Cancer-testis antigens PRAME and NY-ESO-1 correlate with tumour grade and poor prognosis in myxoid liposarcoma

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Abstract

Myxoid liposarcoma is the second most common liposarcoma. Although myxoid liposarcoma is relatively chemosensitive and thus a good candidate for chemotherapy, cases with relapsed or metastatic disease still have poor outcome. Here, we performed a gene microarray analysis to compare the gene expression profiles in six clinical myxoid liposarcoma samples and three normal adipose tissue samples, and to identify molecular biomarkers that would be useful as diagnostic markers or treatment targets in myxoid liposarcoma. This showed that the cancer-testis antigen PRAME was up-regulated in myxoid liposarcoma. We then performed immunohistochemical, western blotting and real-time polymerase chain reaction analyses to quantify the expression of PRAME and another cancer-testis antigen, NY-ESO-1, in clinical samples of myxoid liposarcoma (n = 93), dedifferentiated (n = 46), well-differentiated (n = 32) and pleomorphic liposarcomas (n = 14). Immunohistochemically, positivity for PRAME and NY-ESO-1 was observed in 84/93 (90%) and 83/93 (89%) of the myxoid liposarcomas, and in 20/46 (43%) and 3/46 (7%) of the dedifferentiated, 3/32 (9%) and 1/32 (3%) of the well-differentiated and 7/14 (50%) and 3/21 (21%) of the pleomorphic liposarcomas, respectively. High immunohistochemical expression of PRAME and/or NY-ESO-1 was significantly correlated with tumour diameter, the existence of tumour necrosis, a round-cell component of >5%, higher histological grade and advanced clinical stage. High PRAME and NY-ESO-1 expression correlated significantly with poor prognosis in a univariate analysis. The myxoid liposarcomas showed significantly higher protein and mRNA expression levels of PRAME and NY-ESO-1 (CTAG1B) than the other liposarcomas. In conclusion, PRAME and NY-ESO-1 (CTAG1B) were expressed in the vast majority of myxoid liposarcomas, and their high-level expression correlated with tumour grade and poor prognosis. Our results support the potential use of PRAME and NY-ESO-1 as ancillary parameters for differential diagnosis and as prognostic biomarkers, and indicate that the development of immunotherapy against these cancer-testis antigens in myxoid liposarcoma would be warranted.

Keywords: PRAME; NY-ESO-1; myxoid liposarcoma; cancer-testis antigen; liposarcoma; cDNA microarray

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Raw data from the microarray analysis are available on the website of the Gene Expression Omnibus (accession no. GSE59568, http://www.ncbi.nlm.nih.gov/geo/).

Introduction

Liposarcoma is the most common type of soft tissue sarcoma, accounting for at least 20% of adult sarcomas [1]. Based on morphological features and cytogenetic aberrations, liposarcomas are classified into four types: (1) well-differentiated liposarcomas (WLSs)/atypical lipomatous tumours, (2) dedifferentiated

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liposarcomas (DDLs), (3) myxoid liposarcomas (MLs) and (4) pleomorphic liposarcomas (PLs) [2].

MLs account for more than one-third of all liposarcomas and 10% of all adult soft tissue sarcomas. Histologically, MLs show a continuous spectrum of characteristics: at one end of the spectrum, the lesions show a loose proliferation of uniform round-to-oval cells and lipoblasts with abundant myxoid stroma, and at the other end of the spectrum they show poorly differentiated round-cell features [2]. Genetically, MLs are characterised by reciprocal chromosomal translocation, most frequently t(12;16) (q13;p11) (FUS-DDIT3) or less commonly t(12;22) (q13;q12) (EWSRI-DDIT3) [3].

The main treatment for localised ML is surgery with or without radiotherapy. Cases with tumour recurrence or metastasis have a poor prognosis. With respect to morphology, the poor prognostic factors that have been identified are: large tumour diameter (>10 cm), >5% round-cell component, tumour necrosis, high mitotic rate and high grade based on differentiation [4–7]. It has been reported that the overexpression of p53, insulin-like growth factor 2, insulin-like growth factor 1 receptor, the ret proto-oncogene and MIB-1 are prognostic biomarkers in MLs, but that they are not critical [8–10]. Further studies are needed to identify potential biomarkers both for prognosis and as therapeutic targets for MLs.

Functional genomics, which permits the simultaneous analysis of the expression of thousands of genes, advanced greatly with the development of DNA microarray systems in the mid-1990s [11,12]. The DNA microarray technique has been used to identify disease-associated biomarkers, create gene-based tumour classifications, distinguish tumour subclasses and predict outcomes and responses to chemotherapy [13].

In this study, we attempted to identify new prognostic markers and therapeutic targets for MLs by comparing the gene expression profiles in MLs and normal adipose tissue samples using a gene microarray analysis. Among the genes up-regulated in MLs compared with normal adipose tissue, we focused on PRAME (PRerentially expressed Antigen of MElanoma) because of its potential use as a target for immunotherapy. PRAME belongs to the family of cancer-testis antigens, which have been described as promising immunotherapy targets because of their low-level or absent expression in normal tissue (except testis, which has no human leukocyte antigen (HLA) class I expression) [14,15].

MLs was recently reported to show a high level of PRAME expression, and other recent studies demonstrated that another cancer-testis antigen, NY-ESO-1 [New York ESOphageal squamous cell carcinoma 1] (CTAG1B), which is also described as a promising immunotherapy target [16,17], was hyper-expressed in MLs [18–21]. Correlation of the expression of PRAME and NY-ESO-1 with tumour grade or poor prognosis has been reported in several cancers [22–32]. However, to our knowledge there have been no studies on the relationship between the expression of PRAME and NY-ESO-1 and clinicopathological parameters, or prognosis, in MLs. A study of the PRAME expression profile in other liposarcoma subtypes was performed using only real-time PCR for three DDLs and three Ws samples [21], and a study of the NY-ESO-1 expression profile in liposarcoma subtypes also used a small number of samples [20].

We, therefore, investigated the relationship between the expression of PRAME and NY-ESO-1 and clinicopathological parameters and disease prognosis in MLs, and studied the expression profiles among liposarcoma subtypes in a large number of clinical samples.

Materials and methods

Patients and tissue samples

We retrieved 93 paraffin-embedded primary ML specimens from the soft tissue tumour files registered at the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan between 1975 and 2013. Each tumour was reclassified by a bone and soft-tissue pathologist according to the most recent World Health Organization classification [2]. The samples had been obtained from open biopsy specimens or surgically resected tumours. Specimens obtained after radiotherapy or chemotherapy were excluded. We also immunohistochemically examined 92 samples of other liposarcoma subtypes: DDLs (n = 46), Ws (n = 32) and PLs (n = 14). The following numbers of frozen samples were also available for the respective tissue types, and were applied to the gene microarray analysis or real-time polymerase chain reaction (PCR) analysis: MLs (n = 20), DDLs (n = 16), Ws (n = 20) and PLs (n = 3). Finally, as controls for the gene microarray analysis, we also examined three samples of surrounding nontumourous adipose tissue that were obtained from patients with various types of sarcoma.

The histological grade of each specimen was evaluated according to the grading system of the French Federation of Cancer Centers [33]. For staging, the system described in the seventh edition of the American Joint Committee on Cancer (AJCC) manual was...
used [34]. The study was approved by the Ethics Committee of Kyushu University (No. 26-49) and conducted according to the Ethical Guidelines for Epidemiological Research enacted by the Japanese Government.

Cell lines

The human MLS cell lines 402-91, 1765-92 and 2645-94 [35,36] were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum plus penicillin and streptomycin.

RNA preparation

RNA was extracted from frozen samples using an miRNeasy Mini Kit (Qiagen, Tokyo) according to the manufacturer’s instructions. RNA was quantitated using a NanoDrop-ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). One microgram of RNA per sample was reverse transcribed using a QuantiTect Reverse Transcription kit (Qiagen).

Fusion gene analysis

The fusion gene analysis was performed as previously described [37] and was based on the original primers that specifically amplify the fusion gene transcripts of FUS-DDIT3 and EWSR1-DDIT3 (Supporting Information Table 1).

Gene microarray analysis

For the Oligo DNA microarray analysis, a 3D-Gene Human Oligo chip 25k (Toray Industries, Tokyo) with 25,370 distinct genes was used. For efficient hybridisation, this microarray has three dimensions and is constructed with a well as the space between the probes and cylinder-stems, with 70-mer oligonucleotide probes on the top. Total RNA was labelled with Cy5 using an Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems, Foster City, CA). The Cy5-labelled aRNA pools and hybridisation buffer were hybridised for 16 h. The hybridisation was performed using the supplier’s protocols (www.3d-gene.com).

Hybridisation signals were scanned using a Scanning Array Express Scanner (PerkinElmer) and processed by GenePixPro version 5.0 (Molecular Devices, Sunnyvale, CA). The raw data for each spot were normalised by subtraction from the mean intensity of the background signal, which was determined by the 95% confidence intervals of the signal intensities of all of the blank spots. The detected signals for each gene were normalised by the global normalisation method (the median of the detected signal intensity was adjusted to 25).

Immunohistochemical staining and evaluation

Immunohistochemical staining was performed as previously described [38]. Antigen retrieval was performed using a pressure boiler with 10 mm sodium citrate (pH 6.0). The rabbit polyclonal antibody PRAME (PReferentially expressed Antigen of MElanoma) [Sigma-Aldrich, St. Louis, used [34]. The study was approved by the Ethics Committee of Kyushu University (No. 26-49) and conducted according to the Ethical Guidelines for Epidemiological Research enacted by the Japanese Government.

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MO] (1:300) and the mouse monoclonal antibody NY-ESO-1 (New York ESOphageal squamous cell carcinoma 1) [E-978; Santa Cruz Biotechnology, Santa Cruz, CA] (1:100) were used as the primary antibodies. The immunohistochemical results were judged by three investigators who were blinded to the clinical status of the patients. A consensus judgment was adopted as the proper immunohistochemical result.

The percentage of immunoreactive cells and staining intensity were evaluated in the most representative areas. The proportion of immunoreactive cells was scored from 0 to 4 as follows: 0, <5%; 1, 5% to <25%; 2, 25% to <50%; 3, 50% to <75%; 4, ≥75%. The intensity was scored from 0 to 3 as follows: 0, negative; 1, weak staining; 2 moderate staining; 3, strong staining. The total score (proportion score + intensity score) were evaluated, and cases with a total score >3 were judged as positive.

Western blotting
We conducted a western blot analysis as previously described [37,39] using rabbit polyclonal antibody for PRAME [Sigma-Aldrich] (1:10,000), mouse monoclonal antibody for NY-ESO-1 (E978; Santa Cruz Biotechnology; 1:250) and mouse monoclonal antibody for β-actin (1:10,000) [C4; Millipore, Bedford, MA]. We also analysed five corresponding normal tissues surrounding the tumour. The densitometric score of each sample was normalised by β-actin, and was further normalised to the expression of the testis tissue in each membrane.

Quantitative real-time reverse transcriptase-polymerase chain reaction
Quantitative real-time reverse-transcriptase PCR (RT-PCR) was performed using TaqMan probes on an ABI Prism 7500 sequence detection system (Applied Biosystems) following the manufacturer’s protocol. The TaqMan probes for PRAME (Hs01022301_m1), CTAG1B (cancer/testis antigen 1B) (Hs00265824_m1), which encodes for NY-ESO-1, and GAPDH (Hs99999905_m1) were purchased from Applied Biosystems. The final numerical value (v) in each sample was calculated as follows: v = tumour (PRAME or CTAG1B mRNA value/GAPDH mRNA value)/testis (PRAME or CTAG1B mRNA value/GAPDH mRNA value).

Statistical analysis
Continuous variables are presented as the means ± standard deviation. The chi-square test, Fisher’s exact test and the Mann–Whitney U-test were used as appropriate to evaluate the association between two variables. The Steel-Dwass multiple comparison test was applied to compare the data of the four groups. The survival correlations are illustrated with Kaplan–Meier curves, and survival analyses were performed using the log-rank test. We conducted a Cox proportional hazards regression analysis to estimate the hazard ratios for positive risk factors for death. A two-sided p-value of <0.05 was considered significant. The data analysis was conducted with the JMP statistical software package (version 9.0.2; SAS Institute, Cary, NC).

Results
Gene microarray analysis
Table 1 provides the results of the comparison of the expression profile of six clinical samples of MLSs and three normal adipose tissue samples, in which we identified 22 genes that were consistently up-regulated by more than 20-fold in the MLS compared to the normal adipose tissue. Raw data from the
microarray analysis are available on the website of the Gene Expression Omnibus (accession no. GES59568, http://www.ncbi.nlm.nih.gov/geo/).

Among the up-regulated genes, we focused on the cancer-testis antigen PRAME. MLS samples were recently reported to show a high level of PRAME expression, and other recent studies demonstrated that another cancer-testis antigen, NY-ESO-1 (CTAG1B), was hyper-expressed in MLS [18–21]. In our microarray results, 111 cancer-testis antigens were included, and eight cancer-testis antigens were up-regulated by more than fourfold compared to their levels in normal adipose tissue (Table 2). NY-ESO-1 (CTAG1B) was not included in the gene analysis.

Patient and clinicopathological parameters

The clinicopathological data and the results of the survival analysis for the 93 primary MLS tumours are summarised in Table 3, and representative H&E staining results are shown in Figure 1a,b. More than 75% of the tumours were located in an extremity, and a lower extremity was more likely to be affected than an upper extremity (68 versus 6 cases). Follow-up information was available for 79/93 patients (85%) with a follow-up duration of 77.5 months (range 9–324 months).

Fourteen tumours (15%) contained more than a 5% round-cell component. The prognostic risk factors identified for overall survival and disease-free survival were male gender, tumour diameter (>10 cm), existence of tumour necrosis, higher FNCLCC grade and higher AJCC stage. Round-cell component >5% was a poor prognostic risk factor for disease-free survival.

Fusion gene transcript findings

Among the 20 patients for whom frozen samples were available, 19 patients showed 18 FUS-DDIT3 and 1 EWSR1-DDIT3 fusion-type genes, respectively. In the remaining patient, these fusion genes were not
detectable. Among the 73 patients for whom only formalin-fixed paraffin-embedded materials were available, large quantities of high-quality total RNA suitable for an RT-PCR analysis could be obtained in 13 patients. A fusion-type gene was detected in all 13 of these patients; 12 patients showed the FUS-DDIT3 fusion, and the other patient showed EWSR1-DDIT3.

Immunohistochemistry in MLS and other liposarcomas

The results of our immunohistochemical analysis of MLSs and other liposarcomas are summarised in Tables 4 and 5. Expression of PRAME and NY-ESO-1 was observed in 84 (90%) and 83 (89%) of the 93 MLS cases, respectively. PRAME was mainly localised in the nuclei, whereas NY-ESO-1 was localised in the cytoplasm and nuclei (Figure 1c–h). In the other liposarcomas, the numbers of cases positive for PRAME and NY-ESO-1 were 20 (43%) and 3 (7%) among the 46 DDLS, 3 (9%) and 1 (3%) among the 32 WLS and 7 (50%) and 3 (21%) among the 21 PLS (Figure 2, Table 5).

The expression levels of both PRAME and NY-ESO-1 were significantly higher in MLS than in the other liposarcoma subtypes (p < 0.001; Figure 3a,b), and PRAME expression was significantly higher in the DDLS and PLS than in the WLS by multiple comparison (p < 0.05). In addition, in the DDLS, the dedifferentiated component showed significantly higher PRAME expression compared to the well-differentiated component (p = 0.0035; Figure 3c). Although NY-ESO-1 expression in the dedifferentiated component was not significantly higher than that in the well-differentiated component in all DDLS cases (p = 0.837; Figure 3d), in the 3 NY-ESO-1-positive DDLS cases, NY-ESO-1 expression was increased in the dedifferentiated component.

Table 4. Immunohistochemical results in myxoid liposarcoma

| Percentage of tumour positivity (%) | PRAME | NY-ESO-1 |
|-----------------------------------|-------|----------|
| ≥75                               | 20/93 (21%) | 17/93 (18%) |
| 50 to <75                         | 36/93 (39%) | 40/93 (43%) |
| 25 to <50                         | 24/93 (26%) | 22/93 (24%) |
| 5 to <25                          | 10/93 (11%) | 5/93 (5%) |
| 0 to <5                           | 3/93 (3%) | 9/93 (10%) |

| Staining intensity | PRAME | NY-ESO-1 |
|--------------------|-------|----------|
| 3                  | 19/93 (21%) | 36/93 (39%) |
| 2                  | 45/93 (48%) | 36/93 (39%) |
| 1                  | 27/93 (29%) | 13/93 (14%) |
| 0                  | 2/93 (2%) | 8/93 (8%) |

The table shows percentage of tumour positivity and staining intensity of PRAME and NY-ESO-1 in MLS.

Table 5. Immunohistochemical results in liposarcomas

| Subtype               | Positive cases (percentage of total) |
|-----------------------|--------------------------------------|
|                       | PRAME  | NY-ESO-1 |
| Myxoid                | 84/93 (90%) | 83/93 (89%) |
| Dedifferentiated      | 20/46 (43%) | 3/46 (7%) |
| Well-differentiated   | 3/32 (9%) | 1/32 (3%) |
| Pleomorphic           | 7/14 (50%) | 3/14 (21%) |

Positive rate for PRAME and NY-ESO-1 in liposarcoma subtypes.
compared to the well-differentiated component (Figures 2d and 3e). No significant difference in PRAME or NY-ESO-1 expression was observed between WLS and the well-differentiated component of DDLS by immunohistochemistry (data not shown). In addition, PRAME expression was significantly associated with coexpression of NY-ESO-1 in the MLS samples ($p < 0.0001$ by Fisher’s exact test; Figure 3f).

Immunohistochemistry and clinicopathological parameters

We determined the correlations between the immunohistochemical results and the clinicopathological parameters, the overall survival and the disease-free survival in MLS. High expression of PRAME and NY-ESO-1 correlated with the existence of tumour necrosis, round-cell component $>5\%$, higher
FNCLCC grade and higher AJCC stage. Moreover, the tumour diameter (\(>10\) cm) correlated with high PRAME expression (Table 6). High PRAME expression was a significant risk factor for adverse prognosis (overall survival and event-free survival; \(p<0.05\); Figure 4a,b). High NY-ESO-1 expression was also a

Figure 3. Immunohistochemical results for the liposarcoma subtypes and the coexpression of PRAME and NY-ESO-1 in MLSs. (a,b) PRAME (a) and NY-ESO-1 (b) expression by immunohistochemistry among the liposarcoma subtypes. The MLSs showed significantly higher PRAME and NY-ESO-1 expression compared to the other liposarcoma subtypes. (c,d) Different expression levels of PRAME (c) and NY-ESO-1 (d) between the dedifferentiated component and the well-differentiated component in DDLS. PRAME expression was significantly higher in the dedifferentiated component \((p=0.0035)\). (e) In the three NY-ESO-1-positive DDLSs, PRAME expression was increased in the dedifferentiated component compared to the well-differentiated component. (f) Coexpression of PRAME and NY-ESO-1. Immunohistochemical expression of PRAME and NY-ESO-1 correlated significantly \((p<0.0001\) by chi-square test). IHC, immunohistochemistry; DDLS, dedifferentiated liposarcoma; MLS, myxoid liposarcoma; PLS, pleomorphic liposarcoma; WLS, welldifferentiated liposarcoma; Low, low expression by immunohistochemistry; High, high expression by immunohistochemistry.
significant risk factor for poor disease-free survival ($p = 0.0188$; Figure 4d).

Western blotting

The western blotting results are shown in Figure 5. PRAME was detected in 10 of the 19 tumour samples. Among the five cases available with non-neoplastic samples, PRAME was detected in four tumour samples, but in no normal tissue samples. The tumour samples showed significantly higher expression (mean, $0.748 \pm 0.304$) than the normal tissue samples (mean, $0.08 \pm 0.046$; $p = 0.0208$ by Mann–Whitney U-test; Figure 5d). The samples that showed high PRAME expression immunohistochemically showed significantly higher scores (mean, $1.18 \pm 0.26$) than the samples that showed low PRAME expression by immunohistochemistry (mean, $0.34 \pm 0.20$; $p = 0.0466$; Figure 5f).

NY-ESO-1 was detected in 18 of 19 tumour samples. In contrast, the normal samples showed no NY-ESO-1 expression. The tumour samples showed significantly higher expression (mean, $4.42 \pm 2.02$) than the normal tissue samples (0.0282 ± 0.0091; $p = 0.0117$; Figure 5e). Much as for PRAME, the samples that showed immunohistochemically high NY-ESO-1 expression showed higher scores (mean, $4.90 \pm 1.43$) than the samples that showed low NY-ESO-1 expression by immunohistochemistry (mean, $1.03 \pm 0.22$), but not significantly so ($p = 0.0801$; Figure 5g). In addition, the MLS cell lines 402-91, 2645-94 and 1765-92 showed varying degrees of PRAME and NY-ESO-1 expression (Figure 5c).

Quantitative RT-PCR and the corresponding immunohistochemistry

The quantitative RT-PCR results and the corresponding immunohistochemical results for all liposarcoma subtypes are summarised in Figure 6a,b. All 20 MLS samples showed the expression of PRAME, and 16 showed higher expression than that in testis. In addition, all 20 of the MLSs showed a higher level of CTAG1B expression than in the testis.

Table 6. Immunohistochemical results and statistical analysis

| Variables        | Analysed groups | No. of PRAME high/low | PRAME, p-value* | No. of NY-ESO-1 high/low | NY-ESO-1, p-value* |
|------------------|-----------------|------------------------|-----------------|--------------------------|-------------------|
| Sex              | Male versus female | 32/20                 | 0.0993          | 33/19                    | 0.3952            |
| Age              | <45 versus ≥45 years | 25/20                 | 0.8358          | 31/14                    | 0.0911            |
| Diameter         | ≥10 versus >10 cm | 22/26                 | 0.0374**        | 28/20                    | 0.6421            |
| Location         | Extremity versus others | 36/38                 | 0.1129          | 47/27                    | 0.067             |
|                  | Proximal versus distal | 13/5                  | 7/11            | 44/32                    | 0.7871            |
| Necrosis         | No necrosis vs Existence of necrosis | 21/37                 | <0.0001**       | 25/33                    | <0.0001**         |
| Mitotic count    | 0–4 versus ≥5/50HPF | 42/40                 | 0.2133          | 47/35                    | 0.5156            |
| Round cell component | <5% versus ≥5% | 38/41                 | 0.0099**        | 43/36                    | 0.0381**          |
| FNCLCC grade     | 1 versus 2,3     | 20/35                 | <0.0001**       | 23/32                    | <0.0001**         |
| AJCC stage       | 1 versus 2.3.4   | 17/27                 | 0.0007 **       | 18/26                    | 0.0001**          |

High expressions of PRAME and NY-ESO-1 were correlated with the existence of tumour necrosis, round-cell component >5%, higher FNCLCC grade and higher AJCC stage. The tumour diameter (>10 cm) was correlated with high PRAME expression.

Abbreviations: FNCLCC, French Federation of Cancer Centers, AJCC, American Joint Committee on Cancer; HPF, high-power fields.
*Fisher’s exact test.
**Statistically significant.
Multivariate analysis

Among the clinicopathological parameters (sex, age, round cell component, mitotic activity, necrosis, diameter, location), we detected male gender, tumour diameter (>10 cm) and the existence of necrosis as independent poor prognostic factors for overall survival or for disease-free survival (Supporting Information Table 2). FNCLCC grade and AJCC stage were excluded from this multivariate analysis because they are determined or affected by other parameters. Each immunohistochemical parameter was adjusted by the above three independent poor prognostic factors (gender, tumour diameter and necrosis; Table 7). Neither high expression of PRAME nor high expression of NY-ESO-1 was detected as an independent poor prognostic factor in the multivariate analysis.

Discussion

Although the function of PRAME in tumourigenesis has not yet been fully elucidated, it is known that PRAME can bind to the retinoic acid receptor in the presence of retinoic acid and thereby repress retinoic acid receptor signalling and TRAIL expression, causing cell proliferation and preventing apoptosis and cell-cycle arrest [40,41]. The function of NY-ESO-1 in tumourigenesis also remains poorly understood. In this study, immunohistochemical analysis revealed the expression of PRAME and NY-ESO-1 in 90% (84/93) and 89% (83/93) of the MLS samples, respectively. Moreover, PRAME and CTAG1B mRNA were detected in 100% (20/20) of the MLSs and, in most of these samples, both mRNAs were expressed at levels higher than in the testis. Regarding the liposarcoma subtypes, PRAME and NY-ESO-1 (CTAG1B) expression were significantly higher in the MLSs than in the other liposarcomas by both immunohistochemistry and quantitative real-time PCR. The immunohistochemical results in MLS were closely related to the real-time PCR results, but not significantly so.

With respect to the DDLs, PRAME mRNA expression was detected in 12/16 (75%) samples, two of which showed approximately one-half and one-tenth of the PRAME mRNA expression level in the testis, respectively; the other 10 samples showed...
Figure 5. (a–c) Western blotting of PRAME and NY-ESO-1 (a) in MLS tumour samples and corresponding normal tissue, (b) in MLS tumour samples and (c) in MLS tumour samples and MLS cell lines. PRAME was detected in 11 of 19 tumour samples and NY-ESO-1 was detected in 18 of 19 tumour samples, but not in any normal tissues. The MLS cell lines showed varying degrees of PRAME and NY-ESO-1 expression. PRAME/Actin and NY-ESO-1/Actin were normalised to the expression of testis tissue in each membrane. (d,e) Western blot analysis was performed to compare the normal tissue with tumour samples. (d) PRAME and (e) NY-ESO-1 showed significantly higher expression in tumour samples than normal tissue (p < 0.05 by Mann–Whitney U-test). (f) The samples that showed high PRAME expression in the immunohistochemical study showed significantly higher quantitative values of PRAME/Actin compared to the low expression samples (p = 0.0466). (g) The samples that showed high NY-ESO-1 expression in the immunohistochemical study showed higher NY-ESO-1/Actin compared to the low expression samples, but not significantly so (p = 0.0801). N, normal tissue; T, tumour; IHC immunohistochemistry; High, high expression by immunohistochemistry; Low, low expression by immunohistochemistry.
Relative fold expression of (a) PRAME and (b) CTAG1B measured by quantitative real-time PCR and the corresponding immunohistochemical results. PRAME and CTAG1B expression were normalised by GAPDH, and further normalised to the expression of a testis sample. PRAME was detected in all MLS samples, and 16/20 samples showed higher expression than the testis sample. CTAG1B was detected in 6/16 DDLS, 14/20 WLS and 2/3 PLS samples. One DDLS and one PLS sample showed higher CTAG1B expression than the testis sample, and the others showed low expression. (c, d) The expression levels of PRAME mRNA and CTAG1B mRNA among the liposarcoma subtypes. The mRNA levels of PRAME and CTAG1B in the MLSs were significantly higher than in the other liposarcoma subtypes (p < 0.05 by Steel-Dwass test). (e, f) The association between the expression level of PRAME mRNA and the immunohistochemical result, and between CTAG1B mRNA and the NY-ESO-1 immunohistochemical result. The samples that showed high PRAME and NY-ESO-1 expression by immunohistochemistry showed higher mRNA expression than the samples that showed low expression by immunohistochemistry, but the differences were not significant. IHC, immunohistochemistry; H, high expression by immunohistochemistry; L, low expression by immunohistochemistry; —, negative expression by immunohistochemistry; ND, not detected, Low, low expression by immunohistochemistry; High, high expression by immunohistochemistry.
**Table 7. Multivariate survival analysis for immunohistochemical parameters**

| Variables | OS     | DFS    | OS     | DFS    |
|-----------|--------|--------|--------|--------|
| Sex       | 0.0228*| 0.0019*| 0.026* | 0.0035*|
| Diameter  | 0.0562 | 0.0158*| 0.0395*| 0.0097*|
| Necrosis  | 0.5898 | 0.0954 | 0.1247 | 0.0394*|
| PRAME     | 0.195  | 0.0658 | 0.335  | 0.8412 |
| NY-ESO-1  |        |        |        |        |

The table indicates p-values. Each immunohistochemical parameter was adjusted by the three independent poor prognostic factors (gender, tumour diameter and necrosis; Supporting Information Table 2).

Abbreviations: OS, overall survival; DFS, disease-free survival.

*Statistically significant.

...even lower levels of **PRA ME** mRNA (<one-hundredth of the expression in the testis). The immunohistochemical findings were roughly in agreement with these results: the former two samples showed low PRAME expression, while 4 of the latter 10 samples showed low expression and the other six showed negative expression. By real-time PCR, four samples were **PRA ME**-negative, although one of these four had shown low **PRA ME** expression by immunohistochemistry. The reason for this discrepancy was unclear, although it may have been due to cross reactivity with the primary antibody for immunohistochemistry, and also may have been due to the low quality of the frozen sample. Real-time PCR revealed **CTAG1B** mRNA expression in 6 of the 16 (37.5%) DDLS samples. One of these cases, which showed high NY-ESO-1 IHC expression, had a **CTAG1B** mRNA expression level threefold higher than that observed in the testis, while the other five cases had **CTAG1B** mRNA levels less than one-thousandth of those in the testis.

In the WLS samples, **PRA ME** mRNA expression was detected in 13/20 cases (65%), but all 13 of these samples showed very low expression levels (approximately one-hundredth to one-thousandth of the **PRA ME** mRNA expression in the testis). Two of these 13 cases showed low **PRA ME** expression by immunohistochemistry. Low **CTAG1B** mRNA expression was also detected in 14/20 samples (70%), but none of the 20 samples showed NY-ESO-1 expression by immunohistochemistry. No frozen samples were included among the immunohistochemically NY-ESO-1-positive WLS cases.

In the PLS samples, **PRA ME** mRNA expression was detected in 2/3 (67%), one of which showed one-fifth of the **PRA ME** mRNA expression seen in the testis, and the other of which showed very low **PRA ME** mRNA expression. These two cases also showed low **PRA ME** expression immunohistochemically. **CTAG1B** mRNA was also detected in two of the three samples (67%) by quantitative real-time PCR, and in one of these two samples the **CTAG1B** mRNA expression was higher than that in the testis. However, these two **CTAG1B** mRNA-positive cases showed no NY-ESO-1 expression by immunohistochemistry.

Recently published studies have reported that NY-ESO-1 was expressed in 100% (25/25) and 89% (16/18) of the MLS samples, and in 10% (1/10) of the DDLS, none (0/10) of the WLS and 50% (3/6) of the PLS samples and that **PRA ME** was expressed in 100% of 37 MLSs by immunohistochemistry [19–21]. Our data are nearly coincident with these results and, as with NY-ESO-1, **PRA ME** was highly expressed in the MLSs among the liposarcomas in this study. Therefore, evaluation of the immunohistoexpression of **PRA ME** and NY-ESO-1 may be a useful diagnostic tool to distinguish MLSs from other liposarcomas.

**PRA ME** and NY-ESO-1 are known to be cancer-testis antigens recognised by specific cytotoxic T lymphocytes, and both are considered promising targets of immunotherapy [15]. In a previous study, cancer-testis antigens were expressed only in tumour tissues of various histological origins but not in normal somatic tissues (except for testis tissue which had no expression of HLA class 1 and was not recognised by cytotoxic T lymphocytes) [14]. Moreover, high expression of **PRA ME** has been reported to correlate with high tumour grade in urothelial carcinoma and head and neck squamous cell carcinoma, and to correlate with poor prognosis in breast cancer, osteosarcoma, neuroblastoma and serous ovarian adenocarcinoma [22–27]. In addition, other studies have shown that high NY-ESO-1 expression correlated with high tumour grade in urothelial carcinoma, breast cancer, malignant melanoma and transitional cell carcinoma, and with poor prognosis in head and neck cancer and nonsmall cell lung cancer [22,28–32]. To our knowledge, however, the correlation between **PRA ME** or NY-ESO-1 expression and clinicopathological data or prognosis had not been evaluated in MLSs prior to this study. Our results showed that **PRA ME** and NY-ESO-1 (**CTAG1B**) were highly expressed in MLSs in a coordinated manner, and that their expression correlated with histological grade, clinical stage and poor prognosis. The previous report showed no difference in NY-ESO-1 expression between the myxoid and round cell components, but the small number of samples in that study may have affected this finding [20]. Hence, our results suggest that these cancer-testis antigens may become a new target of immunotherapy and may serve as potential prognostic markers in MLS.
Among the clinicopathological parameters, we found that male gender, tumour diameter (>10 cm), the existence of necrosis, higher histological grade and advanced clinical stage were poor prognostic factors for overall survival and disease-free survival. In contrast, round-cell component >5%, which is often used as a prognostic factor, correlated with poor prognosis only in disease-free survival [5–7]. The relatively small number of cases enrolled in this study could have affected this result.

Male gender was a poor prognostic factor in the multivariate analysis. A recent study was the first to report that male gender was an independent poor prognostic factor in MLS. The same study went on to examine the expression of hormone receptors in MLS, and found that they were negligible [42]. Our present findings indicated that men were significantly more likely to have a round-cell component (13/39 versus 1/40; \( p = 0.0026 \) by Fisher’s exact test) and higher histological grades (Grade 1 versus 2/3: 26/26 versus 12/29; \( p = 0.0435 \) by chi-square test). The expression of PRAME and NY-ESO-1 was also higher in males than females, but the difference was not significant. Further studies will be needed to elucidate the relation between gender and MLS. We also found that the presence of necrosis and >10cm tumour diameter were independent prognostic factors, as had also been observed in previous reports [4–7].

It is still unclear whether PRAME and NY-ESO-1 are involved in the tumourigenesis of MLS, and what mechanisms underlie the expression of these antigens in this tumour. Regulation of the expression of PRAME, NY-ESO-1 and other cancer-testis antigens by promoter region hypomethylation has been described [43–46]. However, further elucidation of the functions and the epigenetic regulation of these cancer-testis antigens in MLS will clearly be needed. In summary, PRAME and NY-ESO-1 (CTAG1B) were expressed in the majority of the MLS samples examined, and their expression in MLS was significantly higher than in the other liposarcoma subtypes. Moreover, the high expression of PRAME and NY-ESO-1 correlated with higher tumour grade and poor prognosis. Our results support the potential use of PRAME and NY-ESO-1 as ancillary parameters for differential diagnosis and as prognostic markers of MLS, as well as the further development of immunotherapy against the cancer-testis antigens in MLS.

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Author contributions

KI and KK performed the experiments and data analysis; KI, YH, TI and AM collected the samples and clinical information; YY and HY interpreted the data; KI, KK and YO drafted the manuscript; and YI and YO conceived and designed the study and interpreted the data. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL ON THE INTERNET**

Additional Supporting Information may be found in the online version of this article.

**Table S1.** Primers used for PCR and sequence analysis.

**Table S2.** Multivariate analysis for clinicopathological parameters.