A Proteomic Approach to Discover Tumor Specific Peptides for Cancer Immunotherapy

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Abstract

One of the greatest challenges in cancer immunotherapy is to identify cancer specific antigens. Intracellular tumor specific proteins are processed as peptides and presented by Human Leukocyte Antigen (HLA) on cell surface as peptide-HLA complexes, which can serve as cancer-specific antigens. MAGE-A3 is an intracellular cancer protein present in about 60-70% of melanoma patients. There are thousands of other intracellular proteins, and the delineation of the specific processes of proteolysis and peptide presentation remains elusive. In order to generate antibodies for immunotherapy, the sequence of the peptide presented in the HLA-peptide complex needs to be known. The objective of this study was to use mass spectrometry to identify and quantify MAGE-A3 tumor-specific peptides presented by HLA. HLA peptides are isolated from xenograft myeloma tumors using an optimized workflow including immunoprecipitation. Reverse phase separation, a discovery method, detects 3,000~ unique HLA peptides per mass spectrometry analysis, and a MAGE-A3 peptide was identified via Mascot Distiller Database. Using a targeted method, more sensitive than discovery method, monitoring specifically the MAGE-A3 peptide, we successfully quantified 720~ peptide copies per cell surface. In summary, tumor specific MAGE-A3 HLA-peptides were successfully isolated, identified and quantified, and are going to be used to generate bispecific antibodies, targeting HLA-peptide complex on cancer cells and CD3+ receptor on T cells, for cancer immunotherapy.
Review of Literature

Cancer is the second leading cause of death in the United States. According to the National Cancer Institute, there will be 18.1 million cancer survivors in 2020, 30% more than 2010, and the costs of cancer care will increase to $157 billion in 2010 (Mariotto AB, et al., 2011). Antibody therapeutics have been used for cancer immunotherapy for over the past two decades. Antibodies that can specifically bind tumor surface antigens, can be applied successfully as guided missiles to deliver potent cytotoxic drugs in the form of antibody drug conjugates (ADCs) and bispecific antibodies (Figure 1). One of the greatest challenges in cancer immunotherapy is the identification of new and safe cancer antigens, which can be targeted by antibodies.

Peptides presented by human leukocyte antigen (HLA) molecules on the cell surface play a crucial role in adaptive immunology, mediating the communication between T cells and antigen presenting cells (Parham et al., 1996). The HLA genes are the human versions of the major histocompatibility complex (MHC) genes that are found in most vertebrates. These peptides are products of degraded proteins that are broken down by proteasomes (Figure 2). In general, HLA-associated peptides are about 8-11 amino acids in length (Ebert et al., 2009). Virtually all the cellular proteins undergo proteolysis in the proteasome and the degraded peptides

Figure 1: Cancer cells are able to surpass the Bispecific antibody is able to bind to both a tumor cell and T cell to stimulate an immune response on the tumor cell.

http://epigeek.com/bispecific-antibodies-cancer-immunotherapy/
are brought by the transporter associated with antigen processing, (TAP) 1 and 2 proteins, to the endoplasmic reticulum (ER), where they are loaded onto the HLA class I molecules for surface presentation (Johnson and Lewis, 2002). However, only a fraction of the degraded peptides are selected and presented on the surface by HLA. The rules for peptide generation and presentation remain elusive (Johnson and Lewis, 2002). Such HLA-peptide complexes can potentially serve as antigens to generate antibodies for cancer immunotherapy (Walz et al., 2015).

Numerous human tumor-associated antigens have been identified through the screening of cDNA libraries from cancer patients containing antibodies to tumor-associated antigens (SEREX), using T lymphocytes specific for tumor peptides presented in the context of specific HLA alleles, and performing reverse-transcription polymerase chain reaction (RT-PCR) for the tumor-associated antigens in tumors cells (Keogh et al., 2001, Snyder et al., 2012, Shore and Kaplan, 2009). One of the identified tumor-associated antigens is MAGE-A3 (Lendvai et al., 2015). It is a cancer/testis antigen expressed in a variety of cancers including melanoma, multiple myeloma, and lung cancer, but not in normal adult tissue with the exception of germ cells of the testis (Pandolfi, 1991, Jia et al., 2011).
MAGE-A3 is found in about 60-70% of melanoma patients and is known to inhibit T cell responses (Rapoport et al., 2015). It is also expressed in a variety of cancers including myeloma, lung, and gastric cancer. The specificity of the antigen’s expressions makes them ideal targets for cancer immunotherapy (Rammensee et al., 2013, Luiten and Bruggen 2000). However, traditional antibody-based therapies are impractical due to the cytoplasmic expression of cancer antigens. HLA class I molecules present a repertoire of endogenously derived peptides on the cell surface for surveillance by immune cells (Darow et al., 1989, Wolfel 1989).

In order to generate antibodies for immunotherapy, the sequence of the peptide presented in the HLA-peptide complex needs to be known. Orbitrap™ and Q Exactive mass spectrometry have been used to detect and sequence specific peptides derived from cancer antigens presented on the cell surface by HLA presentation (Eliuk and Makarov 2015, Bassani-Sternberg et al., 2010 and 2015). There are two approaches to detect HLA-associated peptides using the Orbitrap and Q Exactive mass spectrometry.

The first approach is the discovery method and is an unbiased analysis of all peptides present in the sample. It consists of liquid chromatography-mass spectrometry (LC-MS) analysis of all peptides present followed by the selection of the top 10 abundant peptides for tandem mass spectrometry (MS/MS) per LC-MS scan. The discovery method detects about 3,000 of the most abundant peptides presented by the HLA on the surface of the cell (Walz et al., 2015). These include both endogenous and exogenous peptides. The limitation of the discovery method is that it is less sensitive and only detects peptides that are in abundance.
The second approach is a hypothesis-driven, targeted method. Prior knowledge of peptides, either from bioinformatics and/or literature must be known, in order to specifically monitor those peptides (Gallien et al., 2012). This method uses selected ion monitoring (SIM) and parallel-reaction monitoring (PRM) to acquire MS/MS results on specific peptides of interest. SIM analysis is optimized to only look for peptide mass over charge (m/z) of interest which results in significantly increased sensitivity, allowing for the detection of peptides that have lower copy numbers presented on cell surfaces (Lesur and Domon 2015, and Gallien et al., 2015). PRM is very similar to SIM but it monitors the daughter b and y ions that are dissociated from the parent peptide of interest. The daughter ions of interest are identified prior to the monitoring of the peptides.

Quantification can be performed with both the PRM and the SIM method. The copy number of a peptide present on cells is a factor attributing to the reliability of that peptide as a target for immunotherapy. Peptides with high copy numbers are more reliable targets because the antibody has more targets (Peterson et al., 2012). Known concentrations of light and heavy isotopes of peptides, with the concentration of heavy isotopes remaining constant with varying concentrations of light isotopes, run using both methods, will generate a standard curve based on the ratio of the light and heavy peak areas (Rauniyar et al., 2015). In a sample with an unknown concentration, heavy isotopes are injected and run on the mass spectrometer. Using the ratio of the peak area between the endogenous sample and the heavy isotope, the endogenous concentration can be determined. PRM provides more confidence for both the detection and quantification of peptides because it monitors several daughter ions as opposed to only the parent peptide that SIM monitors (Shi et al., 2016). These cancer specific peptides presented by HLA
molecules on the surfaces of cells are attractive targets for generating specific antibodies for cancer immunotherapy.

**Research Objective**

The objective of this study is to use mass spectrometry to identify peptides derived from cancer specific antigens presented on the surface of tumor cells by HLA molecules. Once the peptides are identified, the copy number per cell will be quantified for showing the reliability of the HLA presenting tumor specific peptide complexes as attractive targets in generating specific antibodies for cancer immunotherapy.

**Methods**

**Cell lines and Xenograft tumors**

IM-9 cells (B lymphoblasts, myeloma, ATCC® CCL-159™) were injected into a severe combined immune deficient (SCID) NSG mouse to as a control arm for a study to determine the in vivo efficacy to anti-tumor biological therapeutics from another experimentation. The xenograft tumors were a byproduct. The tumor was later removed from the mouse and used for this experiment. The extracted tumor was then lysed with 1% NP-40 lysis buffer at a 1:10 ratio to the protein concentration determined with the BioRad Protein Assay (Bio-Rad Laboratories, PA).

IM-9 cells were grown in RPMI-1640 (ATCC Catalog No. 30-2001) with 15% fetal bovine serum. 5 x 10⁸ cells were lysed with 1% nonyl phenoxypolyethoxylethanol (Sigma), 5mM EDTA, 50mM of Tris-HCl pH 8.0, and 150mM of NaCl in water (1% NP-40 lysis buffer). 10mL of the lysis buffer with HALT protease and phosphatase inhibitor (Thermo) were added to cell pellets of 5 x 10⁸ cells and rotated at 4°C for 1
hour. Additionally an ultrasonic cell disruptor was used to break down cell membrane on ice for 30 seconds. Lysates were cleared by 30 min centrifugation at 40,000g.

**Immunoblot analysis**

To verify the presence of HLA complexes, immunoblots were performed on the cell lysates. Protein concentration was determined for the lysates with the Bio Rad Protein Assay (Bio-Rad Laboratories, PA). Cell lysates (20 μg) were separated on SDS-PAGE gels and transferred to a PVDF membrane in a semi-dry blotting chamber at 15V for 30 minutes. The membrane was blocked with 5% non-fat powdered milk and incubated with primary antibody overnight at 4°C. The primary antibody for the detection of HLA molecules was an anti HLA Pan Class I w6/32 at a concentration of 0.2 ug/ml. Following wash with Tris-buffered saline containing 0.05% Tween 20, the membrane was then probed with HRP-conjugated anti-mouse IgG secondary goat antibody for 1 hour at room temperature. Immune complexes were visualized using a western lightning chemiluminescence plus kit and the signals were captured on X-ray film.

**Isolation and Purification of HLA Class I complexes**

Anti- Pan HLA Class I W6/32 antibody was covalently linked to NHS-Sepharose 4 fast flow beads (GE Health Care, United Kingdom). To create a 1 ml affinity column, anti- Pan HLA Class I W6/32 antibody NHS linked beads were packed into a pierce centrifuge column (Thermo). The affinity column was washed with 4 ml of dilution buffer (10 mM Tris-HCl with 0.15 M NaCl, pH 7.4). Approximately 40 mg of lysate was passed through the affinity column using gravity flow (Figure 3). The affinity column was then washed three times with 4 ml dilution buffer and then twice with 4 ml 20 mM Tris-HCl (Teknova, Inc. California). 4 ml of glycine pH 2.5 (GE Healthcare Bio-Science,
United Kingdom) was added to the columns to elute the HLA class I complexes using gravity flow. The elution was collected and 1mL of 0.1% trifluoroacetic acid (TFA) (Honeywell Burdick & Jackson) was added to assist the binding of peptides to the tC-18 Sep-Pak Cartridges. The affinity column was neutralized with 1 M Tris-HCl, pH 8.0, and stored in dilution buffer for further use.

**Isolation and Purification of HLA Class I Peptides**

The eluted HLA complexes were loaded on Sep-Pak tC18 (Waters, MA) cartridges that were washed once with 1mL of 100% acetonitrile (ACN) and 3 times with 1mL 0.1% trifluoroacetic acid (TFA). The peptides were separated from the HLA complexes in the tC-18 cartridges by selectively eluting them with 30% ACN in 0.1% TFA into glass vials. The eluted peptides were concentrated using vacuum centrifugation. The peptides were re-suspended with 20 μl of 0.1% TFA and were used for LC-MS/MS analysis. HLA molecules and β2 microglobulin were eluted using a second elution of 70% ACN in 0.1% TFA of the tC-18 cartridge. HLA molecules were analyzed by immunoblots.

![Image](image_url)
LC-MS/MS analysis of HLA Class I peptides (Discovery Method)

HLA peptides, as mentioned above, were loaded onto a nanoViper Acclaim PepMap100 C18 trap column (75 μm i.d. × 2 cm, 3 μm, 100 Å, Thermo) and separated using a nanoViper Acclaim PepMap RSLC C18 column (75 μm i.d. × 25 cm, 2 μm, 100 Å, Thermo). The gradient was delivered by an EASY-nLC 1000 HPLC system (Thermo) at 250 nL/min. A 90-minute elution gradient with mobile phase A (0.1% formic acid in H₂O) and B (0.1% formic acid in acetonitrile) was as follows (percentage B): 2 to 10% at 10 min, linear to 30% at 80 min, and linear to 45% at 90 min. The peptides eluted from the column were ionized via Flex ion source at 1.5 kV and analyzed by the Q Exactive Plus mass spectrometer (Thermo). Survey scans were carried out in the high field Orbitrap analyzer with resolution at 70,000 and automatic gain control targeted at 10⁶ ions over a mass range of m/z 300-1500 and maximal ion fill time of 20 ms. MS² of the top 10 ions was performed with Higher-energy collisional dissociation (HCD), and scanned in the Orbitrap with dynamic exclusion window of 30 s. For each MS² scan, 10⁴ ions were accumulated with a maximal fill time of 100 ms.

Mascot Distiller software (version 2.4) was used to process the raw MS files prior to searching against the Swiss prot database using Mascot search engine (Version 2.4). The peptide false discovery rate (FDR) was set at 5% and the protein ID was accepted with an ion score of 20 or above.

Predicted the Binding Affinity of the peptide to the HLA

Net MHC 3.4 server (http://www.cbs.dtu.dk/services/NetMHC/) was used to predict the binding affinity of the peptide to the HLAs present on the surface of IM9
cells. HLA genotyping for IM9 cells comprise of HLA-A02, HLA-B49, HLA-B56, and HLA-C01, HLA-C07. The LC-MS/MS detected peptides were analyzed by entering their peptide sequences and peptide lengths into the net MHC server to obtain specific HLA predicted binding affinities. The resulting affinity values are indication of strength of predicted binding to specific HLA molecules. An affinity score \( \leq 50\text{nM} \) indicates that the peptide is a strong binder and an affinity \( \leq 500\text{nM} \) shows that it is a weak binder. If a score is \( >500\text{nM} \), then that peptide is considered a non-binder.

**Targeted Analysis on Q Exactive Instrument**

Based on the results from the discovery method, peptides that fulfilled the criteria for HLA, based on literature (Albert *et al.*, 2015), were monitored in the targeted method for higher sensitivity detection and quantification. 50 fmols of the heavy isotope for each peptide of interest was injected into the endogenous sample for quantification. Targeted selected-ion monitoring using high resolution and accurate mass was performed with the Q Exactive Plus Orbitrap LC-MS/MS (Thermo) to monitor specific peptide m/z’s using methods similar to Gallien *et al.*, 2012 for both SIM and PRM. For SIM, HLA peptides were separated by a nanoflow HPLC as described above. The identification of the peptides was based on high resolutions, accurate masses (<5ppm), isotope patterns, and elution time/retention times (< 10 seconds). The PRM method employed the isolation of target ions corresponding to the daughter ions monitored for the peptides in the 2-Th window, a resolution of 70,000 at \( m/z \ 200 \), a target AGC value of \( 3 \times 10^6 \), and maximum fill time of 3 s. Fragmentation was performed with a normalized collision energy of 25 and MS/MS scans were acquired with starting mass \( m/z \ 100 \).
The analysis of the isotope-labeled peptides in the samples was performed with a LC separation with a linear gradient. For the acquisition method in SIM mode, a full scan method with time-scheduled multiplexed SIM method targeting the isotopically labeled peptides/endogenous peptides in ± 2-min retention time windows. Doubly charged ions within each pair of peptides were multiplexed using 2-Th individual isolation windows. PRM used a time-scheduled multiplexed method with resolution 35,000 (m/z 200) and fixed fills times of 400 ms.

Both the analyses and quantification of SIM and PRM were performed like Gallien et al., 2012 did on Xcalibur (Thermo Fischer Scientific) and Skyline (MacCoss Lab, University of Washington). The peptides for SIM were identified based on the mass over charge ratio. In PRM, the retention time was monitored for both the heavy isotope and endogenous sample. Using a comparison of the peak areas for the SIM and PRM results, the quantity of the peptide was determined.

**Results**

**Optimized HLA-peptide Enrichment**

Using the workflow illustrated in Figure 1, lysate generated from IM-9 cells was passed over a pan-HLA-affinity column capturing HLA-peptide complexes. HLA-peptide complexes were eluted with glycine and the glycine elution was passed through Sep-Pak C18 columns. Using selective elution, 30% ACN eluted HLA-associated peptide, was analyzed by either a discovery or targeted LC-MS/MS approach. (Figure 1)

The immunoblot results showed the presence of HLA complexes (46 kDa)
detected from IM-9 cells (Figure 2, lane 1). The flow through from the affinity column was collected (Figure 4, lane 2). Since there were nearly no detected HLA-molecules in the flow through, the affinity columns were successful in capturing the HLA-molecules from the sample. We were able to further enrich HLA-molecules with our optimized workflow with the analysis of 70% ACN selective elution blot for HLA molecules. Only 10% of total elution was used for blotting. (Figure 4, lane 3).

![Image](image-url)

**Figure 4**: Cancer cells are able to surpass the surveillance of T cells. Bispecific antibodies are able to bind to both a tumor cell and T cell to stimulate an immune response on the tumor cell.

**MAGE peptides was detected during LC-MS/MS analysis**

Following selective elution, the 30% ACN fraction, LC-MS/MS analysis was performed on the peptide fraction detecting on average more than 3000 peptides per analysis. Numerous peptides derived from different families of MAGE were detected among the ~3000 peptide in multiple runs (Table 1). These families were MAGE-A1, MAGE-A10, MAGE-A12, MAGE-A3, MAGE-A4, and MAGE-C1. Due to confidentiality, the molecular mass, the m/z value, and the peptide sequence cannot be released. The peptides were given numeric names instead. The binding affinity of the peptides to HLA-A2, the most common human HLA, was determined with NetMHC 3.4 server. A score lower than 50nM was considered a strong binder and a score lower than 500nM was considered a weak binder. If there was no score, then the peptide was not an HLA-A2 binder. Based on the results of the discovery method, peptide MAGE-A3 #1 was selected to be further monitored in targeted SIM and PRM methods for sensitivity.
Spike-in Stable Isotope Peptide Allows Copy Number Estimation of MAGE-A3 #1

From IM-9 Cells and Mouse Xenograft Tumors using SIM

250 fmols isotopically heavy MAGE-A3 was spiked into the endogenous xenograft pulled tumor lysate sample for quantification. The sample was run on the mass spectrometer using the SIM method. This method uses the Q Exactive Orbitrap to detect peptides based on retention time, high resolution and accurate mass. The quadrupole mass filter allows for the accumulation of only targeted ions prior to MS detection, thus delivering better sensitivity. Orbitrap technology provides increased resolving power to...
discriminate 0.005 atomic mass units. The same procedure was performed on $1 \times 10^8$ cells for quantification of copies/cell (Figure 5).

The ratio between the peak area of the spike-in heavy peptide with known concentration and the peak area of the endogenous peptide was used to calculate the amount in femtomoles (fmols) present in the endogenous sample. The peak area ratio for the cell sample was about 20% and there are about 50.0 fmols of MAGE-A3 #1 peptide present on the surface of $1 \times 10^8$ cells. For tumor 1, the ratio was about 25% and about 62.5 fmols of MAGE-A3 #1 peptide on the surface. For tumor 2, the ratio was about 24% and about 60.0 fmols of MAGE-A3 #1 peptide on the surface.

Since the amount of cells in the tumors cannot be calculated, the copies/cell on the cell sample was calculated using the equation in Figure 6. Based on the calculation, there are 602 copies per cell, with the assumption that there is a 50% recovery rate of eluting HLA from the affinity column. We can assume that there are around the same number of copies of the tumor cells.

<table>
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<tr>
<th>Abundance Calculation</th>
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<tr>
<td>$\frac{1.94 \times 10^7}{9.66 \times 10^7} \times 250 \times 10^{15} \text{ mole} \times 6.02 \times 10^{23} \text{ copy/mole} \bigg/ 1 \times 10^8 \text{ cell} \times 50%^*$</td>
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<td>= 602 copy/cell</td>
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* Assumes 50% recovery from affinity column purification using pan HLA antibody

**Figure 6:** Calculation of the abundance of MAGE-A3 #1 peptides on the surface of IM-9 cells. It is calculated by multiplying the concentration by the Avogadro’s number and divided by the number of cells.
PRM Monitoring of Spike-in Stable Isotope Peptide Allows Copy Number Estimation of MAGE-A3 #1

PRM monitors the daughter b and y ions that are dissociated from the parent MAGE-A3 #1 peptide. This monitors several daughter ions and allows for more sensitivity and confidence in the detection of the peptide. Prior to experimentation, the daughter ions that will be monitored need to be selected. To select the daughter ions, synthetic heavy isotope MAGE-A3 #1 peptide was monitored. Based on the results, y5, y6, and y7 ions were monitored because they contained the heavy amino acid and were consistently detected in several runs (Table 2). Due to confidentiality, the masses were rounded to the nearest whole number and the sequence of the ion is not released. The mass spectrum of the heavy peptide is shown in Figure 7.

<table>
<thead>
<tr>
<th>Ion</th>
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<td>Y2</td>
<td>246</td>
</tr>
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Table 2: Daughter b and y ions detected during the monitoring of heavy peptide. These daughter ions are used to monitor during PRM

A standard curve was generated with 3 standard runs with 50fmols of heavy peptide remaining constant in all 3 runs and the light peptide varying by 5fmo, 50fmols, and 500 fmols. Their peaks area were calculated using Skyline (MacCross Lab Software) in Figure 8A. Based on the ratios, a standard curve was generated (Figure 8B).
The endogenous sample had a 50fmol spike in it and the mass spectrum is shown in Figure 9A. The peak area was calculated with Skyline (Figure 9B). Using the standard curve generated, it was calculated that there were 59.9fmols of MAGE-A3 #1 on $1 \times 10^8$ IM-9 cells (Figure 9B). Using the equation in Figure 4, it was calculated that there are 702 copies/cell with the assumption that there was a 50% loss from the elution of HLA complexes in the affinity column.

**Figure 8**: A. Peak areas of synthetic heavy and light peptides. B. Standard curve generated based on the ratios between the peak areas of the light and heavy peptides

**Figure 9**: A. y5, y6, and y7 ions monitored and detected using PRM method. B. Peak areas of synthetic heavy and light peptides


**Discussion and Conclusion**

In this experiment, HLA-associated, MAGE-A3 tumor specific peptides were successfully isolated, detected, identified, and quantified using an optimized workflow and mass spectrometry. The identification and detection started with the general discovery method that surveys all of the peptides that were presented on the surface of the cell by the HLA and only identifies 3,000 of the most abundant peptides (Walz et al., 2015). The majority of the peptides identified and detected in the IM-9 cell line were also detected in the IM-9 xenograft tumors. The exception was MAGE-A10 #4. This peptide was not detected in the IM-9 cells, but was detected in the xenograft tumor. Further investigation will be needed to determine why, but we speculate that it is because of the cell growth in the tumor form as opposed to the cell form. Of the 11 MAGE protein associated peptides that were detected in both the cell line and the xenograft tumor by discovery method, MAGE-A3 #1 was selected to be further monitored and quantified using the SIM and PRM method.

In the experiment by Jia et al., 2011, such MAGE peptide stimulate T cell response. It was proven by generating bi-specific antibodies with a synthetic version of MAGE-A10 peptide on one of the long chains and targeting the CD3+ receptor on T cells. T2 cells were used because they have a deficiency in the Tap 1 and 2 proteins, failing to load the degraded peptides from proteolysis in proteasome on to the HLA complexes. Therefore, the HLA complexes on the surface of T cells are empty. Jia et al., inserted the generated antibodies to target the T cell and the synthetic MAGE-A10 peptide was inserted into the empty HLA complexes. The peptide MAGE-A10
had shown to stimulate the T cell response and killed the T2 cells. However, the synthetic version used did not establish that MAGE-A10 peptide would be generated and selected for presentation in the elusive rules of the proteolysis process and selection for presentation. The optimized workflow and the use of mass spectrometry in our experiment validated that MAGE-A10 is naturally presented on the surface of tumor cells.

The discovery mass spectrometry examines all peptides in the tumor samples and selects the top ~3,000 most abundant. Cancer peptides may be present in sample, but are not detected by the mass spectrometry because of their abundance on the cell surface (Bassani-Sternberg et al., 2010). Discovery mass spectrometry is non-biased but is less sensitive than the targeted method, SIM and PRM. For cancer immunotherapy, antibody targets with greater abundance are more reliable targets (Mariotto AB, et al., 2011). MAGE-A3 peptides identified in discovery mass spectrometry were show to qualified as reliable targets. SIM and PRM were performed to quantify the abundance of MAG-A3 #1 to determine its reliability as an antibody target.

SIM and PRM allow for a more sensitive detection of peptides than that of discovery. These targeted methods specifically monitor peptide of interest. These methods were used because tumors are very unlikely to be large enough to contain $1 \times 10^8$ cells, so a more sensitive method is needed. SIM only monitors the peptides based on the parent peak (Gallien et al., 2012). The peptide was identified based on retention time and molecular weight. Due to confidentiality, these cannot be released. Based on the ratio between the peak area of the heavy spike in isotope and the peak area of the endogenous
sample, the fmols of MAGE-A3 #1 in the sample was detected. The SIM method quantified 602 copies per cell, which makes it a reliable target for antibodies. The quantity that was calculated was based on the assumption that there was a 50% recovery rate when the HLA complexes were eluted from the affinity column with glycine. Further investigation will be needed to quantify the recovery rate. However, chances are that there is more than a 50% recovery rate, which means that there are more copies/cell than calculated.

The PRM method monitors the daughter b and y ions that are fragmented from the parent peptide in the HCD cell. The daughter ions must contain the heavy isotope amino acid of the peptide because the molecular weight difference is used in quantification (Rauniyar et al., 2015). Ions monitored were identified by analyzing 5 runs of only the heavy isotope peptide. In the runs, the y5, y6, and y7 ions were consistently detected and all contained the heavy isotope. Therefore, these ions were monitored in PRM for the endogenous sample with heavy spike in isotopes samples. PRM provides more confidence because it monitors several ions as opposed to SIM, which monitors only one ion, the parent peptide. The quantification based on the PRM method was 720 copies/cell, which is greater than that calculated by SRM. The difference in the two quantifications is most likely due to the difference in cell sample. SIM and PRM were performed on different samples of IM-9 cells.

The copies/cell calculations were done on IM-9 cells because the number of cells in a tumor cannot be determined. The copies/cell for the IM-9 cells are relative to that in the tumors, and so the copy numbers can be assumed for the tumors. Future research
would be to identify a method to determine the numbers of cells in the tumors and quantify the abundant copies/cell on the tumors as well.

Quantification of the abundance of MAGE-A3 #1 peptide was important for its determination as a target. The chance for the binding of the antibody to the peptide target is greater when the presence of the peptide on the cell surface is greater. From the SIM, it was determined that there are 602 copies/cell of MAGE-A3 #1. PRM determined that there were 720 copies/cell. Both of these quantifications show that MAGE-A3 #1 has very high abundance on the cell surface. According to Lendväi et al., 2015, a peptide with an abundance of ~500 copies/cell is a reliable target for antibodies. The quantification from both SIM and PRM surpass the requirements of a reliable target. Therefore, MAGE-A3 #1 can be determined to be a reliable antibody targets in cancer immunotherapy.

In this experiment, we successfully identified, detected, and quantified a tumor specific peptide: MAGE-A3 #1 peptide. CD3 bi-specific antibodies specifically targeting MAGE-A3 #1 peptide complex are going to be generated and tested for cancer treatment in preclinical studies and probably in clinical trials.
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