP* fusion type was type 5-2, identified in 55 cases (67%), followed by type 7-2 in 16 cases (20%) and type 8-2 in 8 cases (10%; Fig. 1). In 2 cases, the *EWS-CHOP* transcript was identified (2%; Fig. 1). There was no evidence of alternative splicing identified by RT-PCR, *i.e.*, in no case did we detect more than one type of *TLS-CHOP* fusion transcript.

A total of 47 (57%) RT-PCR products were sequenced as follows: 20 cases (40%) of type 5-2 fusion and all type 7-2 and 8-2 *TLS-CHOP* transcripts, and other fusion variants. From the 20 type 5-2 cases sequenced, only one case showed a variation in size and sequence, having two amplified bands (Fig. 1). The smaller band (169 bp) showed a fusion of the 5′ portion of exon 5 of *TLS* with a 26-bp sequence from intron 1 of *CHOP* (nucleotides 2358–2383; GenBank accession no. Y09999), which functioned as a cryptic exon spliced to exon 2 of *CHOP*. This fusion transcript was in-frame (Fig. 2). The *TLS* portion of the fusion transcript lacked 107 nucleotides of exon 5. The break had occurred in exon 5 of *TLS* (at position 497; GenBank accession no. S62138). The larger band (782 bp) was a transcript consisting mostly of *CHOP* intron 1 sequences, which contained stop codons in all frames. The possibility of this latter product arising from genomic DNA was excluded by a negative result on RT-PCR lacking reverse transcriptase. Because the shorter in-frame fusion product in this case was most similar to a "5-2," it was included in the type 5-2 group for further analysis.

Another case showed a novel and unique variant fusion transcript (Fig. 1), joining the 5′ portion of exon 6 of *TLS* to exon 2 of *CHOP*. The *TLS* break was located within exon 6 (position 733). Therefore, a portion of exon 6 (lacking 129 bp) was fused in-frame with exon 2 of CHOP, resulting in a product size of 382 bp (Fig. 2).

Direct sequencing of the two *EWS-CHOP* cases showed the same 179-bp product with an in-frame junction of exon 7 of *EWS* to exon 2 *CHOP*. One of these cases was reported previously (3).

LR DNA-PCR Results. Because the distribution of transcript types in the present study differed from some previous smaller studies (13, 22, 23) in that the proportion of type 5-2 cases was higher, we sought to independently confirm the *TLS-CHOP* fusion type by LR DNA PCR. Among the 61 cases studied by LR DNA PCR, an amplified product in the expected size range was identified in 9 of 9 (100%) type 7-2 (Fig. 3), 6 of 7 (85%) type 8-2, and 28 of 45 (62%) type 5-2 cases tested (Fig. 4), using appropriate primer pairs, as described in "Patients and Methods." In the 28 positive type 5-2 cases, the *TLS-CHOP* genomic junction fragment was amplified in 8 cases, and the reciprocal *CHOP-TLS* fragment was amplified in 20 cases (Fig. 4). Because of case-to-case variation in both genomic junction fragment size and DNA quality, the occurrence of some negative cases with LR DNA PCR assays is not unexpected. As stated in "Patients and Methods," all cases were previously confirmed to harbor *CHOP* rearrangement by Southern blotting or by conventional cytogenetic demonstration of a t(12;16), including those negative by LR DNA PCR but positive by RT-PCR.

To exclude the possibility that some cases with *TLS* intron 7 or 8 breaks were resulting in type 5-2 transcripts through alternative splicing, we also studied 44 type 5-2 cases with the LR TLS7-CHOP2 primer set (used for types 7-2 and 8-2). These assays were all negative, arguing against the possibility of more downstream breaks with alternative splicing. Thus, taken to-

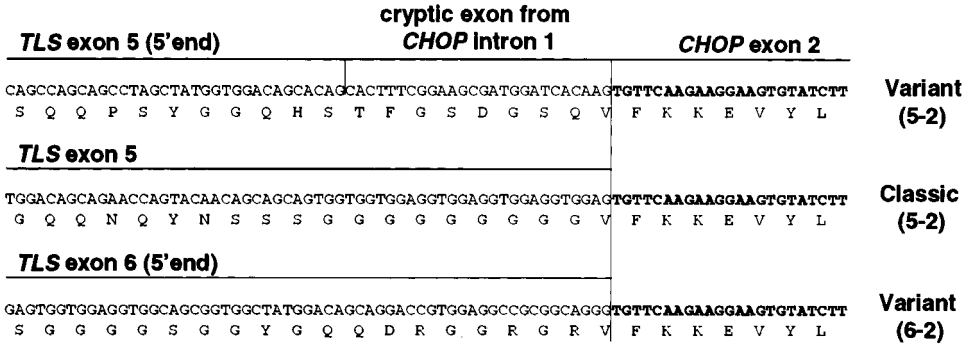

Fig. 2 Junction sequences of the most common type 5-2 fusion and the two novel (6-2) and (5-2 variant) *TLS-CHOP* fusion transcripts, with predicted amino acid sequences.

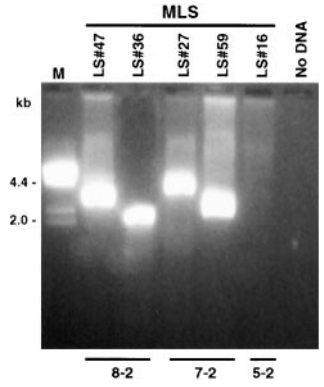

Fig. 3 Long-range DNA PCR for detection of types 7-2 and 8-2 *TLS-CHOP*, using TLS exon 7/CHOP exon 2 primers. *M*, size marker (PhiX174RF DNA/*Hae*III); *LS#47*, 36 type 8-2 *TLS-CHOP* cases; *LS#27*, 59 type 7-2 *TLS-CHOP* cases; *LS#16* type 5-2 *TLS-CHOP* case.

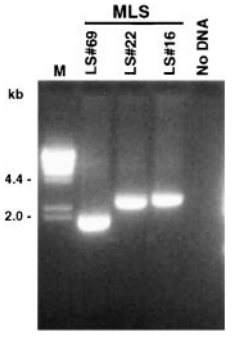

Fig. 4 LR DNA PCR for detection of type 5-2 *TLS-CHOP* genomic junction fragments, using CHOP exon 1/TLS exon 6 primers. *M*, size marker (lambda DNA *Hin*dIII); *LS#69*, *LS#22*, and *LS#16*, type 5-2 cases.

gether, the LR DNA PCR results were entirely consistent with the RT-PCR results, thereby validating the fusion typing results.

P53 Immunohistochemical Results. From the 71 cases available for immunohistochemical analysis, 12 (17%) showed nuclear immunoreactivity for P53 in ≥10% of tumor cells. Except for one case, the nuclear reactivity was predominantly restricted to the RC component or the hypercellular areas (Fig. 5). Eight of the 29 (27%) high-grade MLS and 3 of 27 (11%) low-grade MLS with increased cellularity showed overexpression of P53. Only 1 of the 15 (7%) pure MLS cases studied showed P53 immunoreactivity.

Clinical Follow-Up. Clinical follow-up was available in 79 of 82 patients (96%). Median follow-up was 44 months (range, 3–288 months). Of the 79 patients with follow-up data, 22 (28%) developed local recurrence, 30 (38%) had distant metastases, and 20 (25%) patients died of disease. At last follow-up, 44 (56%) patients were alive with no evidence of disease; 15 patients (19%) were either alive or dead with disease; and 20 (25%) were dead of disease. The 5-year DSS in this group was 73%. Among the metastatic sites, an overrepresentation of unusual locations was noted, such as soft tissue (63%) and bone (37%), with only 33% spreading to the lung. Furthermore, in a significant number of patients, soft tissue and bone metastases occurred early in the disease course, before clinical dissemination or pulmonary metastases.

In the subgroup of patients presenting with localized disease at diagnosis and available follow-up data (70 cases), 19 (27%) had local recurrences and 21 (30%) distant metastases. Thirteen (19%) patients died of disease. In this group, 44 (63%) patients were alive with no evidence of disease, 13 (19%) were alive or dead with disease, and 13 (19%) were dead of disease at last follow-up. The 5-year LRFS was 73%, 5-year MDFS 66%, and 5-year DSS 80%. If the analysis was restricted to cases with histologically low-grade tumors, the 5-year rates were 86% MDFS and 83% DSS for the MLS cases with increased cellularity and 79% MDFS and 100% DSS for pure MLS.

Survival Analyses. By univariate analysis, high histological grade (defined as ≥5% RC) was a strong predictor of MDFS in the localized group ($P < 0.01$) and of DSS both in the entire ($P < 0.01$) and localized groups ($P = 0.01$; Fig. 6). By multivariate analysis, localized tumors with ≥5% RC were independently associated with a poorer DSS ($P = 0.02$). The second cutoff point used to define high histological grade (≥25% RC) did not reach statistical significance in any of the groups or survival functions tested. Also, the presence of increased cellularity in the cases with <5% RC did not reach statistical significance for DSS or MDFS, although a trend ($P = 0.08$) was identified in predicting DSS in the localized cases.

The presence of necrosis (≥5% of tumor mass) correlated with DSS in the entire group ($P < 0.001$ by univariate and $P <$

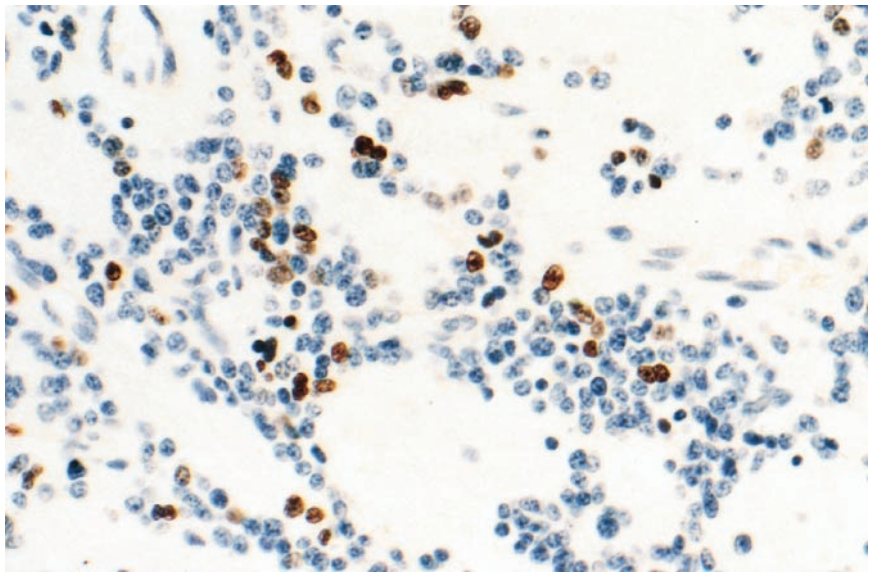

Fig. 5 P53 nuclear immunoreactivity in an area of increased cellularity of a low-grade MLS (LS#4).

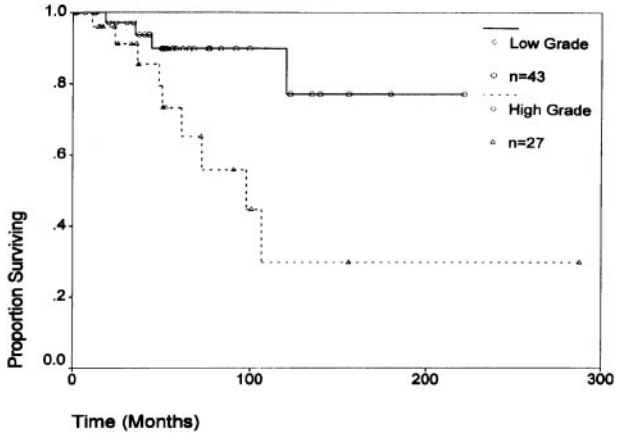

Fig. 6 Kaplan-Meier curve showing a correlation between high histological grade (≥5% RC) and disease-specific survival in a localized MLS group of patients ($P = 0.01$).

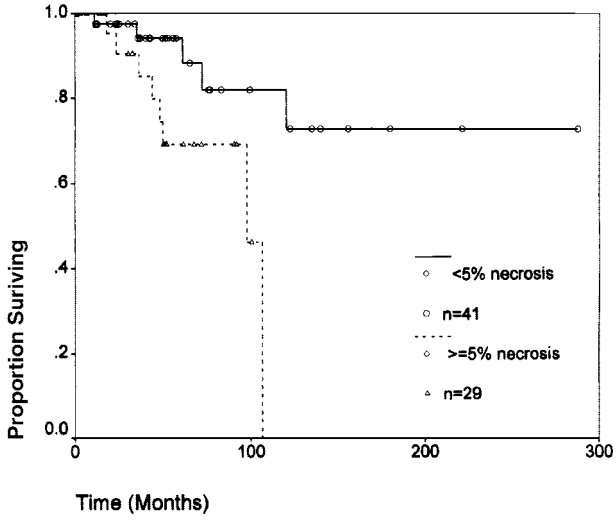

Fig. 7 Kaplan-Meier curve showing a statistically significant association between presence of tumor necrosis (≥5% of tumor mass) and DSS in the localized MLS patients ($P = 0.01$).

0.01 by multivariate analysis) and in the localized group ($P = 0.01$, by univariate analysis; Fig. 7). In addition, the presence of necrosis predicted MDFS in the patients with localized disease at presentation by univariate analysis ($P < 0.05$; Table 2). The tumor size was not found to predict clinical outcome in the present cohort, presumably because the majority of cases (73%) were >10 cm.

Of the two biological factors tested, *TLS-CHOP* fusion type and P53 status, only the latter showed an impact on survival. There was no significant association between *TLS-CHOP* fusion transcript type and histological grade or survival (MDFS, DSS). Overexpression of P53 (defined as nuclear staining in ≥10% of cells) was associated with an unfavorable outcome, predicting MDFS in localized tumors ($P < 0.001$ by univariate and $P = 0.02$ by multivariate analysis; Table 3 and Fig. 8) and DSS in the entire group ($P = 0.001$ by univariate and $P < 0.05$ by multivariate analysis; Table 3). Interestingly, there was an association between *TLS-CHOP* fusion transcript type and P53 status. The majority of P53-positive cases contained the type 5-2 *TLS-CHOP* fusion, and none of the type 7-2 or type 8-2 fusion cases overexpressed P53 protein ($P < 0.01$). Of 12 P53 positive cases, 11 had a type 5-2 *TLS-CHOP* fusion transcript and 1 case had a *EWS-CHOP* fusion.

The LRFS was significantly related to negative surgical margins ($P < 0.01$) and extremity location ($P = 0.02$) in the entire group, by multivariate analysis. The same adverse factors were found to be significant in the localized cases, both by univariate and multivariate analysis. By univariate analysis, the presence of increased cellularity (defined as ≥5%; $P < 0.01$) or tumor location other than thigh ($P = 0.05$) also correlated with

Table 2 Statistical correlation of adverse factors and MDFS in the localized MLS group

	Log-rank test	Cox regression[a]
>5% RC	$P < 0.01$	$P < 0.01$; RR, 3.47; CI, 2.17–5.56
Necrosis	$P < 0.05$	$P = 0.4$
P53 (+) cases	$P < 0.001$	$P = 0.02$; RR, 4.03; CI, 2.14–6.15
"5-2" *TLS-CHOP*	$P = 0.57$	

[a] RR, relative risk; CI, confidence interval.

a higher risk of local recurrence in the localized group of patients.

DISCUSSION

The most common *TLS-CHOP* fusion type detected in our study was type 5-2, followed by type 7-2 fusion, a finding in concordance with data from previous smaller series (13, 24, 25). However, in contrast to some studies (22, 26), we and Kuroda *et al.* (23) found a higher frequency of type 8-2 fusion (10%) and a very small number of fusion variants (1%). Two cases exhibited the *EWS-CHOP* transcript, bringing the number of reported cases to six (3, 8, 9).

The different combinations of *TLS* and *CHOP* genomic breakpoints produce in-frame transcripts because the splice junctions of exons 5, 7, and 8 of *TLS* and exon 2 of *CHOP* occur at the same codon nucleotide position. The two main types, fusion of *TLS* exon 5 to *CHOP* exon 2 (type 5-2) and fusion of *TLS* exon 7 to *CHOP* exon 2 (type 7-2), account for ~85% of *TLS-CHOP* fusions. In contrast to a study that found that 3 of 13 cases contained two transcripts (types 7-2 and 5-2), presumably as a result of alternative splicing (13), none of our 82 cases showed more than one in-frame transcript type, indicating that this phenomenon is uncommon in MLS.

This study represents the first large scale molecular analysis of the genetic heterogeneity of *TLS-CHOP* fusion transcript and its relationship to survival in MLS patients. In contrast to some other sarcomas characterized by specific translocations, such as Ewing sarcoma, synovial sarcoma, or rhabdomyosarcoma (27–30), our results indicate that the molecular variability of *TLS-CHOP* fusion transcripts in MLS does not appear to have a clear impact on clinical outcome. This would suggest that within *TLS-CHOP*, *TLS* exons 6–8 do not contribute an additional functional domain relevant to the biology of MLS. In the *EWS-FLI1* fusion, functional variability seems to be determined primarily by the inclusion of additional portions of FLI1, rather than of portions encoded by *EWS* exons 8–10 (27, 31). In terms of exon structure and domains encoded, *TLS* exons 6–8 correspond to exons 8–10 of *EWS* (32, 33), but there is actually only limited sequence similarity, either at the DNA or protein level (6, 33). This portion of TLS and EWS contains a domain of uncertain function, the RGG domain (defined by multiple copies of the sequence Arg-Gly-Gly) on the NH_2-terminal side of the RNA-binding domain.

In contrast to other liposarcoma variants or myxoid sarcomas of the extremities, MLS tends to metastasize to unusual soft tissue locations, such as the retroperitoneum, opposite extremity, and axilla, among others (2, 3, 34). In the present study, we found a high incidence of soft tissue metastases, 24% for the entire group and almost two-thirds of those with metastases. A significant number of these soft tissue implants were identified early in the disease course, before the manifestation of disease in sites where sarcomas usually metastasize, such as the lung. MLS is the most common type of soft tissue sarcoma presenting with either synchronous or metachronous multifocal disease (3, 34, 35). We have recently demonstrated the monoclonal origin of multifocal MLS by the analysis of *TLS-CHOP* and *EWS-CHOP* genomic rearrangements, establishing the metastatic nature of distant soft tissue lesions in these cases (3).

Histological grading in MLS is based on the extent of RC component. The transition from morphologically low-grade to higher grade areas (whether hypercellular or RC) can be either abrupt or, more often, gradual (36). Therefore, accurate assessment of histological grade in ML is not only biased by sampling error but also by difficulty in objective quantitation of the RC component, particularly in those cases with gradual transition to a RC component. The distinction between the "RC" phenotype and a hypercellular focus can be histopathologically challenging. According to some studies, the presence of any hypercellular areas seems to correlate with an increased risk of recurrence (16, 17, 37), whereas the presence of ≥25% RC shows strong statistical correlation with poor outcome (2). Furthermore, Evans (37) stressed that hypercellularity in recurrent lesions, rather than primary tumors, was that which correlated with poor outcome. He also noted that nearly one-half of MLS with <5% hypercellularity eventually metastasized. Azumi *et al.* (38) noted that no case developed metastasis unless it showed hypercellular areas either in the primary or recurrent tumor. In our study, similar to the study by Smith *et al.* (17), the presence of ≥5% RC strongly correlated with an unfavorable outcome, by both univariate and multivariate analysis, whereas the presence of increased cellularity in low-grade tumors showed only a tendency to predict DSS in localized cases. Our findings indicate that the 5% cutoff for the presence of a RC component is statistically more powerful than 25%, suggesting that it also may be more biologically meaningful. This is best reflected by the high 5-year disease-specific survival rates, 100% for the pure MLS cases and 83% for the low-grade MLS with increased cellularity. Similar to the study by Killpatrick *et al.* (2), our findings also suggest that the presence of tumor necrosis correlates with a more aggressive behavior.

We and others have confirmed the strong specificity of *TLS-CHOP* for MLS in a large number of myxoid sarcomas (19, 39). Because of overlapping morphological features with other myxoid neoplasms, the differential diagnosis of MLS can be sometimes quite challenging. Myxofibrosarcoma in the extremities and WDLS with extensive myxoid change in the retroperitoneum are the most common diagnostic pitfalls (40). Detection of *TLS-CHOP* (or *EWS-CHOP*) fusion transcripts can serve as a useful diagnostic adjunct in such cases. The availability of this large series of MLS with confirmatory translocation data allows us to further comment on two clinicopathological issues: putative mixed or dedifferentiated forms of MLS, and the occurrence of MLS in the pediatric age group. There have been occasional reports of apparent mixed-type liposarcoma, with features of both WDLS and MLS, and a recent description of three cases of MLS with areas of dedifferentiation or a high-grade nonlipo-

Table 3 Statistical correlation of adverse factors and DSS in both the entire and localized MLS groups

	Log-rank test		Cox regression[a]	
	Localized group	Entire group	Localized group	Entire group
>5% RC	$P = 0.01$	$P < 0.01$	$P = 0.02$; RR, 4.10; CI, 2.24–7.51	$P = 0.1$
Necrosis	$P = 0.01$	$P < 0.001$	$P = 0.3$	$P = 0.05$; RR, 3.16; CI, 1.73–5.57
P53 (+) cases	$P = 0.02$	$P = 0.001$	$P = 0.09$	$P < 0.05$; RR, 3.23; CI, 1.92–5.95
"5-2" *TLS-CHOP*	$P = 0.93$	$P = 0.63$		

[a] RR, relative risk; CI, confidence interval.

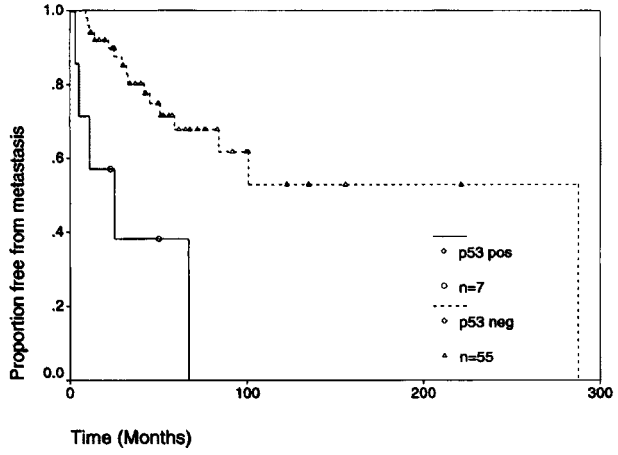

Fig. 8 Kaplan-Meier curve showing a significantly higher metastatic rate in P53-positive localized MLS cases ($P < 0.001$).

genic component, a phenomenon typically associated with WDLS (41–43). In the present study, no cases with *TLS-CHOP* rearrangement showed either areas of well-differentiated or dedifferentiated liposarcoma histologically, further supporting the concept that MLS and WDLS are distinct entities and suggesting that the aforementioned "mixed" types of liposarcoma might be attributable to incomplete histopathological and/or molecular analysis. MLS is a disease of younger adults, with the age at presentation (present series, 45.8 years) on average a decade lower than other histological subtypes of liposarcoma. In the pediatric age group (<18 years), MLS is an extremely rare occurrence (44, 45) and can be confused morphologically with the more common lipoblastoma. Few if any cases reported as pediatric MLS have been confirmed to contain the *TLS-CHOP* fusion. In our study, a single case of MLS occurred in this age group. This was a 13-year-old boy presenting with a histologically low-grade MLS of the thigh, which showed a type 5-2 fusion.

Few prior studies have analyzed the incidence of P53 alterations in the myxoid and round cell forms of MLS (46–48), and they did not examine associations with survival. In these studies, the P53 overexpression varied from <5% (46) up to 30% of cases (47). In the present study, we found that overexpression of P53, typically associated with underlying missense mutation, although present in only a minority of tumors (17%), was associated with poor outcome. In the study by Smith and Goldblum (48), 7% of MLS cases analyzed immunohistochemically were positive for P53 and were present only in high-grade tumors (≥5% RC) and localized within the RC component. These findings are in concordance with our results, in which the majority of P53-positive tumors were seen in high-grade tumors and the nuclear immunoreactivity present in the RC to hypercellular areas. P53 alterations have a similar strong negative impact on survival in at least two other translocation-associated sarcomas, Ewing's sarcoma (14) and synovial sarcoma (15). We also observed an association between the type 5-2 fusion transcript and P53 overexpression, suggesting a nonrandom relationship between fusion transcript type and P53 mutation. This needs to be confirmed in future studies.

In summary, we find that high histological grade (≥5% RC), presence of necrosis, and P53 overexpression are independent predictors of unfavorable outcome in localized MLS.

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