Development of an RNA Aptamer against GP100 to enhance immunogenicity in malignant melanoma cells.

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N36 Jay Pool
GP100

Abstract

Melanoma, or skin cancer, is the most common form of cancer in the United States, accounting for nearly 50% of all cases [1]. Cutaneous malignant melanoma (CMM), causes the majority of skin cancer deaths (9,000 out of the 12,000 per year in the U.S.) yet only represents five percent of all skin cancers [2]. Whereas the mortality rate for skin cancer has been decreasing due to early detection and removal, there is still a need for treatment, especially when the tumor has been discovered after metastasis [3,4].

Cancerous melanoma tissues over-express the biomarker glycoprotein GP100, making it an effective diagnostic marker, though GP100 is also expressed at low levels on the surface of normal melanocytes [5]. On its own, GP100 elicits a weak immune response; recognizable by tumor-infiltrating lymphocytes suggesting the target is accessible to the immune system [5]. Current intravenously administered immune chemotherapies and radiation specifically target GP100 however, have they increased toxicity likely due to attack of healthy tissues [6].

Aptamers are oligonucleotides enriched through iterative rounds of nucleic acid in vitro selection from a starting library containing roughly 10^{13}. The SELEX (Systematic Evolution of Ligands by Exponential Enrichment) method is designed to create greater specificity and binding affinity towards target (protein) for each subsequent round by getting rid of the species that do not bind and amplifying those that do [7].

An aptamer specific to GP100 has two main benefits over traditional therapies. First, drug delivery is relatively easy through subcutaneous methods against melanomas as opposed to intravenous administration mentioned above. The drug could be formulated in a rub on cream that penetrates the skin. Second, using an immune redirection technique [8], the aptamer could direct an immune response specifically to cells expressing targets over a certain threshold such as GP100 on melanoma compared to healthy melanocytes [9]. It is hoped that this kind of aptamer therapy could greatly decrease the mortality rate associated with CMM skin cancer.

Specific Aim # 1 - To develop an aptamer through iterative rounds of nucleic acid enrichment that will specifically and tightly bind to human GP100.

Once enrichment is detected, the selection will continue with GP100+ melanoma cells such as human GP100 transfected mouse melanoma B16 cells [10] and assayed using flow-cytometry (FCM) to determine binding affinity (using a negative-GP100 B16 cell line as the control).

Specific Aim #2 - Next-generation sequencing will be utilized to interrogate the selected pool for specific binding variants.

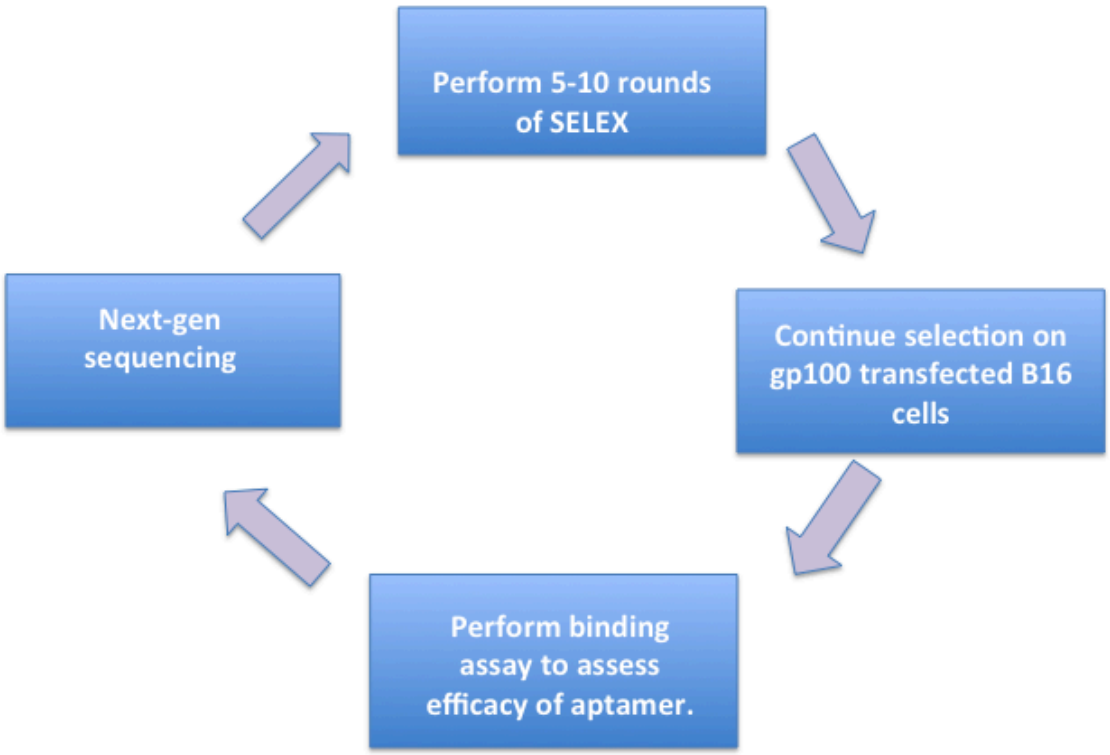

Fig. 1. This figure outlines the specific aims of the project as well as the general outline of the overall procedure.

Working with Altermune Technologies, GP100 has been expressed and purified by

GenScript (phone #: 732-885-9188/lot. No.: 163193S01/P20011204) with a poly-HIS tag for bead based selection. The molecular weight of GP100 is 77 kDa and about 3 mg of GP100 was obtained for $2653 from GenScript. The cost per round is roughly $40 and the estimated number of rounds to be performed is between five and twelve.

Introduction/Background:

Comprising the outer-most layer of the body, melanocytes are accustomed to having to adapt to harsh environmental factors (primarily, ultraviolet radiation). Their adaptive nature is hypothesized to be a main reason that melanoma is one of the most (if not the most) aggressive types of human cancer [11]. Melanoma tumors are also among the largest (in size) cancerous tumors, presenting a great risk for metastasis. Once the cancer has spread, the patient is usually given an estimated 6-9 months to live, with about 14% of metastatic melanoma patients surviving for five years. [11,12]. The aggressive nature of melanoma, due to rapid growth and metastatic propensity (as well as certain mechanisms), makes the tumor cells a hard target for chemotherapy. With no particularly effective form of immunotherapy for these metastasized tumors, the mortality rate for this specific type of dermatological cancer has plateaued.

The "self" peptide, GP100 (glycoprotein 100), is an immunogenic biomarker, recognized by tumor-suppressing lymphocytes. GP100 is found on both melanocytes and melanoma cells, although it is much more abundantly expressed on melanoma cells [5]. Due to the fact that normal skin cells are not targeted by such lymphocytes and that the mortality rate for malignant melanoma is so low, it can be inferred that GP100 does not elicit a strong immune response. Its abundance and inherent immunogenicity make GP100 the perfect target for immunotherapy.

So far, the main forms of immunotherapies that have been employed are chemotherapy and vaccination. These therapies have not been notably successful, especially after metastasis. With regard to chemotherapy, there is a need for more specific targeting. Chemotherapy has proven toxic to healthy tissues when administered, which proves a significant problem as the affected area (area containing melanoma cells) increases [6]. Vaccination (using GP100) was tested to elicit anti-HGP100 antibodies but

did not slow the proliferation of the tumor cells [13].

Therefore, due to the need for increased immunogenicity and specificity against GP100, an aptamer would be an ideal form of immunotherapy. Aptamers are specific oligonucleotide sequences, identified through iterative rounds of selection. The goal is, after 5-10 cycles of SELEX (Systematic Evolution of Ligands by Exponential Enrichment, shown in Fig. 2), to identify a specific sequence of RNA that will tightly and specifically bind to GP100. To develop the therapeutic, this aptamer will be coupled with an immunogen such as Alpha Gal or chemotherapy for specific drug delivery to melanoma cells. With only melanoma cells being targeted, it is hoped that healthy tissue will not be affected and that malignant melanoma (especially metastatic malignant melanoma) can be controlled and/or regressed.

Fig. 2 Depicts the SELEX process with both filter and bead-based binding and selection methods.

Materials and Methods:

The target, GP100 (mol. Weight: 77 kDa, dimer, also found in mice) was obtained from, and His-tag modified as well as purified by, GenScript Technologies and will be targeted in iterative rounds of RNA enrichment. Protein immobilization will be done with a HIS-tag modified nickel beads and the initial R0 pool of 10^{13} unique species will come from the N36 Jay pool with a starting ratio of 400:400pmol (pool:protein). The buffer used to wash the beads is 1X PBS (137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4) and the buffer used during binding and selection is 1X PBS KMT (Selection Buffer) (PBS with 1 mM $MgCl_2$, 3mM KCl, and 0.1% Tween 20). The aptamer is being developed for administration (ultimately) to humans, therefore buffers with a pH similar to that of a human is necessary. The starting incubation time and temperature (for the protein to anneal to the beads and the RNA to anneal to the protein) is 30 minutes at 25°C on a Labquake rotator. Six washes of 700ul selection buffer will be done to remove the non-binding RNA. The first and last

washes will be PCR amplified to analyze the nature of the binding. After the remaining (or binding) RNA is eluted off of the protein (using 80°C diH_20), both washes (first and last) as well as the eluted RNA (elution) will be concentrated using isopropanol precipitation, reverse-transcribed (200 uM N36R24 primer, .1M DTT, 5X FS Buffer, 4mM dNTP), and then PCR amplified for analysis. The initial PCR amplification is performed using real-time PCR and is carried to 30 cycles (of replication) to determine how many cycles are needed for optimum amplification (for the elution) as well as if the binding and selection is stringent enough (based on how much amplification shows up in each of the washes). Initial PCR amplification is performed using: reverse-transcribed ssDNA, 25 uM N36 R24 primer, 25 uM N36 T7F primer, and 2X iTaq SYBR Green PCR Supermix (from Bio-Rad). From this, the initial conditions for the following round may be determined, as well as the number of cycles needed to amplify the reverse-transcribed ssDNA. After the eluted RNA is amplified, it is transcribed using SuperScript III RNA polymerase and a Durascribe transcription kit for six hours and any remaining DNA is removed using Baseline zero DNase. The RNA is then PAGE purified and the RNA is eluted using 0.3M NaOAc (pH 6) diluted with 1X TE (10mM Tris-Cl, pH 7.4, 1mM EDTA, pH 8). The RNA is then quantified using nanodrop spectrophotometry to determine the volume needed for the starting amount (pmol) of the following round.

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Results:

Round 1 was repeated (not all the way through) about four times before binding and selection was successful. The first time Round 1 was performed, there was no visible pellet in the bottom of the tube containing the isopropanol-precipitated RNA eluted from the bead-immobilized GP100. This was not realized, however, until after half of the

supernatant was discarded (as it appeared, at first that there was a pellet) and the round was not completed due to the potential error that this may have caused. The second time round 1 was performed, the same problem occurred, however, upon more freezing and centrifugation, a pellet did form on the bottom of the tube containing the RNA eluted from the bead-immobilized GP100 (binding RNA). It should be noted that every other tube (containing the elution from naked beads, and the washes from both naked beads and GP100 positive beads) contained a visible pellet after just one normal isopropanol precipitation. Due to the formation of a pellet after the second precipitation, the round was completed as normal. Unfortunately, the resulting picomolar amount of RNA from round 1 was not enough to carry on to round 2 (which is very unusual for a first round. Another observation from the first two attempts of round 1, was that the GP100-positive beads were sticking together in the 1X PBS+KMT while the naked beads were not. Due to this and the extremely low yield of RNA, it was decided that the selection might improve if the buffers were changed to 1XPBS+$MgCl_2$ for washing the non-binding RNA off of the GP100-immobilized beads and 1XPBS+$MgCl_2$+BSA+tRNA for the "binding reaction" (when the RNA is added to the GP100-immobilized beads). These changes were made based upon the suggestion of post-doc Altermune employee, Dimitri Van Saemays based on prior experience. The advantages are that these buffers (unlike PBS+KMT) do not use Tween20, which has been said to denature proteins. When the buffers were switched, the bead-immobilized GP100 no longer clumped together, however, they did stick to the plastic tubes and pipet tips. The Tween20 had prevented this.

There were two possible solutions considered: either switch using glass tubes and tips instead of plastic or to biotinylate the GP100 and use M280 Streptavidin beads. Due to the fact that the nickel beads with CD20 immobilied on them behaved the same way in this buffer, it was possible that there was a problem with the beads. Therefore, the protein GP100 was biotinylated and a fourth round 1 was performed. This round was done with little stringency as GP100 is slightly negative and may not bind RNA as well as a positive protein such as lysozyme. Therefore, a side by side selection was performed using lysozyme (+beads), naked beads, and GP100 (+ beads). The stringency was reduced in that

the wash number and volume were fairly low with three 400ul washes as well as no negative selection. The real-time PCR results (Fig. 3) support that GP100 does bind RNA almost as well as lysozyme and better than naked beads:

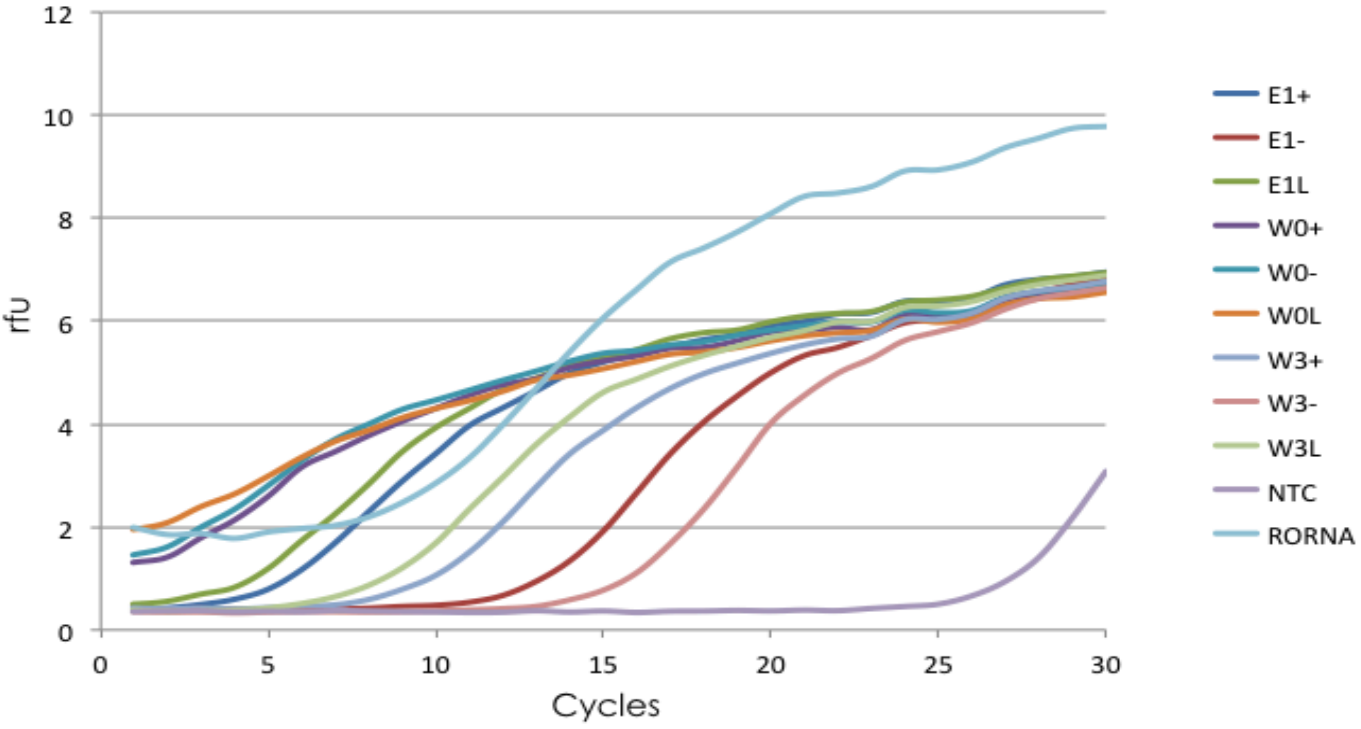

Fig. 3 Depticts the relative amplification by comparing the graphs for each sample for the amount of fluorescence (rfu) at each cycle from 0 to 30 cycles.

In addition, wash 5 of the elution amplified after the elution, which shows that there was more RNA (that bound to the protein) eluted off of the bead-immobilized GP100 than RNA washed off of the protein before elution (non-binders). Additionally, the NTC amplified after any of the samples which means that there was minor contamination, but not enough to skew the results whatsoever. The R0RNA, though much more concentrated than the other samples, was tested for DNA contamination and, while there appeared to be more amplification in the sample containing R0RNA than the others, it amplified much later. This would suggest that the contamination is not significant enough to skew results. A mini-PAGE (fig. 4) was run to determine that the dsDNA was not over or under-amplified, as well as was just over 100 basepairs long, as expected.

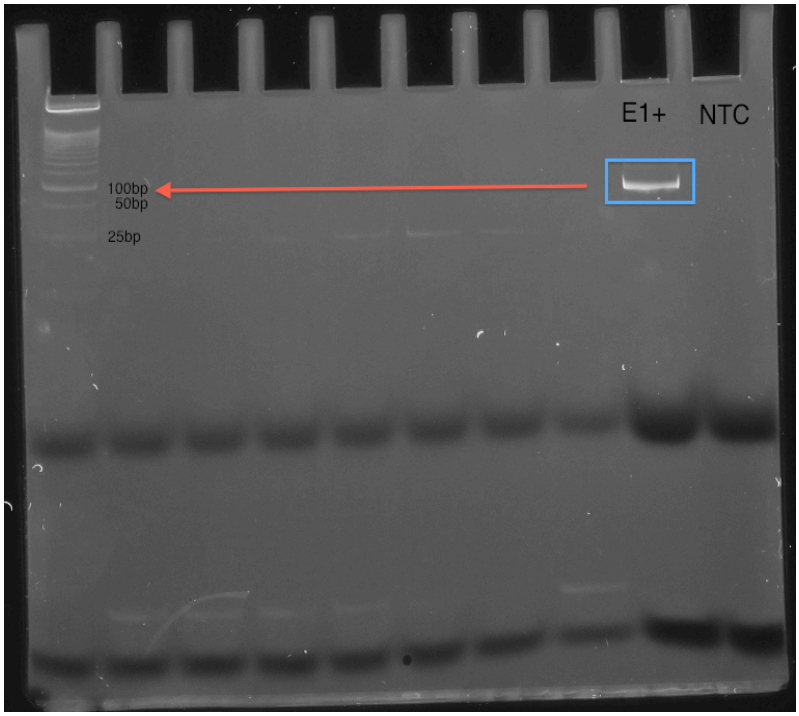

Fig. 4 Is a an image under UV light of the mini-PAGE using 5ul (out of 500ul) dsDNA from large-scale real-time PCR

Figure 4, the digital image of the mini-PAGE shows that the dsDNA was slightly over-amplified (as expected) because the PCR was left to amplify until the rfu reached 15 instead of the normal 10-12 rfu (human error). This is not much of a problem in round 1.

The concentration at the end of round 1 was 2084.7 ng/uL with a yield of 1319.7 pmol of RNA. This is way more than enough to begin round 2. Therefore, round 2 was perfomed with more stringency; increasing wash number and volume to five washes of 500ul. In addition, a negative selection on naked beads was added to reduce bead-binding RNA from being added to the bead-immobilized GP100. This should allow for more specific binding sequences without losing too much RNA variety by being overly-stringent early in the selection.

Round Number	Concentration (ng/ul)	Amount of RNA (pmol)
1	2084.7	1319.7

Future Work:

The protein is available and the initial conditions as well as reagents are prepared. Five to ten rounds will be performed before considering a binding assay. The PCR results as well as quantification of the RNA during each round will help determine the (hypothetical) specificity of the pool. From there, the aptamer will be tested on cells and the selection may be continued using whole-cell SELEX. It is hoped that the ultimate result is an aptamer specific to malignant melanoma cells that can be coupled with an immunogen for enhanced immunotherapy. This may drastically increase the mortality rate associated with malignant melanoma.

In the immediate future, however, more rounds of selection will be performed using the same conditions with possible increases in stringency such as a greater wash number and volume, or negative selection using protein ClfB (clumping factor for staphylococcus). Once a binding assay has been performed and specific binding has been detected, the selection may be switched from using bead-immobilized GP100 to using cells expressing GP100. As well, when specificity has been detected, the resulting RNA may be sent off for NextGen sequencing to identify any repeating sequences, sequence families, or motifs.

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