Analysis of *CHOP* rearrangement in pleomorphic liposarcomas using fluorescence *in situ* hybridization

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Pleomorphic liposarcoma (PLS) is an aggressive subtype of liposarcoma composed of high-grade sarcoma with pleomorphic lipoblasts. PLS usually exhibits a heterogeneous histology and sometimes has a myxoid or round cell area similar to myxoid/round cell liposarcomas (MLS/RCs). Using fluorescence *in situ* hybridization (FISH) analysis, we investigated the existence of *CHOP* split signals in various histological areas of PLS including the MLS/RC-like feature and also estimated the distribution of various signals with polyploidy and amplification. Moreover, to detect *CHOP* fusion transcripts we performed nested reverse transcription-polymerase chain reaction (RT-PCR). Seven PLSs and three MLS/RCs were selected for FISH analysis using the locus-specific indicator *CHOP* (12q13) dual color, break apart probe (Vysis, USA). The FISH analysis was applied to formalin-fixed, paraffin-embedded tissue sections of representative areas in all cases. Six of seven PLS cases showed the *CHOP* split signal ranging from 0.5% to 3% of counted nuclei, while all cases of MLS/RC exhibited *CHOP* rearrangement in more than 50% of counted nuclei. All cases of PLS showed a varied distribution of extra signals with polyploidy and amplification in each histological area. No *CHOP* fusion transcript was found in any case of PLS by nested RT-PCR. A *CHOP* rearrangement in PLS should be recognized only as a representative part of complex karyotypes, because the number of cells with split signals was minute compared with that of MLS/RC, and the signals were found in any area despite their histological differences. The cytogenetic background of PLS and that of MLS/RC are obviously different despite histological similarity. (*Cancer Sci* 2009; 100: 82–87)

Pleomorphic liposarcoma (PLS) is a rare aggressive subtype of liposarcoma, and is characterized by a varying number of pleomorphic lipoblasts in a background of high-grade sarcoma. PLSs are often composed of pleomorphic cells, fascicles of spindle cells, and epithelioid cells admixed with multinucleated giant cells.[1,2] In some cases of PLS, a myxoid or small round cell area similar to the myxoid/round cell liposarcoma (MLS/RC) is observed with various numbers of pleomorphic lipoblasts. In such a situation, we sometimes experience diagnostic difficulty in distinguishing PLS from MLS/RC. Cytogenetically, complicated abnormal karyotypes have been reported in many cases of PLS.[3–6] Recently *FUS-CHOP* fusion transcripts specific for MLS/RC were detected in some cases of PLS using the reverse transcription-polymerase chain reaction (RT-PCR) method.[7,8] In the present fluorescence *in situ* hybridization (FISH) analysis study, we thus investigated the *CHOP* rearrangement in morphologically different areas of PLS including an MLS/RC-like myxoid or round cell area on histological sections.

Material and Method

Sample selection. The archival pathological files of the Laboratory of Pathology, National Cancer Center Hospital, Tokyo, Japan were searched for patients with a diagnosis of PLS. Seven cases of PLS were selected for the study, consisting of primary tumors in four cases, and recurrent tumors in three. Three cases of MLS/RC were also chosen as the positive control group with *CHOP* slit signals. In addition, 13 non-PLS tumors (4 malignant peripheral nerve sheath tumors, 3 synovial sarcomas, 3 myxofibrosarcomas, and 3 leiomyosarcomas) were selected as the negative control.

Pathological evaluation. We reviewed all hematoxylin and eosin (HE) sections containing whole parts of the maximum dimension in each tumor and reclassified the histological heterogeneity of the tumor areas into four patterns including the typical PLS area, myxoid area, round cell area, and non-lipogenic sarcoma area. The typical PLS area consisted of a high-grade sarcoma with a varing amount of pleomorphic lipoblasts (Fig. 1a,b). The myxoid area showed round to spindle cell sarcoma with abundant myxomatous background similar to MLS/RC or myxofibrosarcoma (Fig. 1c). The round cell area exhibited a proliferation of monotonous round cells which resembled round cell liposarcoma (Fig. 1d). The non-lipogenic sarcoma area was composed of high-grade spindle to pleomorphic sarcoma without an apparent lipogenic differentiation (Fig. 1e).

Fluorescence *in situ* hybridization. The most representative sections of seven PLSs and three MLS/RCs were examined with the FISH assay. The *CHOP* FISH studies were performed using formalin-fixed, paraffin-embedded specimens sectioned into 4 μm-thick tissue slices. Briefly, after dewaxing and dehydration, the sections were immersed in 0.2 N HCl for 20 min followed by a pretreatment solution (Abbott Molecular International, 50 mL) at 80°C for 30 min. After digestion in protease for 60 min at 37°C, the sections were washed in phosphate-buffered saline for 5 min at room temperature, fixed in 10% formaldehyde for 10 min at room temperature, washed in phosphate-buffered saline for 5 min at room temperature, and placed into a prewarmed solution (Vysis) for 5 min at 72°C. They were then dehydrated in an ethanol series (70, 85 and 100%) at room temperature for 1 min each and air-dried. Ten microliters of

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Abbreviations: PLS, pleomorphic liposarcoma; MLS/RC, myxoid/round cell liposarcoma; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcription-polymerase chain reaction.

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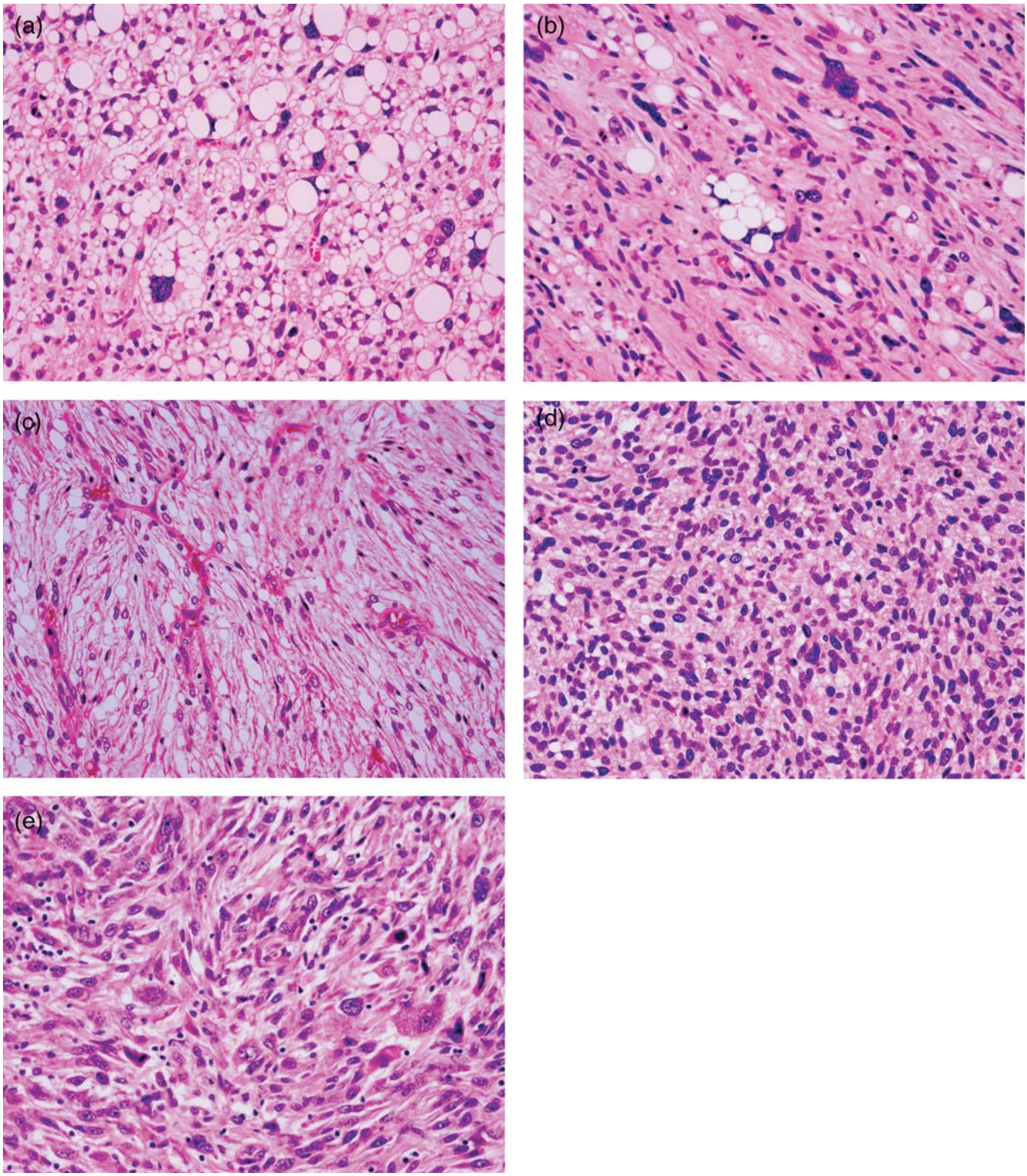

Fig. 1. Various histological areas of pleomorphic liposarcoma (PLS). (a, b) Typical PLS area with high-grade sarcoma and scattered pleomorphic lipoblasts (original magnification ×200). (c) Myxoid area consisting of short spindle and occasional pleomorphic cells with an abundant myxoid material (original magnification ×200). (d) Round cell area characteristic of monotonous round tumor cells without lipoblasts (original magnification ×200). (e) Spindle to pleomorphic sarcoma area without apparent lipogenic differentiation (original magnification ×200).

solution containing a locus-specific indicator (LSI) *CHOP* (12q13) dual-color, break-apart rearrangement probe (Vysis) was added to the sample area of the slides at 45°C. According to the manufacturer's instructions, the probe was composed of a mixture of two FISH DNA probes. The first probe was a 700-kb probe labeled in spectrum orange at the proximal site of the *CHOP* gene. The second probe was an approximately 660 kb probe labeled with spectrum green at the distal site of the *CHOP* gene.

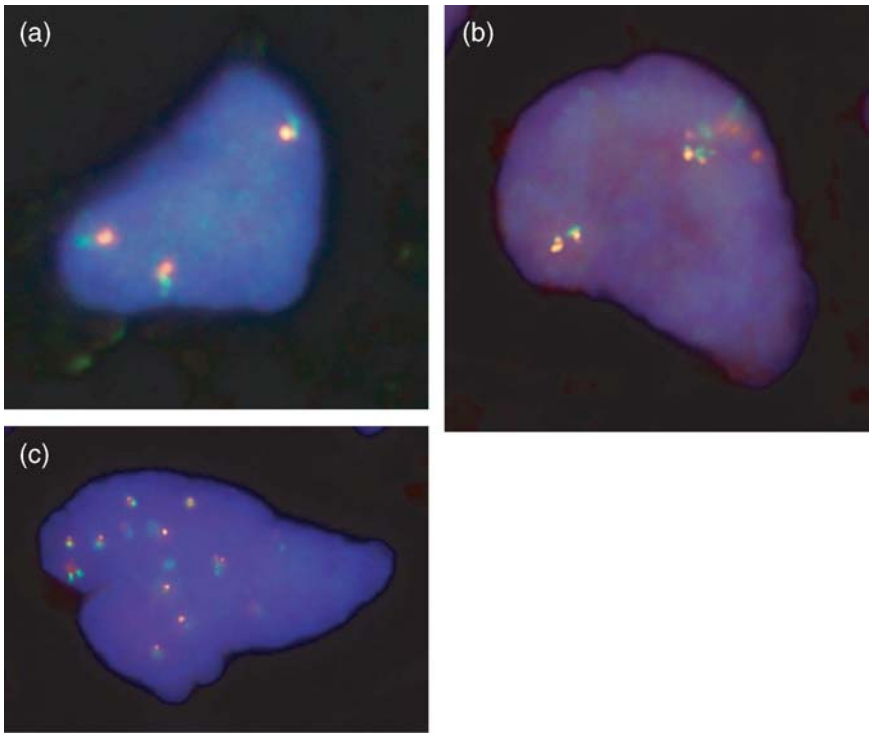

Fig. 2. Pleomorphic liposarcoma analyzed by fluorescence *in situ* hybridization using CHOP probes. (a) Polyploidy pattern with three fusion signals (original magnification ×100). (b) Amplification pattern with differently increased number of orange and green signals (original magnification ×100). (c) Amplification pattern with numerously increased numbers of orange and green signals (original magnification ×100).

We counted 200 nuclei that showed at least two pairs of green and orange signals and the percentage of the split signals was calculated. We defined the signals as being split when the distance between the orange and green signals was at least two times the estimated signal diameter. We also estimated some types of extra signals which represented the polyploidy and amplification pattern. The polyploidy pattern demonstrated an increased number of fusion signals (Fig. 2a). The amplification pattern showed numerously or differently increased numbers of orange and green signals (Fig. 2b,c). A normal signal with two pairs of orange and green signals was also examined. We excluded any nuclei which had widespread defects of the nuclear areas, showed a crowded nuclear appearance, or exhibited an obscure nuclear contour.

RNA extraction. RNA was extracted from five 4 μm-thick formalin-fixed, paraffin-embedded sections sliced from each representative paraffin block in all cases of PLS. In brief, deparaffinized tissue sections were minced in 200 μL of lysis buffer (20 mM Tris-HCl, pH 8.0; 20 mM ethylenediaminetetraacetic acid [EDTA]; and 2% sodium dodecyl sulfate) and incubated with proteinase K (100 mg/mL) overnight at 55°C. After incubation, 1.5 mL of Trizol reagent (Gibco BRL, Gaithersburg, MD, USA) was added to the sample, and total RNA was extracted according to the manufacturer's instructions.

RT-PCR for *TLS/FUS-CHOP* and *EWS-CHOP*. The extracted RNA was promptly reverse-transcribed into cDNA using 200 units of reverse transcriptase (SuperScript II, Gibco BRL) and 1 μL of random primers (Gibro BRL). A nested PCR was performed using the outer and inner primer sets designed specifically to amplify any of the junctional regions of three variant messages of the *TLS/FUS-CHOP* fusion transcript reported previously.[9,10] A nested PCR of *EWS-CHOP* was carried out using the same outer/inner reverse primer of *CHOP* side, and 22.3 (5′-TCCTACAGCCAAGCTCCAAGTC) and 22.3N (5′-CCAACAGAGCAGCAGCTACG) were used for the *EWS* side primers which had a complementary sequence of exon 7 of the *EWS* messages. The PCR profile of the first-round PCR consisted of 40 cycles of denaturation at 94°C for 40 s, annealing at 61°C (60°C in analysis of *EWS-CHOP*) for 45 s, and extension at 72°C for 1 min. The second-round PCR was performed using 1 μL of aliquot of the first PCR product as a template with the following cycling conditions: denaturation at 94°C for 40 s, annealing at 65°C for 45 s, and extension at 72°C for 1 min. After 40 cycles, an aliquot of the second PCR product was electrophoresed on a 2% agarose gel and stained with ethidium bromide. As positive controls for the integrity of mRNA isolated from each sample PCR, amplification of ubiquitously expressed *protein kinase G* (*PKG*) and *porphobilinogen deaminase* (*PBGD*) genes were examined.

Results

The clinicopathological features and results of FISH analysis are summarized in Table 1. Six cases of PLS showed a typical PLS area with scattered pleomorphic lipoblasts. Case 5 was entirely composed of non-lipogenic pleomorphic cells although the primary tumor had a small focus of lipogenic component with a few pleomorphic lipoblasts. Five cases of PLS varied in histology among the different intratumoral areas (cases 1, 2, 3, 6, 7). Four cases of PLS had myxoid or round cell areas (cases 1, 3, 6, 7). In the myxoid area, small round to spindle tumor cells without notable nuclear atypia proliferated in an abundant myxoid background. Monomorphous proliferation of small round to oval cells was observed in round cell areas. Two cases of PLS had a pleomorphic sarcoma area (cases 2, 5).

FISH analysis revealed a split signal of CHOP rearrangement in various areas of PLS which was not limited to the MLS/RC-like myxoid areas (Fig. 3). The incidence of cells with a CHOP split signal ranged from 0.5% to 3% among PLSs. Split signals were often observed in all MLSs, and the percentage of split signals in each case was 60% (30/50), 58% (18/31), and 55% (11/20). All PLSs showed various distributions of normal and extra signals. In all cases of PLS, about half of the tumor cells exhibited various extra signals showing polyploidy and amplification pattern in addition to normal signals. No CHOP split signal was detected in any case of 13 non-PLS tumors (4 malignant

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Table 1. Clinicopathological summary and results of FISH analysis in pleomorphic liposarcoma tissues

Case	Age (years)	Sex	Site	Histological area	Percentage of individual signal pattern (%)			
					Normal	Split	Polyploidy	Amplification
1	49	M	Arm	Typical PLS area	50.5	0	21.5	28
				Round cell area	49	0	33	18
2	54	M	Thigh	Typical PLS area	46.5	1.5	26.5	25.5
				Sarcoma area	21.5	0	52.5	26
3	49	F	Thigh	Typical PLS area	51.5	3	22	23.5
				Myxoid area	56	3	27.5	13.5
4	57	F	Thigh	Typical PLS area	53	0.5	37.5	9
5	52	M	Forearm	Sarcoma area	60.5	3	13.5	23
6	76	M	Thigh	Typical PLS area	42.5	1	18.5	38
				Myxoid area	39	0.5	22	38.5
7	65	F	Thigh	Typical PLS area	29.5	0	31.5	39
				Myxoid area	58	2	27.5	12.5

PLS, pleomorphic liposarcoma; FISH, fluorescence *in situ* hybridization.

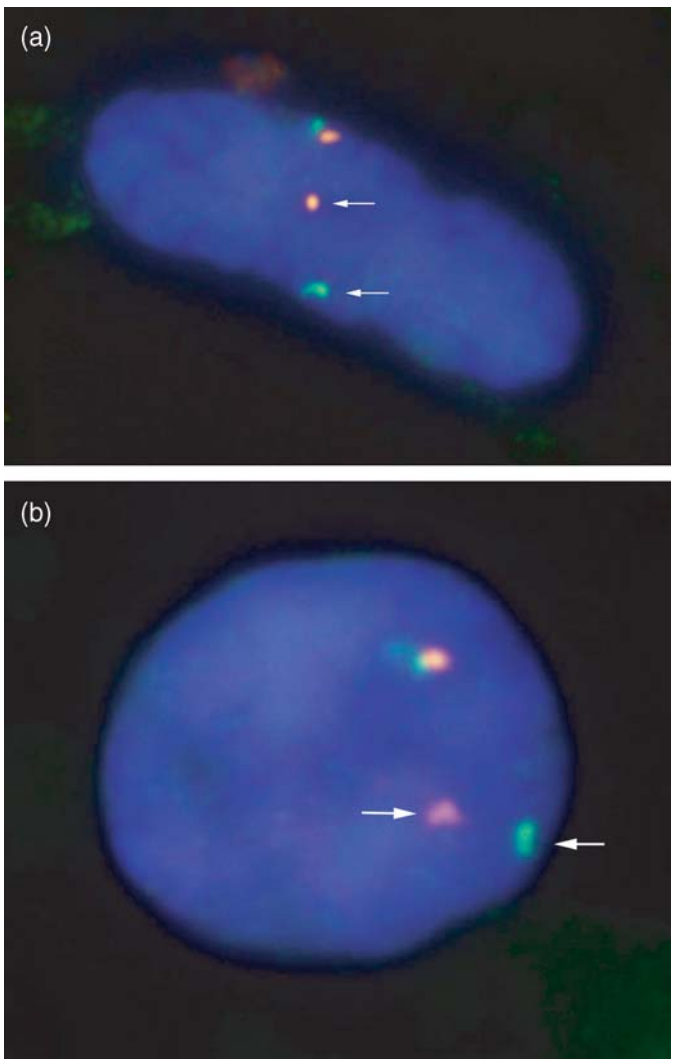

Fig. 3. (a) Split signals (arrows) in a typical pleomorphic liposarcoma area in case 2 (original magnification ×1000). (b) Split signals (arrows) in myxoid area in case 3 (original magnification ×1000).

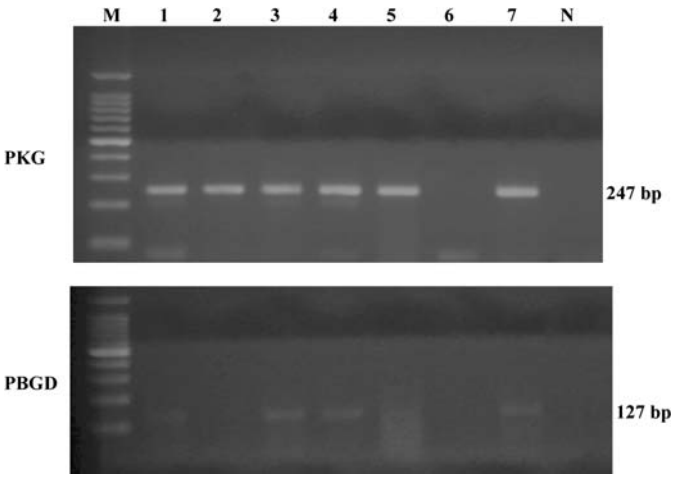

Fig. 4. Reverse transcription-polymerase chain reaction of housekeeping gene products of *PKG* and *PBGD* gene. These products were observed in six cases of pleomorphic liposarcoma (PLS) (lanes 1 to 7 correspond to cases 1 to 7). Four cases of PLS exhibited both of gene transcripts. PKG, protein kinase G; PBGD, porphobilinogen deaminase; M, 100-bp DNA ladder; N, negative control.

peripheral nerve sheath tumors, 3 synovial sarcomas, 3 myxofibrosarcomas, and 3 leiomyosarcomas).

In nested RT-PCR, at least one *PKG* or *PBGD* gene product was observed in six cases of PLS (excluding case 6), and four cases of PLS exhibited both gene transcripts (Fig. 4). Although the quality of nested RT-PCR was ensured by the confirmation of these housekeeping gene transcripts, no *TLS/FUS-CHOP* or *EWS-CHOP* fusion transcript was detected in any case of PLS (Fig. 5).

Discussion

Some types of soft tissue and bone tumors frequently express translocated chimeric genes specific for the individual histological subtypes and an identification of such genes has important implications for confirmative diagnosis. Both the RT-PCR and FISH methods have been used to detect chimeric genes, and the FISH method is advantageous because of its availability for use in routine formalin-fixed and paraffin-embedded sections. Recently, many kinds of FISH probes have become commercially available, although the interpretation of the cutoff value of the positive signal differs individually probe by probe.

Several studies using the same type of FISH probe have been reported, and a cutoff value for some FISH probes has been established. For example, in FISH analysis of Ewing's sarcoma/

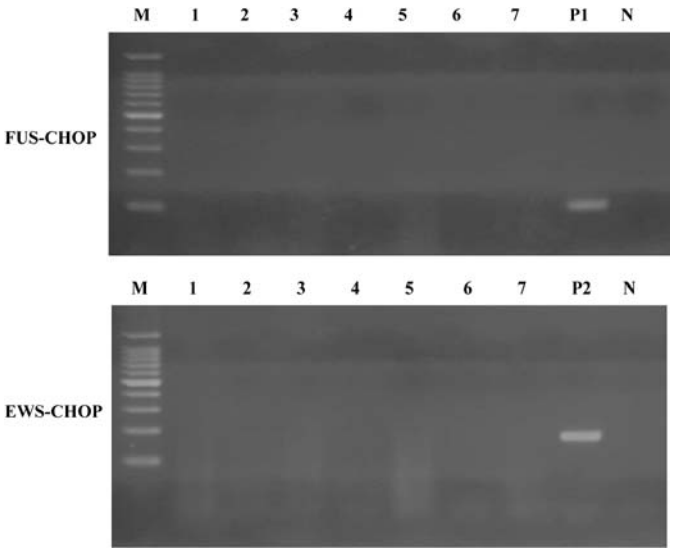

Fig. 5. Reverse transcription-polymerase chain reaction detection of transcripts of *TLS/FUS-CHOP* and *EWS-CHOP* fusion gene. No fusion gene transcript was detected in any case of pleomorphic liposarcoma (lanes 1 to 7 correspond to cases 1 to 7). M, 100-bp DNA ladder; P1 and P2, positive control; N, negative control.

primitive neuroectodermal tumor (EWS/PNET), some investigators defined the cutoff value of positive signal as at least 10% and 20% of counted tumor nuclei.[11,12] Yamaguchi *et al*. have reported that *EWS-FLI1* translocated signals were found in 50–90% of EWS/PNET tumor cells.[13] Recently, 100% of MLS/RC has shown *CHOP* gene rearrangement analyzed by the FISH method using the same probe as in our present study and the diagnostic utility of the *CHOP* rearrangement in the differential diagnosis of myxoid tumors mimicking MLS/RC has been established.[14] However, no study has been reported which has attempted to detect a *CHOP* rearrangement using FISH but not the RT-PCR method in other subtypes of liposarcoma, especially in pleomorphic liposarcomas. In the present study, because the number of split signals in PLS was extremely scarce compared with that of MLS/RC, we thought that the cases of PLS with a few split signals should not be recognized as a feature of MLS/RC.

In some types of soft tissue and bone tumor, a chimeric fusion gene has been revealed which was not specific for an inherent histology.[15,16] Scotlandi *et al*. using the nested RT-PCR method, reported that 13 of 15 cases of giant cell tumor of bone (GCT) exhibited *EWS-FLI1* fusion transcripts. They also performed FISH analysis in two cases of GCT. Both of the GCTs exhibited split signals of *EWS-FLI1* rearrangement and the frequency of the signal was less than 1% of tumor nuclei.[15] On the other hand, Panagopoulos *et al*. studied 10 cases of GCT, and they could not detect any *EWS-FLI1* fusion transcripts with the nested RT-PCR method.[17] They concluded that detection of *EWS-FLI1* fusion transcripts in GCT was due to technical artifacts and such a minute cell population with fusion transcripts did not have any influence on tumor development.

The commercial FISH probe of *CHOP* rearrangement has recently been available, although no study of *CHOP* rearrangement in PLS using FISH has been reported. There have been three reports on the investigation of *CHOP* rearrangement in PLS. Willeke *et al*. found *CHOP* fusion transcripts in 4 of 14 cases of PLS with the nested RT-PCR method.[7] De Cecco *et al*. also identified *FUS-CHOP* transcripts in an epithelioid variant case of pleomorphic liposarcoma using the nested PCR method.[8] On the other hand, Meis-Kindblom *et al*. detected no *CHOP* fusion transcript in five cases of PLS with the ordinary RT-PCR method.[6] FISH analysis was not performed in these studies. According to our results, a few cells with *CHOP* rearrangement certainly existed in some cases of PLS. However, we could not detect either *TLS/FUS-CHOP* or *EWS-CHOP* fusion transcripts in any case of PLS by nested-RT-PCR even in the presence of only a few cells with a *CHOP* rearrangement signal. Our FISH analysis also showed that the *CHOP* split signals were not observed in any case of non-PLS tumors. The frequency of cells with *CHOP* rearrangement in PLSs was significantly lower than that of MLS/RCs. Moreover, the split signals were detected not only in MLS/RC-like myxoid areas but also in variable histological areas. We therefore postulated that the *CHOP* rearrangement in PLS should be recognized only as a representative part of complex karyotypic features.

In some tumors, the criteria of cutoff value and morphological patterns of FISH signals have been examined in detail. In a study of Burkitt lymphoma, two types of FISH probe, the dual color, break apart type and the dual color, double fusion type, were used for FISH analysis. The authors explained various abnormal karyotypes of lymphoma cells by investigation of not only fused or split signals but also many patterns of background extra signals.[18] Matsumura *et al*. described various signal patterns including not only split signals but also patterns of polyploidy and amplification in the analysis of rhabdomyosarcoma.[19] Some reports of G-band analysis in PLSs have shown normal and variously abnormal karyotypes including aneuploidy, polyploidy, complex translocation, and also normal diploidy.[3–6] In the present study, all cases of PLS had many extra signals concerning polyploidy and amplification. We supposed that the various patterns of background signals corresponded with the complex abnormal karyotypes of PLS.

In summary, only a few *CHOP* split signals were clearly found in PLS, although the signal was not limited to MLS/RC-like areas. Moreover, a considerable variety of signals with polyploidy and amplification was observed in PLS. We conclude that the split signal associated with *CHOP* rearrangement may be recognized as one of the complex karyotypes of PLSs and the cytogenetic background of PLS and that of MLS/RC are obviously different despite histological similarity.

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