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NY-ESO-1 is a Ubiquitous Immunotherapeutic Target Antigen for Patients with Myxoid/ Round Cell Liposarcoma

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Abstract

Background—Myxoid/ round cell liposarcoma (MRCL) is the second most common liposarcoma subtype, accounting for more than one third of liposarcomas and approximately 10% of all soft tissue sarcomas. Although MRCL is a chemosensitive subtype, patients with metastatic disease have a poor outcome. NY-ESO-1 is a cancer-testis antigen (also known as cancer germ cell antigen) that has been successfully targeted in vaccine and also adoptive T cell therapy trials for the treatment of several solid tumors.

Methods—We examined the feasibility of targeting NY-ESO-1 in patients with MRCL by evaluating the prevalence of NY-ESO-1 expression among tumors using immunohistochemistry and qRT-PCR. We also analyzed NY-ESO-1 specific tumor recognition by NY-ESO-1 specific T cells using chromium release assay.

Results—A search of the University of Washington Sarcoma Tissue Bank revealed paraffin embedded tumor samples from 25 patients with MRCL. NY-ESO-1 expression was observed in every MRCL tumor assessed (100%); in 18 (72%), staining was homogenous. In all but 2 cases, staining was sufficiently robust (2+) that such patients would be eligible for clinical trials of NY-ESO-1 directed therapy. Using NY-ESO-1 specific CD8+ T cells, we demonstrate *in vitro* sensitivity of myxoid liposarcoma cell lines to antigen-specific lysis.

Conclusions—These results establish NY-ESO-1 as an important target antigen for the treatment of patients with MRCL.

Keywords

NY-ESO-1; Sarcoma; Myxoid; immunotherapy; cancer testis antigens

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Introduction

Based on its immunogenicity, NY-ESO-1 is considered to be among the most attractive antigens for immunotherapy. It has been targeted in a number of clinical studies including several vaccine trials that have induced serologic, CD4+ and CD8+ T cell responses. Delayed type hypersensitivity responses following NY-ESO-1 vaccination have been associated with long-term survival [1, 2]. Objective clinical responses have been observed in melanoma patients following vaccination against NY-ESO-1, including one complete response [3].

NY-ESO-1 has also been successfully targeted in trials of antigen specific adoptive T cells therapy. For example, transfer of NY-ESO-1 specific CD4+ cells have been effectively used to treat patients with metastatic melanoma [4]. NY-ESO-1 has also been targeted using a class I TCR retrovirally transfected into T cells [5] inducing complete responses in melanoma patients. To date there have been no known grade III or grade IV autoimmune toxicities associated with anti-NY-ESO-1 therapy.

NY-ESO-1, a member of the family of Cancer Testis Antigens (CT antigens), was first discovered through serological analysis in esophageal cancer patients and was subsequently found to induce a strong cytotoxic T-cell response [6-8]. As their name implies, CT Antigens (also sometimes referred to as cancer germ-cell antigens), are expressed on a protein level in various malignant tumors and germ cells of the testis but not other adult tissues.

Soft tissue sarcomas are a heterogeneous group of malignancies of mesenchymal origin with poor prognosis in the metastatic setting and a median overall survival less than one year [9, 10]. Liposarcomas account for approximately 10-20% of soft tissue sarcomas and can be classified into three subtypes each with their own distinct clinical behaviors: pleomorphic, well/de-differentiated and myxoid/ round cell liposarcoma (MRCL). MRCL accounts for 40-50% of liposarcomas and is almost always associated with a chromosomal translocation, most commonly t(12;16)(q13;p11) though a number of less common translocations have also been described [11]. The resultant fusion proteins have an activity that is not well understood [12]. MRCL is a relatively sensitive to front line chemotherapy and trabectedin as second line treatment for metastatic MRCL is promising [10, 13]. However, mortality remains high for patients with metastatic disease, suggesting the need for novel approaches.

The discovery that over 80% of synovial sarcomas express NY-ESO-1 [14], often homogenously, established synovial sarcoma as a malignancy with one of the highest rates of NY-ESO-1 expression and led to the perception that synovial sarcoma is a model disease for the study of NY-ESO-1 directed therapy. Supporting this concept, a recently published adoptive therapy trial [5] using retrovirally transfected NY-ESO-1 specific TCR, documented 4 partial responses out of 6 synovial sarcoma patients treated.

Here we report that another soft tissue sarcoma subtype, MRCL, ubiquitously expresses the cancer testis antigen NY-ESO-1, most often homogenously, raising the possibility of NY-ESO-1 directed therapy for this challenging disease with limited treatment options in the metastatic setting.

Methods

Tumor Samples

Both paraffin embedded and flash frozen human MRCL samples were obtained through the University of Washington sarcoma tumor bank (IRB approved protocol #21369).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. MAb E978 was used to detect NY-ESO-1 as previously described [15]. For all assays, appropriate positive (normal testis with preserved spermiogenesis) and negative controls (omission of primary antibody and replacement with phosphate buffer saline, pH 7.4) were included.

Tissue sections were deparaffinized in xylene and rehydrated in a series of graded alcohols. Endogenous peroxidase was blocked by incubating slides for 30 minutes at room temperature in 99.7% methanol containing 0.3% hydrogen peroxide. Slides were washed with Tris-buffered saline solution and blocked in 2% bovine serum albumin (BSA) at room temperature for 5 minutes to prevent unspecific protein interactions. A heat-based antigen retrieval technique using a commercial vegetable steamer was used by heating slides in a buffer solution (Table1) for 30 minutes at approximately 96°C. Tissue slides were incubated with primary antibodies overnight at 4°C in a wet chamber. Primary antibody detection was performed by use of a Novolink polymer detection kit (Leica Microsystems Inc., Bannockburn, IL) in accord with the manufacturer's instructions. 3,3-Diaminobenzidine (DAB) served as a chromogen and counterstains were done with Harris hematoxylin. Finally, slides were dehydrated in a series of graded ethanols and cover slipped.

Slides were examined under a light microscope by two pathologist including an experienced bone and soft tissue pathologist. Characteristic morphological features of myxoid liposarcoma were confirmed, including myxoid stroma, primitive mesenchymal cells, lipoblasts and an arborizing capillary vasculature. Cases with staining present in less than 5% of cells were considered focally positive. 1+ was considered 5-25%. 2+ was 25-50%. 3+ was considered 50-75%. Tumors with staining >75% were considered 4+.

qRT-PCR

RNA was extracted from frozen tumor samples using Trizol (invitrogen) and from cell lines using RNeasy kit (Qiagen). Due to varying tissue collection conditions, RNA quality was recorded prior to analysis, using either a gel or bioanalyzer. Samples with poor quality RNA were not analyzed further. One non-myxoid liposarcoma sample was invading into the spermatic cord and was thus not included in the analysis because of concern for false positive.

RNA samples were converted to cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Results were analyzed using GAPDH as a house keeping gene and were calculated relative to testis using the standard curve method. NY-ESO-1 primers were TGCTTGAGTTCTACCTGCCA and TATGTTGCCGGACACAGTGAA [16]. GAPDH primers were GAAGGTGAAGGTCGGAGTC and GAAGATGGTGATGGGATTTC [17]. In all MRCL tumors, NY-ESO-1 expression was also confirmed and quantitated using primers from SA Biosciences. Amplification was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, Ca) on an ABI 7900HT (Applied Biosystems, Foster City, Ca).

Antigen Specific T cells

NY-ESO-1 specific effectors were generated from a HLA -A*0201+ synovial sarcoma patient who was leukapheresed under established protocols (Fred Hutchinson Cancer Research Center, protocol #1246). PBMC derived dendritic cells [18] were pulsed with the NY-ESO-1 peptide SLLMWITQC. PBMC were depleted of $CD25^+$ T cells using CliniMACS CD25 MicroBeads (Miltenyi Biotech, Auburn, CA) according to manufacturer's instructions and were stimulated using IL-21 as previously described [19]. NY-ESO-1+ cells were sorted using NY-ESO-1 tetramer then cloned with limited dilution

and expanded using a Rapid Expansion Protocol [20]. MART-1 specific T cell clones were used as control effector cells.

Cell Lines

The human myxoid liposarcoma cell lines 402 and 1765 (gifts of Pierre Aman) have been previously described [21]. They were maintained in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA), and 10% FBS.

The T2 cell line is a TAP-deficient T–B cell hybrid expressing the HLA-A2 allele which was used (unpulsed) as a negative control and pulsed with target peptide as a positive control.

Chromium Release Assay Using Recombinant Vaccinia – HLA-A2 Transfected Targets

Because the target cells (402 and 1765) did not express the HLA-A*0201, we used recombinant vaccinia to endow target cells with the presenting HLA restricting element according to established methods[22]. NY-ESO-1+ MRCL tumor lines, 402 and 1765 were transfected with the HLA-A*0201 gene using a vaccinia vector at a multiplicity of infection (MOI) 2.5, found to be optimal in titration experiments. As a control against cell lysis resulting from the vaccinia infection, cell lines were also treated with wild type Vaccinia virus. For the chromium release assay, cell lines were labeled with 100 μCi ^{51}Cr and cocultured with effector cells for 4-6 hours at 37°C plus 5% CO_2.

Results

MRCL samples expressed NY-ESO-1 in 100% of cases; homogenous expression was observed in over 70% of patients

We stained 25 MRCL tumors for NY-ESO-1, all were positive for NY-ESO-1 expression. In two cases, only 1+ staining was observed (5-25% positive for staining). In the remaining 23 cases at least 2+ staining was observed (2+ staining has been the cut off for clinical trial eligibility in some trials of NY-ESO-1 directed therapy [5]). In 6 of 25 cases, flash frozen tissue was available for RNA extraction and qRT-PCR analysis (see supplemental table 1). In all of these cases, NY-ESO-1 expression was confirmed (Table 1).

In 18/25 patients (72%), staining was homogenous (3+ or 4+), ie. present in more than 50% of the tumor. Nine of these patients had 4+ staining. In all but 3 cases, the primary tumor was stained from a resection specimen, however, in the 3 cases that were from metastatic disease staining appeared to have at least similar pattern of expression (4+ in cases, 2+ in one case). At least eleven patients subsequently developed metastatic disease (a number of patients were lost to follow up) but we were unable to observe a clear correlation between disease out come and staining intensity.

Importantly, although most patients had the classic t(12;16)(q13;p11) translocation there were four patients with different cytogenetics: t(10;16;12), t(12;22), t(15;17) as well as one patient with normal cytogenetics that was repeated and confirmed (growth of normal host cells could not be excluded). All of these patients had NY-ESO-1 staining regardless of karyotype.

To determine if NY-ESO-1 expression was limited to the MRCL subtype, we tested seven non-myxoid liposarcoma specimens by IHC and frozen samples from three (additional) patients by qRT-PCR. These ten tumors included 2 pleomorphic liposarcomas, four well-differentiated and four de-differentiated tumors. None of these tumors expressed NY-

ESO-1. While these negative results are not definitive, they do suggest that the high prevalence of NY-ESO-1 expression seen in MRCL tumors is not shared amongst the other liposarcoma subtypes.

Of note, although both myxoid and round cell areas of the tumor tended to stain positive for NY-ESO-1, a more uniform and intense appearance of the staining was observed in the round cell component. Also of interest was that the well-differentiated elements of some tumors stained less homogenously (1+ to 2+) than the more typical Myxoid and round cell components within the same tumor. (Figure 1).

MRCL Cell Lines Expressing NY-ESO-1 can be recognized and specifically lysed by NY-ESO-1 specific effectors

The MRCL cell lines 402 and 1765 were analyzed by qPCR and found to express NY-ESO-1 mRNA transcripts at levels even higher than testis normalized to GAPDH (2.5- and 3.5-fold higher). Both cell lines under went class I typing at the Puget Sound Blood Center and were found to be negative for HLA-A*02. Since HLA-A2-restricted NY-ESO-1 specific CTL were used to evaluate antigen recognition, 402 and 1765 were pre-infected with a recombinant vaccinia virus expressing HLA-A*0201 (Vac-A2). When treated with Vac-A2, cells were lysed at > 30% after 4 hours with an E:T ratio of 20:1. Control MART-1-specific effectors were unable to kill either cell line. Similarly, MRCL cell lines transfected with wild-type vaccinia virus were not sensitized to lysis by NY-ESO-1 specific effectors (Figure 2).

Discussion

NY-ESO-1 is widely considered an attractive target for immunotherapy. Complete responses have been seen in melanoma trials targeting NY-ESO-1 using both vaccines as well as adoptively transferred T cells. The discovery that 80% of synovial sarcomas express NY-ESO-1 was rapidly translated into a clinical trial; the NCI surgery branch treated six synovial sarcoma patients using T cells transfected with a retrovirus expressing the NY-ESO-1 TCR. Partial responses were observed in 4 of 6 patients [3-5].

Here we report another soft-tissue sarcoma subtype that demonstrates a pattern of NY-ESO-1 expression that is even more prevalent than synovial sarcoma. Although other reports have included NY-ESO-1 expression in liposarcomas including MRCL generally, this is the first study to specifically examine NY-ESO-1 protein expression in MRCL [23-28]. Based on our analysis of 25 samples, MRCL appears to express NY-ESO-1 with a frequency that is unmatched by any other malignancy studied to date. Furthermore, a high proportion (9 of 25) had 4+ (>75%) staining and an additional 9 of 25 patients had 3+ (>50%) staining. Interestingly, patients with the histopathologic phenotype of MRCL, possessing a variety of chromosomal translocations were included in this analysis and all expressed NY-ESO-1. Furthermore, we demonstrated that MRCL cell lines are capable of presenting *in vitro* NY-ESO-1 peptide such that it can be recognized by NY-ESO-1 specific effectors initiating cell mediated lysis of tumor cells.

MRCL is generally associated with a characteristic fusion protein, however it is not clear what role the mutation plays in oncogenesis. Most cases contain t(12;16) (q13;p11) producing the FUS-CHOP fusion protein although a notable minority contain the t(12;22) (q13;12) translocation associated EWSR1-CHOP (containing EWSR1, the Ewings sarcoma (EWS) breakpoint region 1). Both FUS and EWS (along with TAF15) are in the FET family (also known as the TET family) of RNA binding proteins [29]. However, although the RNA binding profiles of the FET family proteins are remarkably similar to one another [30],

Ewing's sarcomas, which typically have translocations of EWS [31, 32] do not generally express NY-ESO-1 [33].

There is evidence to suggest that murine adipocyte derived mesenchymal stem cells transfected with a FUS-CHOP gene develop an MRCL phenotype; however the translocation alone was insufficient to induce a MRCL tumor like phenotype using human adipocyte derived mesenchymal stem cells transfected with the FUS-CHOP suggesting the need for additional genetic "hits" [34-37]. Similar to MRCL, in synovial sarcoma models the presence of SYT-SSX alone appears insufficient on its own to cause oncogenesis [38]. Mesenchymal stem cells have also been postulated as a potential cell of origin in synovial sarcoma [39].

There has never been a study of NY-ESO-1 specific serologic response in MRCL patients [28, 40]. An analysis of the serologic response to a number of CT antigens including NY-ESO-1 was reported from 54 sarcoma patients including 5 synovial sarcoma patients and an MRCL patient. Serology was negative except for two patients (one with pleomorphic sarcoma and another with fibrosarcoma) [41]. Serology was also analyzed in the study by Ayyoub et al, which included one patient with liposarcoma, though histologic subtype was not mentioned [28].

Expression of CT antigens has been correlated with outcomes in a number of malignancies [42-44]. Although the numbers in this study would be underpowered to perform an adequate analysis assessing a difference in outcome pattern between strong and weak NY-ESO-1 expression, we are assessing the feasibility of this approach in our patient population. We are also assessing ways to apply this knowledge to preclinical models such as MRCL xenografts in order to advance NY-ESO-1 directed immunotherapy for sarcoma patients [45].

No other malignancy, including synovial sarcoma, has been described having NY-ESO-1 expression in 100% of cases or with such a high proportion homogenous expression. We believe that like synovial sarcoma, these results will establish MRCL as a model disease for the study of NY-ESO-1 directed therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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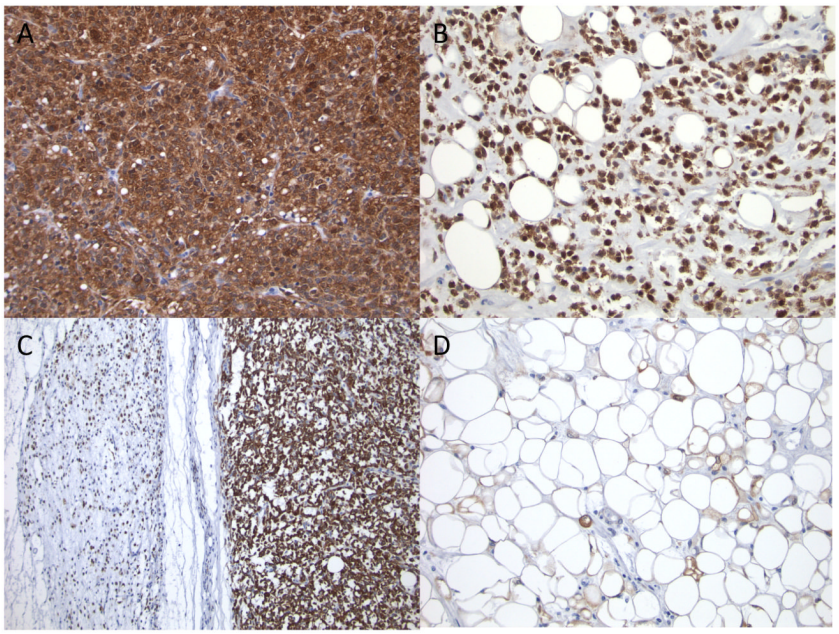

Figure 1.

Round cell components often had intense and uniform staining (A) but myxoid components were still frequently homogenously stained (B). In tumors with both myxoid and round cell components, both segments stained homogenously but sometimes more intensely in the round cell compartment (C). In some of the lesser staining tumors, well differentiated areas of mature appearing fat were sometimes present (D).

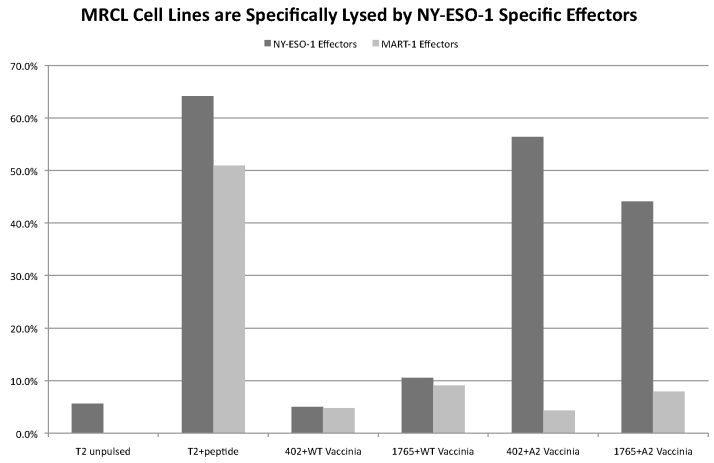

Figure 2.

NY-ESO-1 Effectors recognize and specifically lyse MRCL cell lines at an effector to target ratio of 20:1 following transfection with vaccinia virus expressing HLA-A*0201. MART-1 effectors and WT vaccinia virus were used as controls.

Patient	Cytogenetics	Site of primary tumor	Primary or Metastatic Specimen	Outcome	NY-ESO-1 Staining	qRT-PCR confirmed
1	cytogenetics unavailable	right thigh	Primary	Metastatic Disease, deceased	3+	Not available
2	cytogenetics unavailable	right hip	Primary	Metastatic Disease	3+	Not available
3	cytogenetics unavailable	left buttock	Primary	Local recurrence, currently disease free	3+	Not available
4	cytogenetics unavailable	right thigh	Metastatic	Metastatic Disease	2+	Yes
5	cytogenetics unavailable	left popliteal fossa	Primary	Metastatic Disease, deceased	4+	Not available
6	t(12;16) with other complex cytogenetics	right groin	Primary	Metastatic Disease, deceased	4+	Not available
7	t(12;16)(q13;p11)	left popliteal fossa	Primary	Disease free	1+	Not available
8	t(12;16) with other complex cytogenetics	left thigh	Primary	Metastatic Disease, deceased	3+	Not available
9	t(10;16;12)(q26;p11;q13)	left calf	Primary	Disease free	2+	Not available
10	cytogenetics unavailable	right thigh	Primary	Disease free	4+	Not available
11	t(12;16)(q13;p11)	right thigh	Primary	Local recurrence, currently disease free	2+	Not available
12	t(12;16)(q13;p11)	right thigh	Primary	Isolated recurrence, currently disease free	4+	Not available
13	t(12;16)(q13;p11)	left popliteal fossa	Primary	Disease free	3+	Yes
14	t(12;16) with other complex cytogenetics	right tibia	Primary	Disease free	4+	Not available
15	t(12;16)(q13;p11)	right thigh	Primary	Disease free	4+	Not available
16	t(12;22)(q13;q12)	right great toe	Primary	Disease free	2+	Not available
17	cytogenetics unavailable	left leg	Metastatic	Metastatic Disease	4+	Not available
18	t(12;16) with other complex cytogenetics	right thigh	Primary	Disease free	1+	Not available
19	t(12;16)(q13;p11)	right pelvis	Primary	Metastatic Disease	4+	Not available
20	normal cytogenetics	left thigh	Metastatic	Metastatic Disease	3+	Not available
21	t(12;16) with other complex cytogenetics	left axilla	Primary	Disease free	4+	Yes
22	t(12;16)(q13;p11)	left gluteal	Primary	Metastatic Disease	3+	Not available
23	t(15;17)(q22;q23)	left thigh	Primary	Disease free	3+	Yes

Patient	Cytogenetics	Site of primary tumor	Primary or Metastatic Specimen	Outcome	NY-ESO-1 Staining	qRT-PCR confirmed
24	t(12;16)(q13;p11)	right thigh	Primary	Disease free	3+	Yes
25	t(12;16) with other complex cytogenetics	left ankle/fibula	Primary	Metastatic Disease	2+	Yes

Scoring of staining is based on percentage of cells staining positive for NY-ESO-1: Focal <5%; 1+ 5-25%; 2+ 25-50%; 3+ 50-75%; 4+ >75%