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Original Article

Optimizing peptide epitope-based autoantibody detection in cancer patients

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Abstract: Autoantibody (autoAb) response is an important arm of endogenously arising anti-tumor immune responses, and has received new attention as a cancer biomarker with the recent success of immune check-point inhibitor therapy. Our laboratory has been focusing on measuring autoAb against B-cell epitopes in order to bypass the necessity to purify a panel of recombinant proteins. In order to optimize peptide-based autoAb measurement and to increase sensitivities to cover more patients, we developed a new approach of using mixed peptides to conjugate on the same microsphere and compared its results with the use of a dominant peptide epitope using Luminex microbead-based multiplex assays. The peptide epitopes of two cancer/germline antigens, New York esophageal cancer antigen-1 (NY-ESO-1) and X antigen family member-1b (XAGE-1b), and cancer/stem cell antigen, sex determining region Y-box-2 (SOX2), were used as prototypes in this study. Our results indicate that using mixed peptides of B-cell epitopes improves the sensitivity of detecting more patients with autoAb responses. Thus, when the full-length protein is not available for conjugating onto microspheres, a mixture of B-cell epitopes is the method of choice for using Luminex multiplex assay to detect autoAb response in cancer patients.

Keywords: Tumor-associated antigen, autoantibody, biomarker, immune monitoring, B cell epitope, multiplex assay

Introduction

Recent successes in immune check-point inhibitors have demonstrated the power of tipping the balance of endogenously arising anti-tumor immune responses in vivo [1-3]. AutoAb response is an important arm of endogenously arising anti-tumor immune responses, and has also received new attention as a cancer biomarker candidate for prostate cancer, lung cancer and others [4]. Due to the significant heterogeneity of tumor-associated antigens (TAA) present in cancer patients, biomarker studies usually rely on measuring autoAb against a panel of TAA using a multiplex approach. The recent development of Luminex microbead-based multiplex assay provides a simple solution to measure autoAb responses against a panel of antigens for cancer, transplantation, infectious diseases, and other indications [5-7]. However, the requirement for purifying a large panel of recombinant proteins is difficult to achieve for most laboratories. Our laboratory has been focusing on bypassing such impediments through the development of a multiplex assay using dominant B cell-epitopes [5, 8, 9].

Even though dominant epitope-based autoAb in tandem with the standard Prostate-specific Antige (PSA) assay has been shown to improve accuracy in prostate cancer diagnosis, one significant hurdle in the dominant epitope-based assay, however, is the loss of autoAb responses against less dominant peptide epitopes or conformational epitopes [5, 8, 9]. Using classic cancer/germline antigens, NY-ESO-1 and XAGE-1b, and a cancer/stem cell antigen, SOX2, as prototypes [10], our laboratory is attempting to answer the question whether a dominant B-cell epitope or a mixture of B-cell epitope peptides may substitute the full-length protein in measuring autoAb against TAA without sacrificing sensitivity and specificity for prostate cancer, lung cancer and others which share these anti-

Table 1. Serum samples used in autoAb studies

Sample Type	Total Number of Sera
Prostate Cancer	101
Lung Cancer	32
Healthy Donor	8
Positive Control to	10
NY-ESO-1	4
SOX2	2
XAGE-1b	4
Total	151

gens. An optimized approach based on dominant B-cell epitopes may have implications in areas such as cancer biomarkers, transplantation, infectious diseases, and others areas requiring multiplex evaluation of Ab or autoAb against a panel of antigens.

Materials and methods

Serum samples

Serum samples were collected from subjects who received informed consent under institutional review board-approved protocols from UCLA and collaborating hospitals, and stored at -80°C until use. Serum samples from healthy donors (HD) were obtained from subjects routinely screened to exclude the presence of concomitant diseases and cancer patient serum samples were collected at time of biopsy and prior to surgery (**Table 1**). Positive controls were based on previous screening results [5].

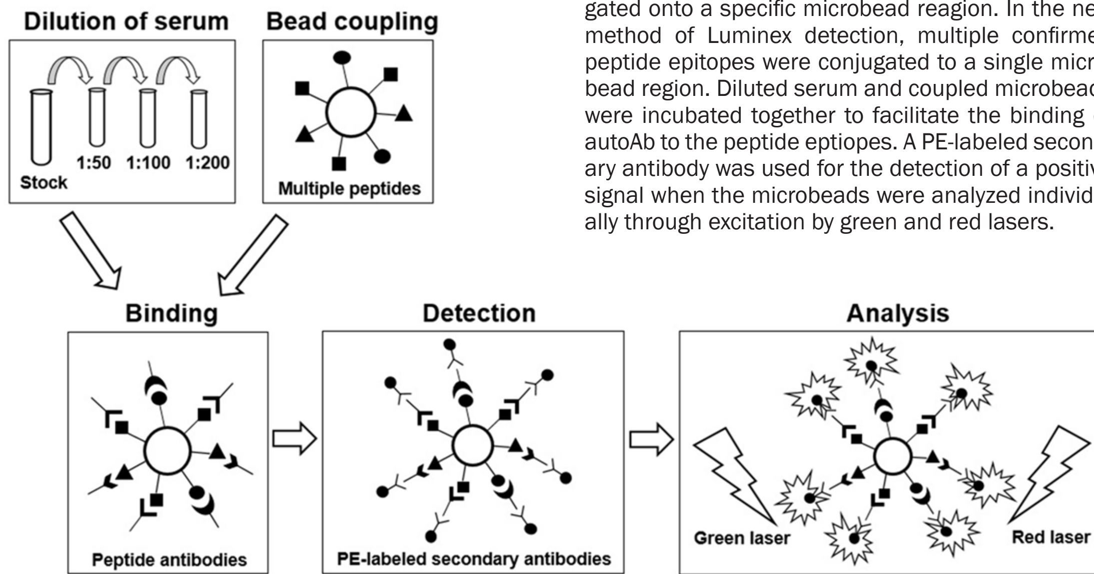
Luminex microbeads-based assay

Serum samples were screened using Luminex microsphere-based assays (Austin, TX). Peptide epitopes from prototype antigens, NY-ESO-1, SOX2, and XAGE-1b (Genemed Synthesis, San Antonio, TX), and a control random peptide sequence (Genscript, Piscataway, NJ) were conjugated onto microbeads (Bio-rad, Hercules, CA) by using sulfo-NHS (Thermo Fisher, Waltham, MA) to convert carboxyl groups on the microbeads to amine-reactive esters, and EDC (Thermo Fisher) to couple the ester to primary amine groups on the peptides (**Figure 1**). Peptides were conjugated onto the microbeads at 20 μg per 1.0×10^6 microspheres (**Table 2**).

Serum samples were diluted at 1:10, 1:50, and 1:200 in assay buffer (PBS, 1% BSA). Then they were incubated with 2,500 beads per region of conjugated microspheres per 100 μl in a well of 96-well plates (Bio-rad) for 1 hour at room temperature with gentle agitation. After, the plates were washed 3 times with wash buffer (PBS, 1.0% Bovine Serum Albumin, 0.1% Sodium azide, 0.05% Tween-20) using a magnetic plate washer (Bio-rad). Secondary antibody, PE-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA), was added to the wells and the plates were then incubated for 1 hour at room temperature with gentle agitation. The plates were once again washed 3 times with wash buffer and fluorescence intensity (FI) was recorded using the Bio-Plex 200 system (Bio-rad). FI of auto-antigen epitope-conjugated microspheres were compared to those of a baseline random peptide control by taking the ratio of fluorescence intensity (RFI). The RFI of patient serums were compared to those of healthy donor serum, and a positive reaction was defined as RFIs that were 3 standard deviations above the mean, same criteria used for ELISA as previously published [11, 12].

ELISA

Positive serum samples detected by Luminex screening were confirmed using ELISA. Antigen-coated Nunc ELISA plates (eBioscience, San Diego, CA) were prepared using 50 ng/well of purified NY-ESO-1, 250 ng/well of SOX2 protein, and 100 ng/well of control BSA protein in 100 μL of carbonate bicarbonate buffer. The ELISA plates were also coated with 60 ng/well of full-length XAGE-1b peptide and 60 ng/well of control randomized synthetic peptide in 100 μL of carbonate bicarbonate buffer. The coated plates were then left to incubate overnight at 4°C to allow for the absorbance of the antigens onto the plates. The next day, the plates were blocked with 5% fetal bovine serum (FBS) in PBS + 0.05% Tween 20 (PBST) for 2 hours, washed with PBST, and loaded with 100 μL of diluted serum samples. Serum samples were pre-diluted at 1:10, 1:20, and 1:50 with 5% FBS in PBST. The serum samples, once loaded onto the pre-coated ELISA plates, were left to incubate for 2 hours at room temperature. The plates were then washed again with PBST and loaded with secondary antibody, HRP-conjugated goat anti-human IgG (Sigma Aldrich,



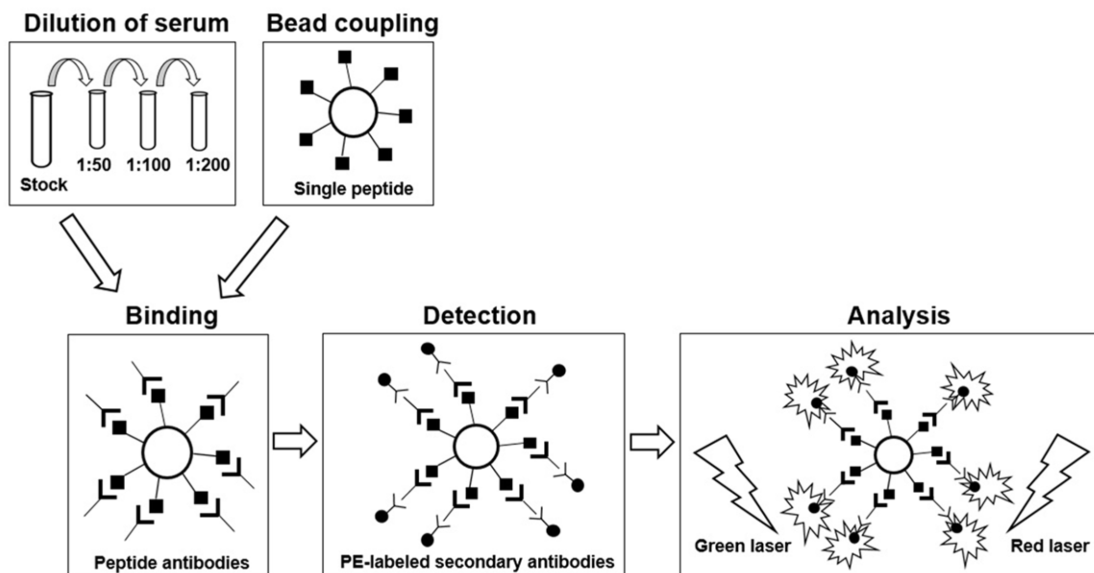


Figure 1. Comparison of the Luminex approaches based on mixed peptides and single peptide conjugated onto a specific microbead reagion. In the new method of Luminex detection, multiple confirmed peptide epitopes were conjugated to a single microbead region. Diluted serum and coupled microbeads were incubated together to facilitate the binding of autoAb to the peptide eptiopes. A PE-labeled secondary antibody was used for the detection of a positive signal when the microbeads were analyzed individually through excitation by green and red lasers.

St. Louis, MO), that was diluted with 5% FBS in PBST. The plates were developed after 1 hour of incubation, after which absorbance (OD) at 450 nm was recorded. The difference in OD was calculated by subtracting the OD of the BSA protein or control peptide from the OD of the proteins or peptide of interest: NY-ESO-1 protein, SOX2 protein, and XAGE-1b full-length protein. Positive reactions were defined as ΔOD that were 3 standard deviations above the mean [11, 12].

Western blot

Serum samples that were positive for autoAb response as determined by Luminex, but not confirmed by ELISA, were tested by Western blotting. Purified NY-ESO-1 protein and NY-ESO-1 expressing cell lysates from transfected 293T cells as well as purified proteins of SOX2 from 2 sources (Genscript and Genemed Synthesis), were run on 4-12% Bis-Tris SDS gels (Thermo Fisher) alongside negative control,

Table 2. Conjugation of peptides of mangetic microbeads with a summary of detection results

Microbead Region	Analyte Peptide Epitope: Amino Acid Residues	Number of positive sera
18	Control peptide of 40 mer random sequence	0
19	NY-ESO-1: 1-40	6
25	NY-ESO-1: 90-130	5
26	NY-ESO-1: 120-160	3
27	NY-ESO-1: 150-180	4
29	NY-ESO-1: 1-40 and 90-130	14
35	NY-ESO-1: 120-160 and 150-180	10
37	NY-ESO-1: 1-40, 90-130, and 120-160	7
43	NY-ESO-1: 1-40, 90-130, 120-160, and 150-180	16
45	SOX2: 52-87	1
46	SOX2: 98-124	2
53	SOX2: 52-87 and 98-124	4
55	XAGE-1b: 1-25	4
62	XAGE-1b: 57-81	2
63	XAGE-1b: 1-25 and 57-81	4
65	XAGE-1b: full-length 1-81	11

Table 3. Patients with positive autoAb responses as detected by Luminex screening

Prostate Cancer Patient (n=101)	Analytes		
	NY-ESO-1	SOX2	XAGE-1b
#11			+
#13	+	+	+
#53	+	+	
#62	+		+
#79	+	+	
#82	+		
#98	+		
#100	+		
#107			+
#110	+		+
#132	+		
#135	+		
#138		+	+
#139	+		+
#143			+
#148	+		+
Total	12	4	9
Lung Cancer Patient (n=32)	**Analytes**		
	NY-ESO-1	SOX2	XAGE-1b
#22			+
#27	+		
#31	+		
#34	+		
#48	+		+
Total	4	0	2

293T cell lysate. Following separation, the proteins were transferred to a PVDF membrane (Thermo Fisher). The membrane was blocked overnight at 4°C using 5% milk in PBST. After, the membrane was washed with PBST and then incubated for 1 hour at room temperature with serum samples diluted at 1:1000 in blocking buffer (5% milk in PBST, 0.1% SDS was added to reduce reaction background). Secondary antibody, HRP-conjugated goat anti-human IgG (Jackson Immuno Research), was diluted in 5% milk in PBST and applied to the membrane for 1 hour at room temperature. The membrane was developed using ECL Western Development Kit (Thermo Fisher).

Results

Combination of peptide epitopes improves sensitivities to detect autoAb by using a single dominant peptide epitope

A single dominant B-cell epitope was previously used to detect autoAb response against NY-ESO-1. However, the use of a single peptide prevents the detection of autoAb against less dominant epitopes. We thus developed an approach using a combination of peptide epitopes to be conjugated onto the same microbeads (**Figure 1**) and compared these results with the results using a single dominant peptide epitope in order to cover more patients. Initial experiments resulted in increased sensitivities against NY-ESO-1 and SOX2 autoAb using the mixture of peptide epitopes over a single domi-

nant peptide epitope (**Tables 2** and **3**). When only the dominant B-cell epitope, NY-ESO-1:1-40, was used, 6 patients were determined sero-positive for autoAb against NY-ESO-1. When using a mixture of peptide epitopes containing the dominant epitope, NY-ESO-1:1-40, as well as 3 others, NY-ESO-1:90-130, 120-160, and 150-180, 16 patients were determined sero-positive for autoAb against NY-ESO-1. Similarly, increased sensitivity, when using a combination of peptide epitopes, was observed with SOX2 (**Tables 2** and **3**).

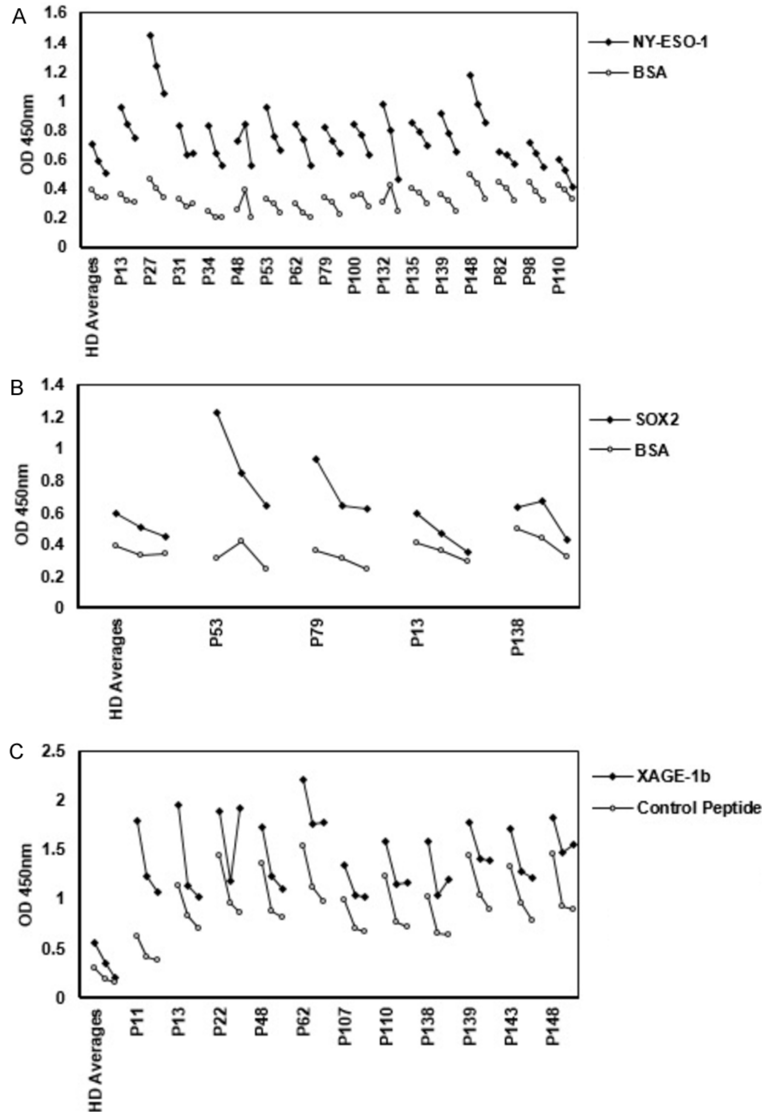


Figure 2. ELISA confirmation of positive sera as determined by Luminex. Patient sera positive for (A) NY-ESO-1, (B) SOX2, and (C) XAGE-1b were diluted at 1:10, 1:20, and 1:50 and the OD at 450 nm was compared to that of control BSA for proteins, NY-ESO-1 and SOX2, and control randomized peptide for XAGE-1b. Shown in the figure are all samples that have exceeded the crtiteria for being positive described in Materials and Methods and previous publications [11, 12].

While the incorporation of less dominant epitopes was able to increase the sensitivity of the assay for NY-ESO-1 and SOX2, the full length protein peptide was most sensitive for XAGE-1b autoAb. The mixture of peptide epitopes detected 16 (10.6%) positives for NY-ESO-1 autoAb and 4 (2.7%) positives for SOX2 autoAb, while the full length protein detected 11 (7.3%) positives for XAGE-1b autoAb out of 151 samples screened (**Tables 2** and **3**). Due to significant aggregation, the full-length NY-ESO-1 and SOX2 proteins failed to conjugate onto the microspheres under the current conditions.

Confirmation of positive sera by ELISA and Western blot

ELISA is the most commonly used assay to detect protein targets in blood sera. To verify autoAb against NY-ESO-1, SOX2, and XAGE-1b determined by the new mixed peptide approach using the Luminex assay, sero-positive samples were also screened using full-length proteins in ELISA (**Figure 2**). Of the 16 sero-positive for NY-ESO-1 autoAb, 13 were also positive against the full-length NY-ESO-1 protein using ELISA (**Figure 2A**). About 2 out of 4 positive sera for SOX2 autoAb were also verified against full-length SOX2 protein by ELISA (**Figure 2B**), and all 11 positives for XAGE-1b autoAb were confirmed by ELISA (**Figure 2C**).

The remaining sera were then tested by Western blot. Purified proteins of NY-ESO-1 and SOX2, as well as the cell lysate of transfected 293T cells for NY-ESO-1 expression and cell lysate for unaltered 293T cells, were loaded and run through SDS-PAGE. Western blots verified the presence of autoAb against NY-ESO-1 (**Figure 3A**) and SOX2 (**Figure 3B**) in the patient sera, and thus independently verified the pres-

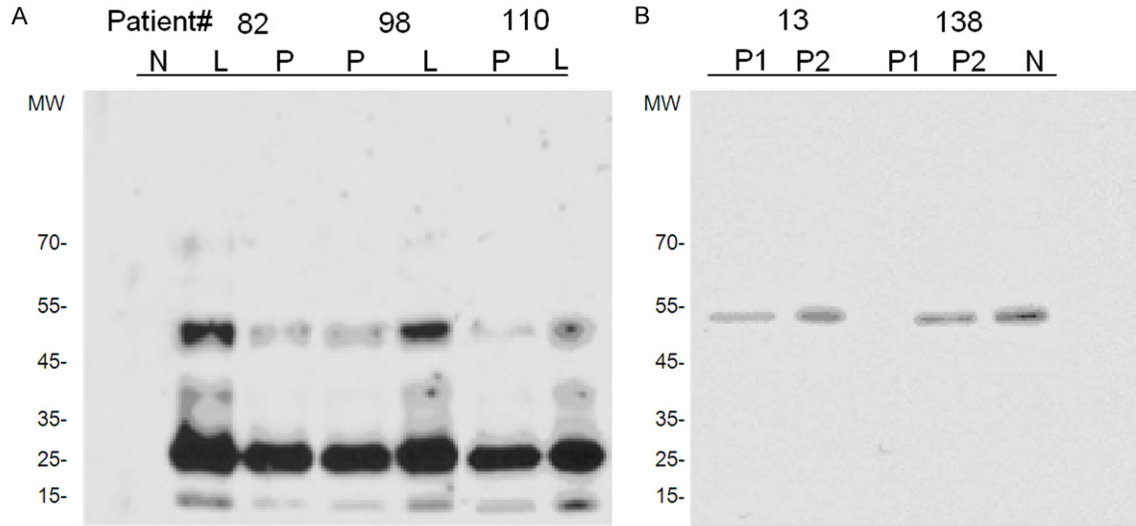


Figure 3. Western blot confirmation of positive sera as determined by Luminex. A: Based on the results from the multiplex Luminex assay, NY-ESO-1 sero-positive patients #82, 98, and 110 were used against NY-ESO-1 transfected 293T cell lysate (L) and purified recombinant protein of NY-ESO-1 (P) on a PVFD membrane. B: Similarly, SOX2 sero-positive patients #13 and 138 were used against SOX2 proteins (P1 and P2 from different resources) on a PVDF membrane. In each panel, a positive serum was used against 293 lysate to show a negative reaction on the far left and far right lanes labeled with N, respectively. Molecular weights for proteins were marked in kDa on the side.

Table 4. NY-ESO-1 and XAGE-1b protein/mRNA levels and spontaneous autoAb responses

Subjects Based on IHC staining	XAGE-1b mRNA Copies	XAGE-1b Ab	NY-ESO-1 mRNA Copies	NY-ESO-1 Ab*
NY-ESO-1$^+$/XAGE-1b$^-$				
Z007	1814	-	771	+/-
Z001	748	-	4170	+
Z012	5218	-	202	-
Z006	7.6e5	-	319	+/-
NY-ESO-1$^-$/XAGE-1b$^+$				
Z008	9.84e5	-	41	++
Z002	1.4e6	-	2	-
Z011	4.39e5	+/-	50	-
Z005	2.85e6	-	1	+
NY-ESO-1$^-$/XAGE-1b$^-$				
Z009	0	-	0	-
Z003	10	-	0	-
Z010	86	-	6	-

*Spontaneous autoAb is indicated with "++, +, and +/-" to show decreased levels of responses; whereas "-"indicates no detectable responses.

ence of autoAb initially identified by the new mixed peptide approach under the Luminex microsphere platform.

Discussion

Previous studies of autoAb against TAA have been limited by both technology [13, 14] and analytes of interest, which were shared with autoimmune conditions rather than being tumor-specific [15-17]. The utilization of the Luminex platform allows for the detection of multiple analytes in a single controlled assay, and is compatible with the detection of both nucleic acids and proteins. Our lab has recently shown the detection of autoAb against 6 TAAs in conjuction with PSA in a single A+PSA assay

with improved sensitivity and specificity over PSA alone [5].

The current pilot study addressed several observations that will require further investigation. Among them was the potential of using peptides, rather than whole proteins, to detect autoAb in human serum. For several of the TAAs on our panel, more than one epitope is recognized by the autoAb, which poses the question of how the peptides of these epitopes should be conjugated onto the microbeads for maximum detection by Luminex.

The addition of less dominant B-cell epitopes to form a mixture of epitopes conjugated to a single microbead was the most sensitive option for detecting autoAb against NY-ESO-1 and SOX2 in patient serum using the Luminex assay. However, the mixture of peptides did not seem to be as effective when compared to the full length protein peptide, as in the case of XAGE-1b. The short length of XAGE-1b, at 81 amino acids, facilitated the synthesis of the full length protein peptide, but the synthesis of full protein peptide of other markers, such as NY-ESO-1 and SOX2, can be difficult. Addition of more peptide epitopes or synthesis of peptide epitopes of varying lengths, such as the combination of 2 epitopes into one peptide, can be further explored to mimic the effects of a full length protein peptide.

Although the ELISA assay failed to confirm a few of the sera deemed positive for autoAb by the Luminex assay, these sera were later verified by Western blotting. The difference in detection between the ELISA and Luminex methods could be the result of heightened sensitivity in the Luminex assay platform. The microbead suspension allows for the three-dimensional coating of the bead with the peptide of interest, as well as three-dimensional access to potential recognition sites of the autoAb to the peptide epitope, another innovation that has improved the sensitivity for the detection of autoAb response in serum samples. Technical advances also allow us to investigate whether development of autoAb responses correlate with expression of TAA. As shown in **Table 4**, serum mRNA levels of TAA correlated well with immunohistochemistry (IHC) staining for both XAGE-1b and NY-ESO-1. However, patients who developed autoAb responses were not necessarily those with higher levels of mRNA and protein expressions.

Besides NY-ESO-1, SOX2, and XAGE-1b, the flexibility of the Luminex assay allows for the incorporation of other peptide epitopes to the panel of analytes. This platform has now been used as part of the "A+PSA" and other immunodiagnostic assays developed in our laboratory.

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Disclosure of conflict of interest

None.

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