

The cancer-testis antigen NY-ESO-1 is highly expressed in myxoid and round cell subset of liposarcomas

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Liposarcomas are a heterogeneous group of fat-derived sarcomas, and surgery with or without chemoradiation therapy remains the main stay of treatment. NY-ESO-1 is a cancer-testis antigen expressed in various cancers where it can induce both cellular and humoral immunity. Immunotherapy has shown promise in clinical trials involving NY-ESO-1-expressing tumors. Gene expression studies have shown upregulation of the gene for NY-ESO-1, *CTAG1B*, in myxoid and round cell liposarcomas. Herein, we evaluated the expression of NY-ESO-1 among liposarcoma subtypes by quantitative real-time PCR, western blot analysis, and immunohistochemistry. Frozen tissue for quantitative real-time PCR and western blot analysis was obtained for the following liposarcoma subtypes ($n=15$): myxoid and round cell ($n=8$); well-differentiated ($n=4$), and dedifferentiated ($n=3$). Formalin-fixed paraffin-embedded blocks were obtained for the following liposarcoma subtypes ($n=44$): myxoid and round cell ($n=18$); well-differentiated ($n=10$); dedifferentiated ($n=10$); and pleomorphic ($n=6$). Full sections were stained with monoclonal antibody NY-ESO-1, and staining was assessed for intensity (1–3+), percentage of tumor positivity, and location. In all, 7/8 (88%) and 16/18 (89%) myxoid and round cell expressed *CTAG1B* and NY-ESO-1 by quantitative real-time PCR and immunohistochemistry, respectively. Western blot correlated with mRNA expression levels. By immunohistochemistry, 94% (15/16) of positive cases stained homogeneously with 2–3+ intensity. Also, 3/6 (50%) pleomorphic liposarcomas demonstrated a range of staining: 1+ intensity in 50% of cells; 2+ intensity in 5% of cells; and 3+ intensity in 90% of cells. One case of dedifferentiated liposarcoma showed strong, diffuse staining (3+ intensity in 75% of cells). Our study shows that both *CTAG1B* mRNA and protein are overexpressed with high frequency in myxoid and round cell liposarcoma, enabling the potential use of targeted immunotherapy in the treatment of this malignancy.

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Liposarcomas, which account for approximately 11% of soft tissue malignancies, are a heterogeneous group of fat-derived soft tissue sarcomas composed of neoplasms with differing histomorphology, cytogenetic and molecular abnormalities, and biological behavior.¹ The subtypes include the more commonly encountered well-differentiated, myxoid

and round cell, and dedifferentiated liposarcomas and the less commonly seen spindle cell and pleomorphic liposarcomas. Surgery with or without chemoradiation therapy remains the main stay of treatment. Unfortunately, tumor recurrence, progression, and metastasis remain a challenge and are largely dependent on subtype and tumor location.²

Cancer-testis antigens are a unique family of antigens, which have restricted expression to testicular germ cells in a normal adult but are aberrantly expressed on a variety of solid tumors, including soft tissue sarcomas, melanoma, and epithelial cancers.^{3,4} Cancer-testis antigens are highly immunogenic and can elicit spontaneous

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T-cell and/or humoral responses upon exposure.³ Among the known cancer-testis antigens, NY-ESO-1 is considered the most immunogenic and has become an attractive target for cancer vaccine development, which would utilize the immune system to selectively target and eliminate the cancer-testis antigen-expressing tumor cells.³

Similar to other sarcomas, gene expression studies have reported the presence of multiple cancer-testis antigens in liposarcomas, including SSX, LAGE-1, CTAG1B, CT-7, CT-10, GAGE, BAGE, PRAME, and various MAGE antigens.^{4–6} Furthermore, Segal *et al.* (2005)⁷ reported increased *CTAG1B* and *PRAME* gene transcripts specifically in the myxoid and round cell subtype, which constitutes approximately one-third of all liposarcomas.^{1,7} Other than a recent report of increased NY-ESO-1 protein expression in the myxoid and round cell subtype, NY-ESO-1 expression has only been minimally evaluated in liposarcomas.^{4,8,9}

NY-ESO-1 expression was initially identified in a case of esophageal squamous cell carcinoma and has subsequently been described in a variety of malignancies, including melanoma, sarcomas, and in various additional carcinomas.^{10,11} Protein expression in normal adult tissue appears to be restricted to the testis, more specifically spermatogonia.⁸ Studies have demonstrated high immunogenicity with the induction of both humoral and specific CD8+ cytotoxic T-cell immune responses against the NY-ESO-1-expressing tumors.^{12–14}

Given the previous reports of increased NY-ESO-1 in liposarcomas, we sought to further characterize NY-ESO-1 expression in the various liposarcoma subtypes using quantitative real-time PCR, western blot analysis, and immunohistochemistry.

Material and methods

Case Material

The liposarcomas ($n=44$) evaluated by immunohistochemistry were retrospectively identified from 2003 to 2011 from the surgical pathology archives at The Ohio State University Wexner Medical Center and included the following subtypes: well-differentiated ($n=10$), myxoid and round cell ($n=18$), dedifferentiated ($n=10$), and pleomorphic ($n=6$). The cases were re-reviewed by a bone and soft tissue pathologist (OHI), and the diagnoses were confirmed per established morphological diagnostic criteria.¹⁵ Notably, regarding the myxoid and round cell liposarcomas, molecular studies, including fluorescent *in situ* hybridization and/or conventional cytogenetic analysis, performed on 12 of the cases demonstrated rearrangement of *CHOP* (*DDIT3*) gene at 12q13 and t(12;16)(q13;p11), respectively, confirming the diagnosis in those cases. The six tumors diagnosed as pleomorphic liposarcoma were histologically consistent with

conventional pleomorphic liposarcoma. None of the six cases of the pleomorphic liposarcoma contained any areas of conventional myxoid and round cell liposarcoma akin to the pleomorphic myxoid liposarcoma variant from the criteria as established by Alaggio *et al.*¹⁶ A representative formalin-fixed, paraffin-embedded block was obtained for each tumor and submitted for NY-ESO-1 immunohistochemical staining. Furthermore, frozen tissue samples of additional cases (total $n=15$) of well-differentiated ($n=4$), myxoid/round cell ($n=8$), and dedifferentiated ($n=3$) liposarcomas were obtained for quantitative real-time PCR and western blot studies. Both paraffin-embedded and frozen tumor samples were obtained after approval through the Institutional Review Board Ohio State University, Department of Pathology (Institutional Review Board-approved protocol number-2002H0089).

RNA Extraction and Quantitative Real-Time PCR

RNA was extracted from frozen sarcoma samples using RiboZol (Amresco) and a modified manufacturer's protocol for RNA extraction using Trizol reagent (Ambion). RNA was quantitated using a NanoDrop-ND 1000. One microgram of RNA per sample was reverse transcribed using the iScript cDNA synthesis kit (Biorad). Quantitative real-time PCR was performed in 10 ml reactions according to recommended conditions by Applied Biosystems. Taqman probes for *GAPDH* (Hs00266705_1) and *CTAG1B* (Hs00265824_m1), which encodes for NY-ESO-1, were used.¹⁷ Each sample was measured in triplicate. No template controls and no reverse transcriptase controls for each sample were included. Cycle threshold values were averaged across triplicate samples. *GAPDH* was used to calculate percentage relative expression of each sample. Samples were further normalized to the expression of the testis positive control. Standard deviations were calculated by comparisons of delta cycle thresholds for each well in a triplicate.

Western Blot

Protein was isolated from frozen sarcoma samples using Ripa buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP40, 0.10% SDS) with protease inhibitor and was quantitated using Quant-iT protein assays (Invitrogen Life Technologies). Forty micrograms of protein per sample was run by electrophoresis on 10% gels and transferred. Antibodies and dilutions were NY-ESO-1 (08-312, 1:2500, Upstate Cell Signaling Solutions) and *GAPDH* (sc-32233, 1:200, Santa Cruz). Anti-mouse secondary was used for both antibodies (115-035-003, 1:10 000, Jackson ImmunoResearch). *GAPDH* was used for loading normalization. Radiographs were digitally scanned and quantified by densitometry using ImageJ software (<http://rsbweb.nih.gov/ij/index.html>).

Immunohistochemistry

NY-ESO-1, clone E978 (Santa Cruz Biotechnology; Santa Cruz, CA), was titrated on normal testis, and testis was used as control tissue. Paraffin-embedded tissue was cut at 4 microns, placed in a 60 °C oven for 1 h, cooled, and then deparaffinized and rehydrated through xylenes and graded ethanol solutions to water. All slides were quenched for 5 min in a 3% hydrogen peroxide solution in water to block for endogenous peroxidase. Antigen retrieval was performed by heat-induced epitope retrieval, in which the slides were placed in a 1 × solution of Target Retrieval Solution (Dako, Carpinteria, CA) for 25 min at 96 °C using a vegetable steamer and cooled for 15 min in the solution. Slides were then placed on a Dako Autostainer Immunostaining System. The primary antibody NY-ESO-1 was diluted with a Dako antibody diluent at 1:100 and incubated for 60 min. Slides were then blocked for endogenous biotin using the Dako Biotin Blocking System. The secondary antibody, a 1:200 dilution of biotinylated goat anti-mouse (Vector, Burlingame, CA) prepared with Vector 2% Normal Goat Serum in Dako antibody diluent, was incubated for 30 min at room temperature. The detection system used was Vectastain Elite (Vector) for 30 min. Staining was visualized with the DAB+ chromogen (Dako; 5-min development). Slides were then counterstained in Richard Allen hematoxylin, dehydrated through graded ethanol solutions.

Quantitation of Immunohistochemistry

NY-ESO-1 immunopositivity was scored semiquantitatively for the percentage of tumor cells staining (0%; <5%; 5–<25%; 25–<50%; 50–<75%; and ≥75%), intensity (0, negative; 1+, weak staining/trace; 2+, moderate staining; and 3+, strong staining), and subcellular location (cytoplasmic and/or nuclear).

Results

Quantitative Real-Time PCR

We performed quantitative real-time PCR on a total of 15 liposarcoma samples, which included eight myxoid and round cell, three dedifferentiated, and four well-differentiated liposarcomas, as well as one testis sample as a positive control for *CTAG1B* expression. The results are depicted in Figure 1 and Table 3. Seven (88%) myxoid and round cell liposarcomas showed expression of *CTAG1B*, five of which were significantly higher than expression found in the testis. One (33%) dedifferentiated liposarcoma showed expression, but it was roughly one-tenth the expression level observed in the testis. None of the well-differentiated liposarcomas were positive for *CTAG1B* expression.

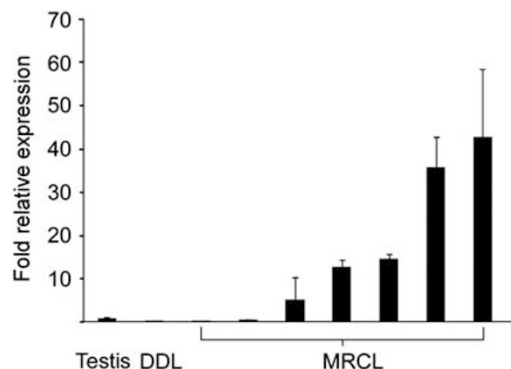


Figure 1 Relative fold *CTAG1B* expression. *CTAG1B* expression of liposarcoma samples was measured by quantitative real-time PCR. *CTAG1B* expression was normalized to *GAPDH*. Expression is plotted as fold expression relative to *CTAG1B* expression in testes. Only samples showing *CTAG1B* expression are plotted. One of the three dedifferentiated samples showed expression and none of the four well-differentiated liposarcomas showed expression. Seven of the eight myxoid/round cell liposarcoma samples were positive for *CTAG1B* expression; five of those at levels significantly higher than the testis sample. DDL, dedifferentiated liposarcoma; MRCL, myxoid and round cell liposarcoma.

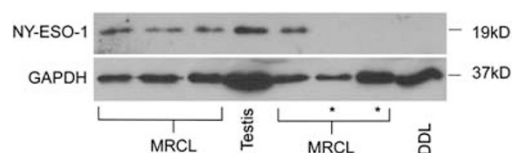


Figure 2 Western blot analysis of NY-ESO-1. The seven myxoid/round cell liposarcomas and one dedifferentiated liposarcoma that showed *CTAG1B* expression via quantitative real-time PCR were analyzed for NY-ESO-1 protein expression by western blot as well as the testis control sample. A GAPDH loading control is shown. MRCL, myxoid/round cell liposarcoma; DDL, dedifferentiated liposarcoma; *, MRCL samples that showed very low expression of *CTAG1B* by quantitative real-time PCR.

Western Blot

We performed western blot analysis on the eight liposarcoma samples that demonstrated *CTAG1B* expression with quantitative real-time PCR, consisting of seven myxoid and round cell liposarcomas and one dedifferentiated liposarcoma. One testis sample was utilized as a positive control for NY-ESO-1 protein expression. The results are depicted in Figure 2 and Table 3. Four of the seven myxoid and round cell liposarcomas demonstrated NY-ESO-1 protein expression. The samples that contained low levels of *CTAG1B* by quantitative real-time PCR (two myxoid and round cell liposarcomas and one dedifferentiated liposarcoma) were all negative for protein expression on western blot analysis. Notably, the samples did not show even loading, with the testis demonstrating the highest total protein levels. However, after normalization to GAPDH expression, four of the five myxoid and

round cell samples that had shown higher expression of *CTAG1B* mRNA by quantitative real-time PCR also had higher NY-ESO-1 protein expression by western blot.

Immunohistochemistry

The results of NY-ESO-1 immunohistochemical staining in liposarcomas are summarized in Tables 1, 2, and 3. Consistent immunoreactivity was noted in myxoid and round cell liposarcomas (89%) and to a lesser degree in pleomorphic (50%) and dedifferentiated liposarcomas (10%). The staining pattern was cytoplasmic and nuclear in all positive instances; however, a cytoplasmic predominance was apparent. All 10 cases of well-differentiated liposarcomas were negative for NY-ESO-1.

Of the 16 immunoreactive cases of myxoid and round cell liposarcoma, 15 (94%) demonstrated strong, homogenous immunoreactivity, as defined by >50% tumor cell positivity (Figure 3a); thus, only one tumor showed heterogeneous staining, characterized as 1+ intensity in 20% of tumor cells. Notably, 11 of the positive tumors showed 3+ intensity in ≥90% of tumor cells.

Table 1 Immunohistochemistry results in liposarcomas

Subtype	Positive cases (percentage of total)
Myxoid/round cell	16/18 (89%)
Pleomorphic	3/6 (50%)
Dedifferentiated	1/10 (10%)
Well-differentiated	0/10 (0%)

Table 2 Immunohistochemistry results in myxoid and round cell liposarcomas

Percentage of tumor positivity	Staining intensity	Number (percentage of total)
≥75%	2–3+	14/18 (78%)
50–<75%	3+	1/18 (5.5%)
25–<50%	NA	0/18 (0%)
5–<25%	1+	1/18 (5.5%)
0%	NA	2/18 (11%)

Table 3 *CTAG1B* and NY-ESO-1 mRNA and protein expression in liposarcoma subtypes

Subtype	qRT-PCR	Western blot	IHC
Myxoid/round cell	7/8 (88%)	4/7 (57%)	16/18 (89%)
Pleomorphic	—	—	3/6 (50%)
Dedifferentiated	1/3 (33%)	0/1 (0%)	1/10 (10%)
Well-differentiated	0/4 (0%)	—	0/10 (0%)

IHC, immunohistochemistry; —, not evaluated.

The myxoid and round cell components demonstrated similar patterns of immunoreactivity in positive cases. Two tumors (11%) were negative for NY-ESO-1.

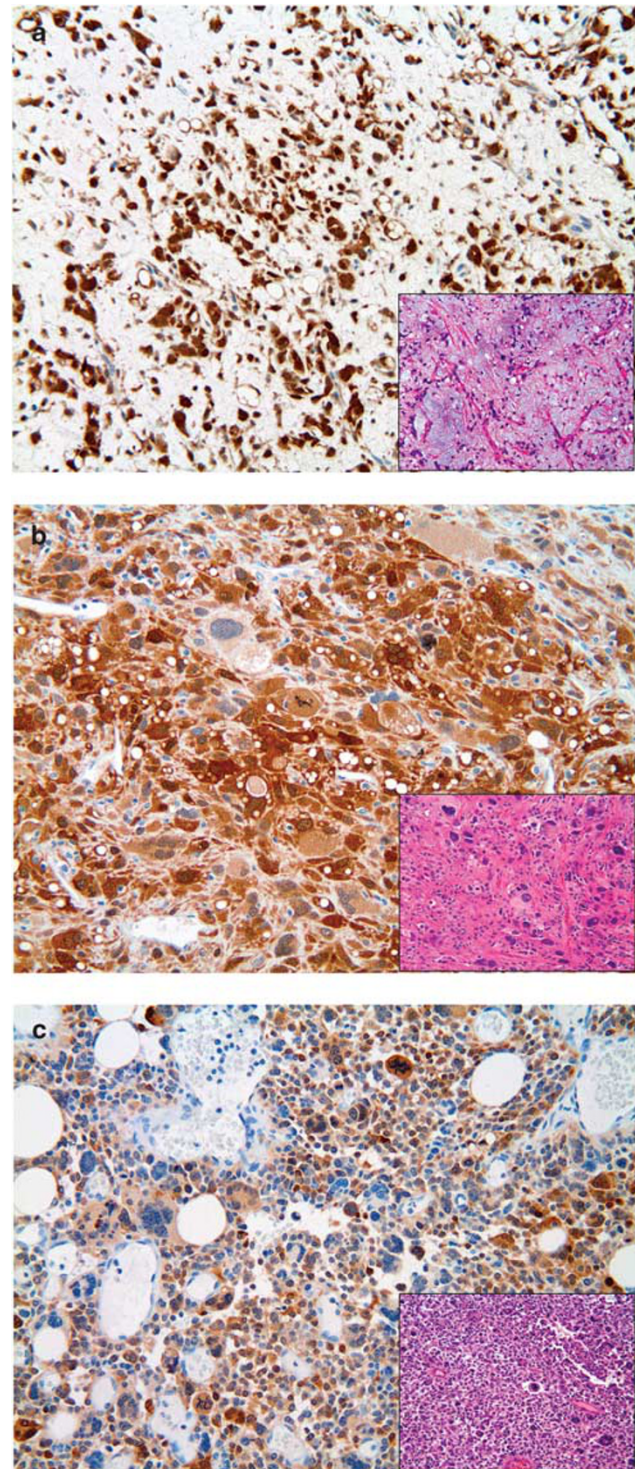


Figure 3 NY-ESO-1 immunoreactivity in liposarcoma subtypes. Diffuse, homogeneous NY-ESO-1 immunoreactivity in (a) myxoid/round cell and (b) pleomorphic liposarcomas (inset, H&E). (c) Diffuse NY-ESO-1 immunoreactivity with variable staining intensity in dedifferentiated liposarcoma (inset, H&E). All figures ×200.

The three NY-ESO-1-positive cases of pleomorphic liposarcoma demonstrated variable staining characteristics as follows: diffuse (90% of tumor cells) with 3+ staining intensity (Figure 3b); patchy (50% of tumor cells) with 1+ staining intensity; and focal (5% of tumor cells) with 2+ staining intensity. The single case of NY-ESO-1 immunoreactivity in dedifferentiated liposarcoma demonstrated diffuse staining (75% of tumor cells) with variable staining intensity (1 to 3+) (Figure 3c).

In addition, the case of myxoid and round cell liposarcoma that did not demonstrate *CTAG1B* mRNA by quantitative real-time PCR underwent formalin processing for further evaluation by H&E and immunohistochemical staining. The presence of myxoid and round cell liposarcoma was confirmed and subsequent staining for NY-ESO-1 showed strong, diffuse tumor immunoreactivity (3+ intensity of 95% of tumor cells).

Discussion

NY-ESO-1, a cancer-testis antigen, is a promising target for cancer immunotherapy, given its limited extra-tumoral expression and its characteristic high immunogenicity. *CTAG1B*, the gene for NY-ESO-1, is located at Xq28 and codes for an intracellular, 18-kDa protein.^{10,17} The function of NY-ESO-1 is unknown; however, it has been postulated that cancer-testis antigens in general are involved in germ cell self-renewal or differentiation, conferring qualities such as immortality, self renewal, migratory ability, and capacity to transform to cancer cells upon expression.^{11,18,19}

NY-ESO-1 was initially identified in esophageal squamous cell carcinoma utilizing 'SERological identification of antigens by recombinant Expression cloning' (SEREX) methodology.¹⁰ Subsequently, protein expression has been described in a variety of malignancies, including melanoma, neuroblastoma, lung, breast, prostate, endometrial, ovarian, uterine, hepatocellular, and bladder carcinomas.¹¹ Increased protein expression in a variety of soft tissue sarcomas has also been reported, including synovial sarcomas, gastrointestinal stromal tumors, uterine leiomyosarcomas, angiosarcoma, malignant fibrous histiocytoma, and liposarcomas.^{4,9,20} The most common tumors to demonstrate NY-ESO-1 expression, as determined by immunohistochemistry with the monoclonal antibodies ES121 and E978, are myxoid and round cell liposarcoma (100%), neuroblastoma (82%), synovial sarcoma (80%), melanoma (46%), and epithelial ovarian cancer (43%).^{9,20-23} Studies involving NY-ESO-1-expressing tumors have demonstrated high immunogenicity with induction of both humoral and specific CD8+ cytotoxic T-cell immune responses.^{9,12-14} Immunotherapy targeting this antigen has shown some promise in clinical trials involving NY-ESO-1-expressing tumors especially in synovial sarcomas and malignant melanomas.²⁴⁻²⁷

In our study, we demonstrated that NY-ESO-1 is highly expressed in myxoid and round cell liposarcoma, which is a commonly encountered liposarcoma subtype. *CTAG1B* mRNA is consistently increased in these tumors (seven of eight samples tested), of which, five cases demonstrated greater levels than that seen in normal testis. Furthermore, the increased mRNA levels correlate with increased protein expression, as determined by western blot. Immunoreactivity by immunohistochemistry was demonstrated in 89% (16/18) of the myxoid and round cell liposarcomas evaluated, and, importantly, was distributed homogeneously in 94% of the positive cases. Fluorescent *in situ* hybridization analysis of the two negative cases of myxoid and round cell liposarcomas was consistent with rearrangement involving *CHOP* gene at 12q13, confirming the diagnosis in both cases. Interestingly, the myxoid and round cell sample that was negative for *CTAG1B* mRNA expression by quantitative real-time PCR demonstrated strong, homogenous immunoreactivity by immunohistochemistry. The reason for this discrepancy is unclear, but the *GAPDH* normalization control for this sample showed very low levels of expression on multiple trials, suggesting low quality or abundance of mRNA for the sample. As different antibodies were used for the immunohistochemical and western blot analyses, it is also possible that epitope variance could explain these results.

Our data parallels a recently published study that reported frequently homogeneous, NY-ESO-1 expression in 100% (25/25) myxoid and round cell liposarcomas evaluated by immunohistochemistry and quantitative real-time PCR.⁹ They did not see NY-ESO-1 immunoreactivity in any of the seven non-myxoid liposarcomas studied. On the contrary, we noted NY-ESO-1 expression in both pleomorphic (3/6; 50%) and dedifferentiated (1/10; 10%) subtypes. The overall staining distribution was more heterogeneous in the pleomorphic subtype compared with the myxoid and round cell subtype of liposarcoma. Notably, fluorescent *in situ* hybridization analysis performed on one of the three NY-ESO-1-positive pleomorphic liposarcomas was negative for rearrangements of *CHOP* (12q13) and *EWSR1* (11q12) genes. Regarding dedifferentiated liposarcoma, we reported a low level of *CTAG1B* mRNA expression in a single case, which did not correlate with protein expression on subsequent western blot analysis. Immunohistochemistry also identified a case of NY-ESO-1 immunoreactivity, characterized as diffuse staining of variable intensity. Overall, it appears that there is an infrequent protein expression in this subtype. *CTAG1B* mRNA and NY-ESO-1 protein expression were not seen in any of the samples of well-differentiated liposarcoma evaluated by quantitative real-time PCR and immunohistochemistry respectively.

Tumor expression patterns of cancer-testis antigens is highly associated with DNA methylation status.^{28,29} In a study of epithelial ovarian cancers

utilizing microdissection techniques, heterogeneous NY-ESO-1 expression correlated with intra-tumoral differences in promoter as well as global DNA methylation status.²⁹ Accordingly, a heterogeneous pattern of expression might attenuate clinical and immunological responses to cancer-testis antigen-directed immunotherapy, which relies on antigen presentation. Thus far, studies have demonstrated a predominantly heterogeneous staining pattern with NY-ESO-1, with only infrequent homogenous staining seen within a particular tumor type.^{8,21,23} Other than myxoid and round cell liposarcomas, the only exception is in synovial sarcomas, where NY-ESO-1 expression was reported to be consistently homogenous, being present in 70% of positive tumors.²⁰ A subsequent clinical trial utilizing adoptive immunotherapy targeting NY-ESO-1 has shown promise in synovial sarcomas.²⁷

In conclusion, we report consistent, homogenous NY-ESO-1 expression within myxoid and round cell liposarcomas using quantitative real-time PCR, western blot analysis, and immunohistochemistry. These findings allow for the rational utilization of immunotherapy in this relatively common subset of liposarcomas, especially in the setting of metastatic disease or in patients who have failed or cannot tolerate conventional therapies.

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Jessica Gillespie performed the quantitative real-time PCR and western analyses of *CTAG1B* and NY-ESO-1 expression.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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