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Brief Communication on MAGE-A4 and Coexpression of Cancer Testis Antigens in Metastatic Synovial Sarcomas: Considerations for Development of Immunotherapeutics

Hélène Vanacker, Robert Connacher,† Alexandra Meurgey,*
Julien Bollard,* Valéry Attignon,* Franck Tirode,* Myriam Jean-Denis,*
Mehdi Brahmi,* Jean-Yves Blay,* Ruoxi Wang,‡ Dennis Williams,†
and Armelle Dufresne**

Abstract: Therapeutic options for synovial sarcoma (SyS) have not evolved for several decades and the efficacy of second-line treatments is very limited. The expression of a large family of proteins known as cancer testis antigens (CTAs) in SyS has spurred the development of targeted T-cell therapies currently in clinical trials, such as those aimed at melanoma-associated antigen (MAGE)-A4 and New York esophageal squamous cell carcinoma 1 (NY-ESO-1), which have shown promising clinical efficacy. Extensive knowledge of the prevalence of expression and coexpression of CTAs is critical to design T-cell therapies with optimal coverage of the patient population. We analyzed the expression of CTAs of the MAGE-A family as well as NY-ESO-1 and preferentially expressed antigen in melanoma (PRAME) by RNA sequencing in a large cohort of 133 SyS samples from patients registered in the French sarcoma database (NETSARC+). Among MAGE-As, MAGE-A4 had the highest prevalence (65%), followed by MAGE-A10 (15%) and MAGE-A9 (13%). Almost all samples (92%) expressing any of the MAGE-As also expressed MAGE-A4. NY-ESO-1 was expressed in 65% of samples, with a large but incomplete overlap with MAGE-A4, whereas PRAME was present in 121 (91%) samples. Complementary immunohistochemical analyses were used to establish the positive correlation between RNA and protein expression for MAGE-A4 and NY-ESO-1. These data inform the strategy for optimal coverage of the SyS patient population with T-cell therapies, offering patients with SyS new options for single or combined second lines of treatment.

Key Words: cancer testis antigens, T-cell therapies, synovial sarcoma

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Synovial sarcoma (SyS) makes up ~5%–10% of soft tissue sarcomas, and patients with metastatic tumors have poor outcomes.¹ Historical 5-year overall survival rate for patients with metastatic disease was <10% in 1 systematic review,¹

and median overall survival from the start of second-line systemic therapy was 16 months in another retrospective study.² Thus, there is an unmet need for more impactful therapies.

SyS is immunologically “cold,” with limited T-cell infiltration, low numbers of antigen presenting cells, and low programmed death-ligand 1 expression,³ making effective use of immunotherapies (eg, checkpoint inhibitors) more challenging.⁴ Notably, SyS are known to exhibit high levels of cancer testis antigens (CTAs), which normally have restricted expression primarily within the testis. These include New York esophageal squamous cell carcinoma 1 (NY-ESO-1), preferentially expressed antigen in melanoma (PRAME), melanoma-associated antigen (MAGE) A4, taxol resistance-associated gene-3/chondrosarcoma-associated gene (TRAG-3/CSAGE), synovial sarcoma X (SSX), and membrane-associated phospholipase A1b [lipase member I (LIPI)].^{5–7} These CTAs provide a promising set of biomarkers for the development of engineered T-cell receptor (TCR) T-cell therapies for the treatment of SyS.

In recent clinical trials, therapies targeting MAGE-A4, MAGE-A3, and NY-ESO-1 have been used to treat patients with advanced/metastatic SyS, with promising clinical responses.^{7–11} Clinical trial data also indicate expression of MAGE-A4 is comparable in SyS primary and metastatic lesions.¹² Data describing the prevalence, expression, and coexpression of CTA levels in SyS are essential to understand potential clinical benefits from the development of CTA-targeting immunotherapy products, but are currently limited. We report CTA expression data from archival tissue samples from a large cohort of French patients with SyS based on RNA sequencing (RNASeq), validated with immunohistochemical (IHC) correlative analysis.

MATERIALS AND METHODS

This retrospective study was performed in accordance with Good Clinical Practice principles; all patients provided written consent according to local laws. Centre Léon-Bérard (CLB) Clinical Trial Review Committee reviewed/approved the study protocol/informed consent form (Ethical code R201-004-120, 18 Dec 2020). A study was performed on formalin-fixed paraffin-embedded (FFPE) samples from adult patients with histologic diagnosis of metastatic SyS confirmed by a sarcoma expert pathologist from Réseau de Référence en Pathologie des Sarcomes (RRePS) network. Patients were identified from the French NETSARC+ database (clinical reference network for soft-tissue/visceral sarcomas, including 28 expert centers). Whole-exome RNASeq of FFPE samples was performed as

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From the *Centre Léon-Bérard, Lyon, France; †Adaptimmune, Philadelphia, PA; and ‡Adaptimmune, Abingdon, Oxfordshire, UK.
Reprints: Robert Connacher, Adaptimmune, 351 Rouse Boulevard, Philadelphia, PA 19112 (e-mail: robert.connacher@adaptimmune.com).

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previously described.¹³ High-quality (HQ) RNASeq was defined as >10 million unique mapped reads. CTAs of interest (and the NCBI reference sequence code for the respective gene) were MAGE-A1 (NM_004988), MAGE-A2 (NM_005361), MAGE-A3 (NM_005362), MAGE-A4 (NM_001011548), MAGE-A6 (NM_005363), MAGE-A8 (NM_001166400), MAGE-A9 (NM_005365), MAGE-A11 (NM_005366), MAGE-A12 (NM_001166386), MAGE-B2 (NM_002364), NY-ESO-1 (NM_001327), PRAME (NM_206953), and MAGE-A10 (NM_001011543). RNASeq values are expressed as log₂(transcripts per million [TPM]+1). Log₂(TPM+1) cutoffs used for positive expression were 1.62 (MAGE-A4), 3.12 (NY-ESO-1), and 4.05 (PRAME) (as described in Supplemental Methods, Supplemental Digital Content 1, <http://links.lww.com/JIT/A887>).

MAGE-A4 and NY-ESO-1 IHC assays based on concordant with the clinical trial assays used to assess enrollment eligibility in clinical trials of MAGE-A4- and NY-ESO-1-targeting TCR T-cell therapies were developed/validated at CLB. A trained/certified CLB pathologist performed MAGE-A4/NY-ESO-1 P-scoring on glass. A tumor was considered MAGE-A4/NY-ESO-1 positive by IHC if P-score met cutoff of ≥30% tumor cells at ≥2+ intensity.

Detailed methods of patient selection, IHC scoring, IHC tumor sample quality control criteria, and RNASeq are shown in Supplemental Methods (Supplemental Digital Content 1, <http://links.lww.com/JIT/A887>).

RESULTS

Expression of MAGE-A4 by IHC/RNASeq

The IHC assay developed at CLB stained sections from 146 FFPE SyS samples. RNASeq data were obtained from 133 FFPE samples, 132 of which also had IHC data (Supplemental Fig. 1, Supplemental Digital Content 2, <http://links.lww.com/JIT/A888>). HQ RNASeq data were available for 105/133 samples. The clinical trial and CLB MAGE-A4 IHC assays displayed very good diagnostic

concordance, both using a positivity cutoff of 30% of tumor cells with staining intensity ≥2+ (not shown).

The correlation between CLB IHC P-score and RNASeq was very good ($R^2=0.4873$; Fig. 1A). Log₂(TPM+1) of 1.62 gave best concordance between IHC and RNASeq, with the same percentage of positive samples, sensitivity 89.3%, specificity 79.1%, positive predictive value 88.2%, and negative predictive value 80.9%.

Expression of NY-ESO-1 by IHC/RNASeq

The correlation between IHC P-score and RNASeq was also very good ($R^2=0.7256$), which resulted in log₂(TPM+1) of 3.12 for best concordance of these assays (Fig. 1B).

Expression of CTAs in SyS

Analysis of RNASeq data showed that PRAME, NY-ESO-1, and MAGE-A4 had highest prevalence of expression in SyS (Fig. 2A). Using defined log₂(TPM+1) positivity cutoffs for MAGE-A4, NY-ESO-1, and PRAME (Supplemental Fig. 2, Supplemental Digital Content 3, <http://links.lww.com/JIT/A889>), and 1.0 for all other CTAs, number of positive samples out of 133 was 121 (PRAME; 91.0%), 87 (NY-ESO-1; 65.4%), 87 for MAGE-A4; 65.4%), 20 (MAGE-A10; 15.0%), 17 (MAGE-A9; 12.8%), 9 (MAGE-A1; 6.8%), 8 (MAGE-A2; 6.0%), 7 (MAGE-A11; 5.3%), 3 (MAGE-A3; 2.3%), 3 (MAGE-A6; 2.3%), and 1 (MAGE-A12, MAGE-B2; 1%). No samples expressed MAGE-A8. Using log₂(TPM+1) positivity cutoff of 1 for MAGE-A4, number of positive samples was 98 (73.7%).

We then looked at the expression of different MAGE-As (Fig. 2B). After samples expressing only MAGE-A4 (n=52; n_{HQ}=42), most abundant sample types only expressed MAGE-A4 and MAGE-A10 (n=10; n_{HQ}=8), followed by those coexpressing MAGE-A4 and MAGE-A9 (n=8; n_{HQ}=5). In fact, most samples positive for any MAGE-As (n=95; n_{HQ}=73) were positive for MAGE-A4 (n=87, 92%; n_{HQ}=68, 93%). Only 7 samples expressed MAGE-A9 (n=5; n_{HQ}=3), MAGE-A2 (n=1; n_{HQ}=1), or MAGE-A11 (n=1; n_{HQ}=1) and not

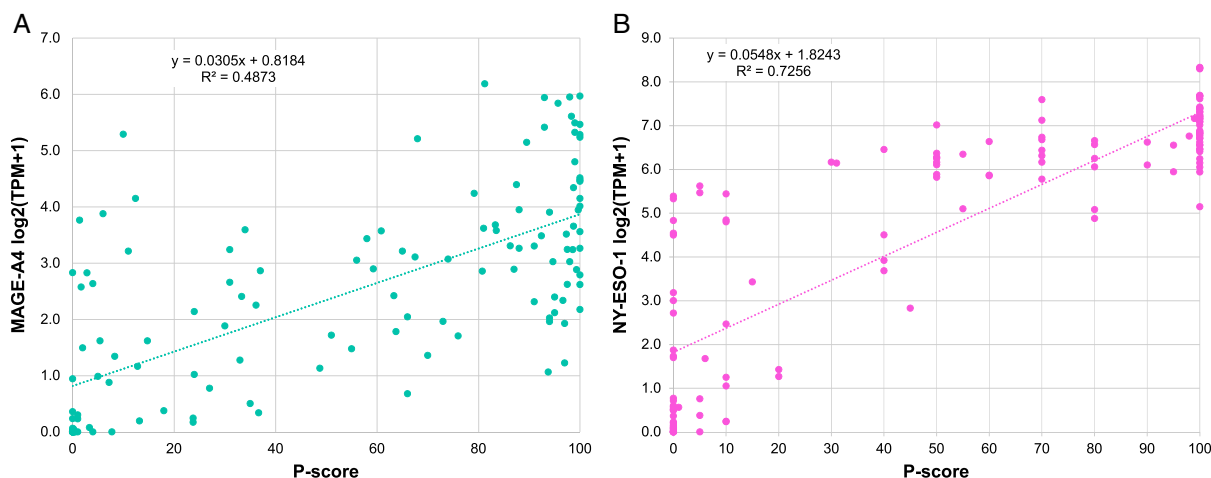


FIGURE 1. Correlation of IHC and RNASeq analysis of serially sectioned tissue from 132 SyS FFPE tissue blocks. (A) Positive correlation of MAGE-A4 IHC scoring (P-score, percentage of tumor cells with staining intensity ≥2+) to corresponding sections assessed for MAGE-A4 expression by RNASeq. (B) Positive correlation of NY-ESO-1 IHC scoring (P-score) to corresponding sections assessed for NY-ESO-1 expression by RNASeq. P score was expressed as a continuous variable for MAGE-A4 but was reported in 5% increments for NY-ESO-1. FFPE indicates formalin-fixed paraffin-embedded; IHC, immunohistochemical; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1; RNASeq, RNA sequencing; SyS, synovial sarcoma; TPM, transcripts per million.

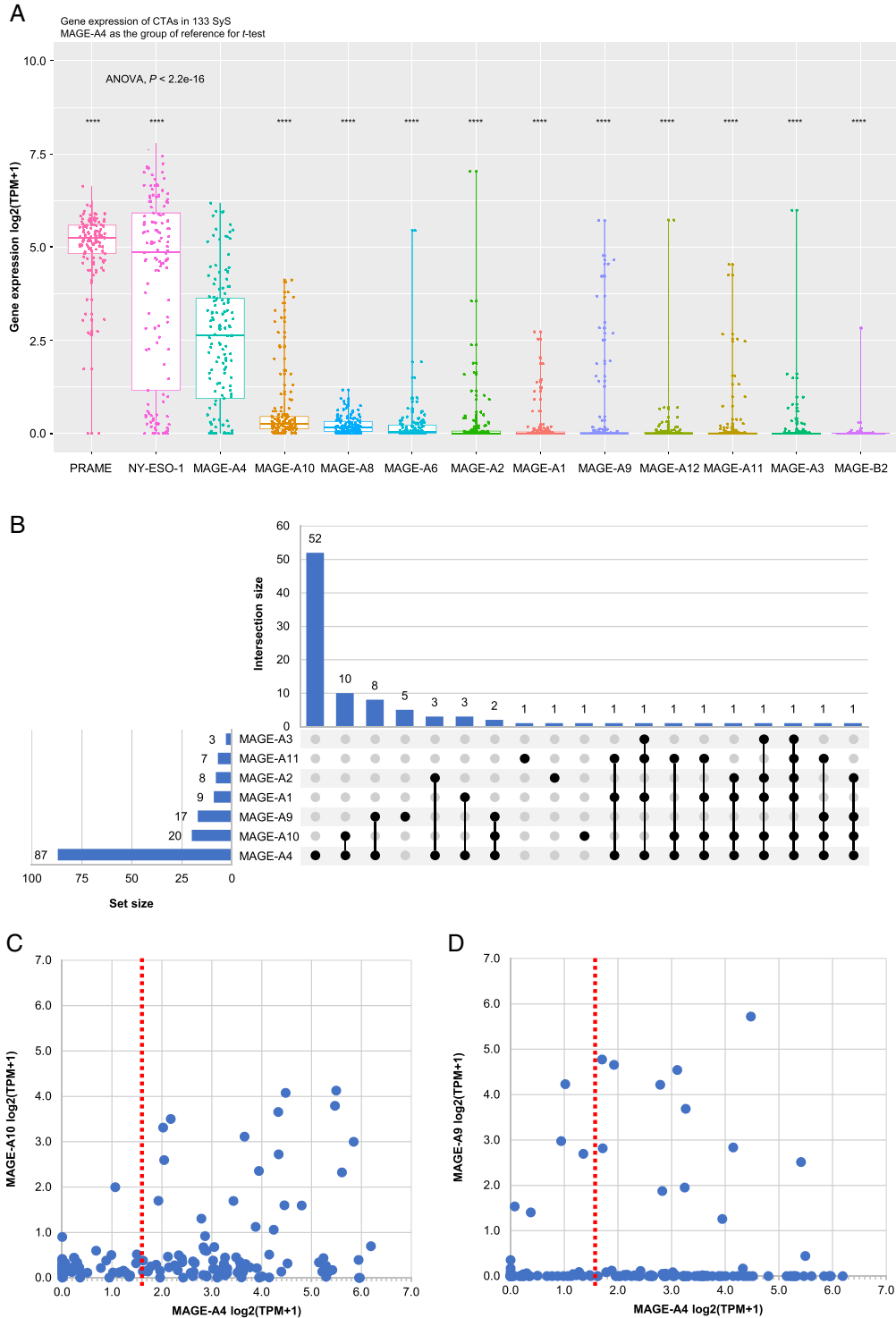


FIGURE 2. (A) Gene expression analysis of CTAs by RNASeq from FFPE tissue of 133 SyS samples. The box plot represents the standard deviation and median of each individual biomarker. Samples with **** above box plot denote significance by ANOVA analysis $P < 2.2e-16$. (B) Upset plot containing a subset of 95 samples with any MAGE family RNASeq expression above defined cutoffs. Cutoffs were 1.62 for MAGE-A4 and 1.0 for all other MAGE genes. Numbers above intersection size bars denote the total number of samples above the cutoff(s) for MAGE gene(s) in the dot plot below. Numbers to the left of set size bars denote the total number of samples above the cutoff for the designated individual MAGE gene. (C and D) Scatter plots of 133 SyS samples comparing relative RNASeq expression of MAGE-A4 with either MAGE-A10 or MAGE-A9. The red dotted line denotes the MAGE-A4 cutoff for positivity at 1.62. ANOVA indicates analysis of variance; CTA, cancer testis antigen; FFPE, formalin-fixed paraffin-embedded; MAGE, melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PRAME, preferentially expressed antigen in melanoma; RNASeq, RNA sequencing; SyS, synovial sarcoma; TPM, transcripts per million.

MAGE-A4 or any other MAGEs. Likewise, the only sample positive for MAGE-B2 did not express any MAGE-As (not shown). Nineteen of 20 samples expressing MAGE-A10 also expressed MAGE-A4, with higher expression of MAGE-A4 in 16 (Fig. 2C). Of 12 samples coexpressing MAGE-A4 and MAGE-A9, 5 had higher expression of MAGE-A4 (Fig. 2D).

Coexpression of MAGE-A4, NY-ESO-1, and PRAME

We next looked at the coexpression of the most frequently expressed CTAs (PRAME, NY-ESO-1, and MAGE-A4). Of the 133 samples, 104 (78.2%) were MAGE-A4 and/or NY-ESO-1 positive using defined cutoffs (Fig. 3A). Seventeen samples (12.8%) were positive for MAGE-A4 but negative for NY-ESO-1, 17 (12.8%) vice versa, 29 (21.8%) were negative for both, and the remaining 70 (52.6%) were positive for both (Fig. 3A). We then looked at the 126 samples that expressed any one of PRAME, NY-ESO-1, or MAGE-A4. Among 121 samples expressing PRAME, 14 coexpressed MAGE-A4, 16 coexpressed NY-ESO-1, and 69 both (Fig. 3B).

DISCUSSION

With several immunotherapies targeting specific CTAs undergoing clinical trials, the question of how to effectively treat the largest percentage of patients with SyS remains. This study provides one of the largest SyS data sets to date, confirming mounting evidence that MAGE-A4, PRAME, and NY-ESO-1 CTAs are widely present and can be coexpressed.^{5,14} In all, 78.2% of samples were positive for MAGE-A4 and/or NY-ESO-1, impacting the number of patients with SyS eligible to receive investigational TCR T-cell therapies in ongoing clinical trials (afamitresgene autoleucel or uzatresgene autoleucel targeting MAGE-A4, letetresgene autoleucel targeting NY-ESO-1) according to

defined expression cutoffs. With TCR T-cell therapies providing promising responses in the clinic, there is potential that targeting multiple CTAs would provide added benefit. Conceptually, a large subset of patients may benefit from sequential and/or simultaneous TCR-based therapy, and if distinct CTAs are expressed in different tumor cells, the benefit to patients could be greater. It is interesting to note that for those samples below the MAGE-A4 cutoff, PRAME and, to a lesser extent, NY-ESO-1, are positive, opening the door to more personalized, patient-specific treatments. Notably, in this study, RNASeq cutoffs used for PRAME were not correlated to any IHC scoring, and prevalence for any CTA could change by a defined cutoff level. When considering sequential TCR T-cell therapies, it may be valuable to balance previous patient response rates and the degree of positivity of each CTA biomarker to determine their priority. Of course, unique assays with specified cutoffs for these CTAs to determine patient eligibility may further shape enrollment decision-making/rationale for how best to treat subpopulations.

Of interest, 4 samples did not exhibit RNASeq expression above defined cutoffs for any CTA measured, which suggests small subpopulations may require novel CTA targeting. To delineate the heterogeneity of sarcoma CTA expression, additional studies examining other CTAs expressed in sarcomas (TRAG-3/CSAGE, SSX, LIPI)⁷ could shed light on these unique cases. Notably, MAGE-A4 was positive in most cases where another MAGE family member was above its cutoff for both the 105 HQ and 133 total RNASeq sample set. Indeed, for MAGE-A10, the second-most prevalent MAGE in these patients, only 1 positive sample was below cutoff for MAGE-A4. This study also shows a good correlation between MAGE-A4 and NY-ESO-1 RNASeq to CLB IHC assay data, justifying the use of IHC as a valid alternative to RNAseq to identify SyS patients eligible for T-cell therapy. Although our study also demonstrates that SyS can be MAGE-A4 negative and

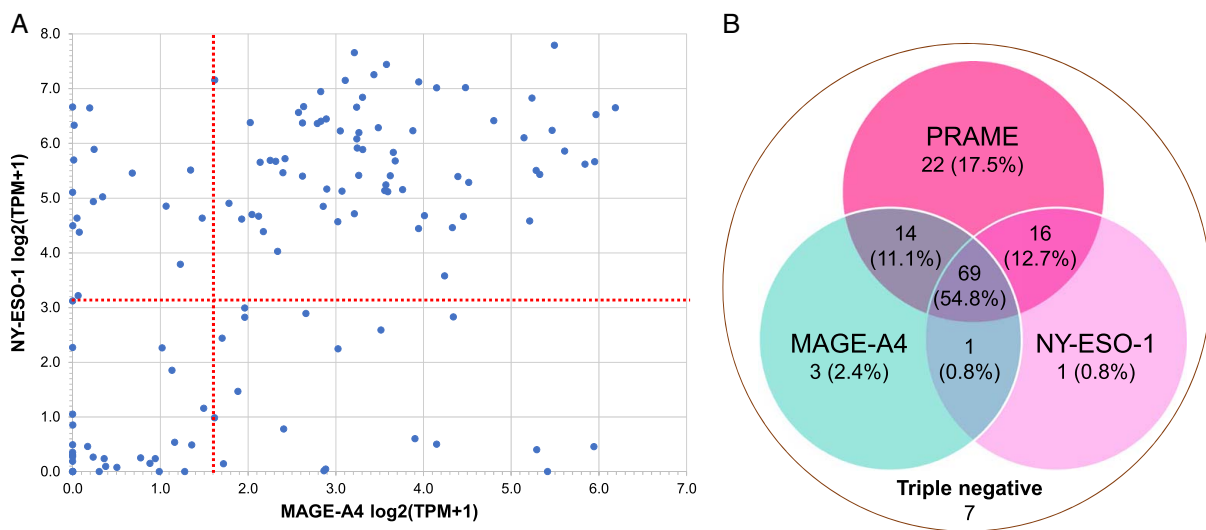


FIGURE 3. (A) Scatter plot of 133 SyS samples comparing relative RNASeq expression of NY-ESO-1 with MAGE-A4. The horizontal red dotted line denotes the NY-ESO-1 cutoff for positivity at 3.12 and the vertical red dotted line denotes the MAGE-A4 cutoff for positivity at 1.62. (B) Venn diagram containing 126 SyS samples above minimum defined log₂(TPM+1) RNASeq cutoffs for either MAGE-A4 (1.62), NY-ESO-1 (3.12), or PRAME (4.05), demonstrating uniquely positive and coexpressing populations. Seven patients were triple negative for MAGE-A4, NY-ESO-1, and PRAME. MAGE indicates melanoma-associated antigen; NY-ESO-1, New York esophageal squamous cell carcinoma 1; PRAME, preferentially expressed antigen in melanoma; RNASeq, RNA sequencing; TPM, transcripts per million.

above the cutoff for another MAGE, developing TCR therapies targeting these other MAGEs may not be warranted due to low volume and because most samples were also NY-ESO-1 and/or PRAME positive. Nevertheless, TCRs with specific cytotoxicity targeting MAGE-A1, MAGE-A3, MAGE-A6, and MAGE-A9 within tumor cell lines have been tested preclinically.¹⁵ In sum, this real-world report provides compelling evidence that TCR T-cell therapies targeting MAGE-A4, NY-ESO-1, and PRAME have the potential to treat most SyS cases, including as combination therapy, either sequentially or simultaneously, in patients positive for more than one of these CTAs. Furthermore, these data suggest the need for future studies examining the dynamics of CTA expression after sequential or simultaneous TCR T-cell therapies, along with clinical responses.

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Conflicts of Interest/Financial Disclosures

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