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Preferential selection of androgen polymorphs within and between the cancer cell lines MDA-MB-468, MCF-7, and SKBR3.

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ABSTRACT

Research was completed to elucidate whether a single X chromosome was preferentially inactivated within cancer cell lines that came from female donors. This was approached with a mind towards using non-essential X-linked proteins as targets for pointed cancer therapy. Research used reverse transcriptase polymerase chain reaction and custom oligonucleotide primers designed to include the poly-Q region of androgen receptor mRNA. Agarose gel electrophoresis (1%) and polyacrylamide gel electrophoresis (6.5%) with ethidium bromide allowed visualization of all mRNA polymorphs produced within a population of approximately 10^6 cells for each replicate. It was found that up to five androgen receptor polymorphs were expressed within a given population of cancer cells from the cell lines SKBR3, MCF-7, and MDA-MB-468. Since many polymorphs are expressed it is reasonable to conclude that there is not only one X chromosome expressed throughout an entire cell culture. This finding conforms to the known mutator phenotype associated with cancer cells. It became apparent that certain polymorphic variants were not only selected for within the same cell line, but also between different breast cancer cell lines. Certain polymorphs being conserved suggests there is a potential selective pressure that causes the production of specific androgen receptor polymorphs. Due to this discovery, future research might be completed regarding assessment of androgen receptor polymorphs in primary tissue samples. Completed research also supports research on the use of androgen ablation therapy in the treatment of breast cancer.

INTRODUCTION

Providing positive patient outcomes with a prudent "standard of care" proves elusive when the patients present with solid tumor metastatic breast cancer. However, an appealing method of approaching this challenge is with treatments that involve using the immune system of the patient. Immunotherapy strategies may involve adoptive T-cell therapy, with concomitant use of standard chemotherapy, or through the use of immune-activating antibodies. Lloyd John Old, who is known as the father of tumor immunotherapy, pioneered the idea that the body's defense mechanism could be used to fight cancer. In the 1960's when Old began research on tumor immunotherapy it was considered an unconventional idea, and he was on the fringe of the scientific community, but that position was not held for long—in contrast to his position as director of the Cancer Research Institute. Old is credited with being the first to discover p53 a protein known as "the guardian of the genome" (DeLeo 1979), the first to discover that killer T-cells and helper T-cells do respond to cancer cells as well as naming them as CD8+ and CD4+ cells owing to his experiment which grouped them according to clusters of differentiation, and that there were antibody receptors on all human cells including cancer cells which are all critical to the idea of using the immune system to try and defeat cancer proliferation.

When cancers do respond to immunotherapy the response is long lasting, on the order of years as compared to other treatments that are effective only for months or weeks. Unfortunately, current immunotherapies exhibit only 5-10% positive response likelihood among all metastatic solid cancers that these therapies

have been tested upon (Rosenberg 2004). Therefore, when immunotherapy is effective the result is often long lasting; unfortunately the probability of this is not very high. The paradigm in immunotherapy has switched away from working to find a vaccine and toward moderation of the immunosuppressive tumor microenvironment using targeted therapies (Begley 2008).

The following research was based on assessing whether every cancer cell carries the same active X chromosome. It was critical to assess the levels and presence of mRNA or transcription using a novel methodology that makes use of reverse transcriptase polymerase chain reaction (RT-PCR). The purpose of assessing the clonality of cells at this level was to establish not only which genes are expressed, but also the rates at which each individual cell expresses these genes. Rates are expected to be equivalent if the cells are truly clonal. The use of RT-PCR for this purpose is novel, but is comparable to other research has been successfully completed in the past (Bukur 2010). Research focused on targeting X-linked non-essential expressed proteins, which can be accomplished using primers for those proteins. The human androgen receptor was targetted for this purpose and has been used successfully by the SickKids Hospital for X-chromosome inactivation studies. The inactive X chromosome does not express the androgen receptor protein. SickKids hospital did not divulge their primer sequence so a custom sequence was developed. The use of MDA-MB-468 and MCF-7 have been used in past research as controls owing to their basal level and moderate levels of HER2 expression respectively (Belsches-Jablonski 2001). These can be compared to the cell line SKBR3, which has a high level of HER2 expression.

By assessing transcription via cDNA products from RT-PCR and size differentiation using polyacrylamide gels for higher resolution of the ~500bp androgen receptor bands it was found that there are preferentially selected androgen polymorphs both within a cancer cell line, but also between cancer lines suggesting there is selective pressure for cancer to develop certain polymorphs of the androgen receptor protein.

METHODS

Cell Culturing Methods

A seven-step procedure is standard for the thawing of cancer cell lines from frozen cultures and was obtained from Thermo Fisher Scientific. An explicitly copied protocol is covered below; the only modification was a removal of process steps that are not pertinent to this study (Thermo Fisher Scientific 2014).

1. A cryovial containing frozen cells was removed from an -80°C freezer and was immediately placed in a 37°C water bath.
2. Cells were thawed quickly (< 1 minute) by gently swirling the vial in the 37°C water bath until there was just a small bit of ice left in the vial.
3. The vial was transferred to a laminar flow hood and was disinfected with 70% ethanol prior to being placed in the hood.
4. Cells were transferred to Nunc-easy flasks that had prewarmed growth media (10%FBS, in RPMI solution).
5. Cell media was changed after 3-5 hours to allow cell attachment and removal of DMSO containing media.
6. Subsequent cell splits involved freezing of cells in excess of the 500,000 used to establish new cultures.

The flasks used in the study were NuncEasyFlasks and were available once again through Concordia University College of Alberta. Media was RPMI from Life technologies (Gibco 22400-089) supplemented with 10% FBS.

Cell cultures were all gifts of the University of Alberta (MCF7, SKBR3, and MDA-MB-468). These were then split three times into new cultures resulting in 3 "generations" of cells to produce the best possible pseudoreplicates for the experiment.

RNA Isolation Methods

The GenEluteTM Mammalian Total RNA Miniprep Kit (Catalogue No. G1N10-1KT) produced by SigmaAldrich contained all the necessary reagents and components for high yield RNA extraction. The kit uses a silica-based system with a microspin format and obviates the use of cesium chloride reagents, and phenols. Cells were lysed and homogenized in a buffer containing guanidine thiocyanate that denatures macromolecules such as: proteins, DNA, and importantly RNases. Addition of ethanol caused the RNA to bind to the media when the lysate was spun through a silica membrane in a microcentrifuge tube. The contents were washed, and RNA was eluted in 75 µL of elution solutionTM. RNA that is less than 200 nucleotides in length was not recovered, therefore selectively enriching for the recovery of mRNA.

Some materials were not included in the kit and were used from Concordia's supply, they included: ethanol (100%), RNase-free pipette tips, microcentrifuge tubes, and a microcentrifuge. The following protocol was used to produce an RNA solution that had 10-30 µg of RNA for each reaction from 10^6 cells taken from each culture. Before beginning, the lysis solution was prepared. 2-mercaptoethanol (ME) was added to a volume of 250 µL of lysis solution ($1 \bullet 10^6$ cells). Cell counts were

determined using standard hemocytometer methods with 4 counts per culture averaged. 2-mercaptoethanol was used to fully inactivate RNases. The 2-ME was added to a concentration of 10 µL/mL of lysis solution. In addition to this preparation the wash solution is also supplied as a concentrate and was diluted using 100% ethanol.

To derive ideal centrifuge rpm values the equation RCF = 1.118 X 10^{-5} • radius (in cm) • rpm^2, where RCF = gravitational acceleration in units of *g* was used. The following protocol was taken explicitly from Sigma-Aldrich.

Cells attached to the NuncEasy Flasks were released with 2 mL of trypsin and pelleted before lysis. Pellets were vortexed with 250 µL of the lysis Solution/2-ME mixture which was added to the 1,000,000-pelleted cells from each replicate.

To remove cellular debris and sheared DNA the lysed cells were pipetted into a GenElute Filtration Column (blue insert with a 2 mL receiving tube). Cells were centrifuged at maximum speed (12,000–16,000 3 *g*) for 2 minutes. The filtration column was discarded. 250 µL of 70% ethanol were added to the filtered lysate and the mixture was agitated via repeated pipetting. The entirety of the lysate/ethanol mixture was pipetted into a GenElute Binding Column (colorless insert with a red o-ring seated in a 2 mL receiving tube). The column was spun at 15,000 rpm for 15 seconds. The flow through was discarded.

The first column wash was done with 500 µL of Wash Solution 1 and was pipetted into the column and centrifuged at maximum speed for 15 seconds. Flow through was discarded once again. The binding column was transferred into a fresh 2 mL collection tube. 500µL of the earlier diluted Wash Solution 2 was pipetted into the

column and centrifuged at maximum speed for 15 seconds. The flow through was discarded. A third column wash was performed using a second 500 µL aliquot of Wash Solution 2 that was pipetted into the column and centrifuged at maximum speed for 2 minutes to dry the binding column. Finally, the binding column was transferred to a new 2mL collection tube. 50 µL of the Elution Solution was pipetted into the binding column and centrifuged at maximum speed for 1 minute. Only 30 µg of RNA were expected from the 10^6 cells used.

RT-PCR Methods

Since the above procedure did not yield sufficient RNA for gel electrophoresis, polymerase chain reaction was used to produce stronger DNA equivalents of the mRNA yielded and amplified their presence. This was done in research by Bukur and colleagues in 2010 and produced an elucidation of one of the components of the antigen processing machinery. Since we are assessing clonality via gene expression, custom androgen and herstatin primers were used in the RT-PCR and are shown in appendix I immediately following this report.

The protocol served as a guideline for one-step RT-PCR. Reverse transcription and PCR were carried out consecutively in the same tube. All components required for both reactions were added during setup. The protocol was optimized for 1 pg – 2 µg of total RNA so only 10 µL of template RNA was used from the previous RNA isolation.

HotStarTaq DNA Polymerase, contained in the QIAGEN OneStep RT-PCR Enzyme Mix, required initial activation by incubation at 95°C for 15 min before

amplification could take place. Incubation also inactivated the reverse transcriptases. It was therefore an important consideration to avoid heating and activating the HotStarTaq DNA Polymerase until the reverse-transcriptase reaction was finished.

The primers used in the RT-PCR reaction are described in appendix I of this report and target the polymorphic (Q) region of the androgen receptor and the herstatin mRNA respectively. These were developed according to common protocols laid out by the NCBI primer design website.

The 5x QIAGEN OneStep RT-PCR Buffer provided a final concentration of 2.5 mM $MgCl_2$ in the reaction mix. Reaction mixtures were set up in an area separate from that used for RNA preparation or PCR product analysis. The mix for each reaction of the 20 described below included 10 µL of Qiagen one step RT-PCR buffer 5x, 2 µL of dNTP mix, forward primer 0.3 µL, reverse primer 0.3 µL, RNase free water 25.4 µL, 2 µL of polymerase enzyme, 10 µL of template RNA from the RNA extraction tube, for a total of 50 µL for each reaction.

Reaction contents:
1-3: Replicates of MDA-MB-468 with HER2 primers
4-6: Replicates of MCF-7 with HER2 primers
7-9: Replicates of SKBR3 with HER2 primers
10: A reaction with no RNA expected to be present (negative control), but including HER2 primers.
11-13: Replicates of MDA-MB-468 with Androgen receptor primers
14-16: Replicates of MCF-7 with Androgen receptor primers
17-19: Replicates of SKBR3 with Androgen receptor primers
20: A reaction with no RNA expected to be present (negative control), but including Androgen primers.

Thermal cycler conditions:

Reverse transcription was carried out at 50 ˚C for 30 minutes; PCR was then activated at 95˚C for 15 minutes. 25 cycles of 1 minute at 94˚C (denaturation), 1 minute at 50˚C (annealing), and 1 minute at 72˚C (extending) were carried out and then the PCR products were stored at 4˚C until samples were used in electrophoresis 18 hours later.

Restriction Digest of Androgen Receptor cDNA

Restriction endonuclease digestion was performed on cDNA products isolated from the androgen receptor mRNA RT-PCR method listed above using *SmaI* restriction enzyme (NEB Cat. R0141s). Since the DNA was estimated to be 0.833 µg/µL and common practice for endonuclease digestion describes the need of 1µg then 1.2 µL of DNA template was required and used. To separate PCR tubes was added 16.3 µL of sterile deionized and nuclease free water, 2 µL of 2.1 10X buffer (NEB Cat. B7002S), 0.2 µL acetylated BSA, 1.2 µL DNA template, and 0.5 µL of *SmaI* restriction enzyme (10 Units total). This restriction mixture was centrifuged for 5 seconds at 13,500 rpm to concentrate the reaction at the bottom of the PCR tubes. The reaction was then left to incubate at 20˚C for 4 hours before the products were run on a 1% agarose gel.

Gel Electrophoresis Methods

The final DNA contents from the PCR as well as the restriction digestion products were analyzed using standard gel electrophoresis on a 1% agarose gel.

The purpose of using the gel was to provide size determination of the mRNA present from the metastatic breast cancer cells. The gel was run using the Biorad gel electrophoresis apparatus available at Concordia University College of Alberta. To a 250 mL Erlenmeyer flask 1.0g of agarose was added to 100 mL of 1X TAE. The solution was microwaved until clear, 3 μL of ethidium bromide was added to the solution at a concentration of 1 μg/mL, and poured into a casting apparatus with a comb. This was allowed to cool until solid. All ethidium bromide was stored in a separate ethidium bromide waste container because the intercalator is toxic and mutagenic to humans. To each of the PCR products 5μL of standard 6X loading dye was added, and a 10 μL 1kb DNA ladder from NEB (Cat. NO468S) was added to various lanes for comparison. The gel was run at 80 V for 30 minutes with some checking using UV light in between to make sure bands didn't run off the end of the gel. Finally the gel was photographed. All surfaces were washed with a 10% bleach solution. The gel was visualized using a UV transilluminator and photographed using a Samsung Galaxy S5 camera. In addition to these results the products of RT-PCR for the androgen receptor polymorphism were also run on a 6.5% polyacrylamide gel for better resolution of the ~500 bp size fragments. The PAGE was accomplished using Ready to Use Gel Matrix (Li Cor cat. 827-05607). Samples were added to the polyacrylamide gel and ran with 1X TBE (995 mL dH_2O, 10.8g Tris base, 5.5 g boric acid, and 0.744g $Na_2EDTA*2\ H_2O$) buffer at 150V for 44 minutes. In addition to the androgen receptor products run on the gel a 1kb DNA ladder was also ran alongside for size determination (NEB 4086S).

Restriction Digest of Androgen Receptor cDNA

Restriction endonuclease digestion was performed on cDNA products isolated from the androgen receptor mRNA RT-PCR method listed above using *SmaI* restriction enzyme. Since the DNA was estimated to be 0.833 µg/µL and common practice for endonuclease digestion describes the need of 1µg then 1.2 µL of DNA template was required and used. To separate PCR tubes was added 16.3 µL of sterile deionized and nuclease free water, 2 µL of 2.1 10X buffer (Cat. B7002S), 0.2 µL acetylated BSA, 1.2 µL DNA template, and 0.5 µL of *SmaI* restriction enzyme or 10 Units total (Invitrogen Catalogue: 14228-018). This restriction mixture was centrifuged for 5 seconds at 13,500 rpm to concentrate the reaction at the bottom of the PCR tubes. The reaction was then left to incubate at 20˚C for 4 hours before the products were run on a 1% agarose gel. Using NEB Cutter 2.0 it was determined that 284 bp and 142 bp bands should be produced if the sequence was the androgen cDNA sequence.

RESULTS

Results on the cDNA from herstatin mRNA transcripts from living cancer cells are minimal with no bands present for each of each of: MCF7, SKBR3, and MDA-MB-468. The gel was produced using standard 1% agarose protocols and 2µL of ethidium bromide for each of the 100 mL poured gels. The gel produced of the cDNA compliments produced by the herstatin primers chosen is shown below in figure 1. The primers used are shown below in the appendices. Herstatin protein was not found to be over transcribed via this method while literature asserts that it is

definitely overexpressed in SKBR3, and moderately expressed in MCF-7 (Cobleigh et al. 1999). Furthermore, pieces found were too small to be the expected cDNA isolated from intact herstatin protein mRNA.

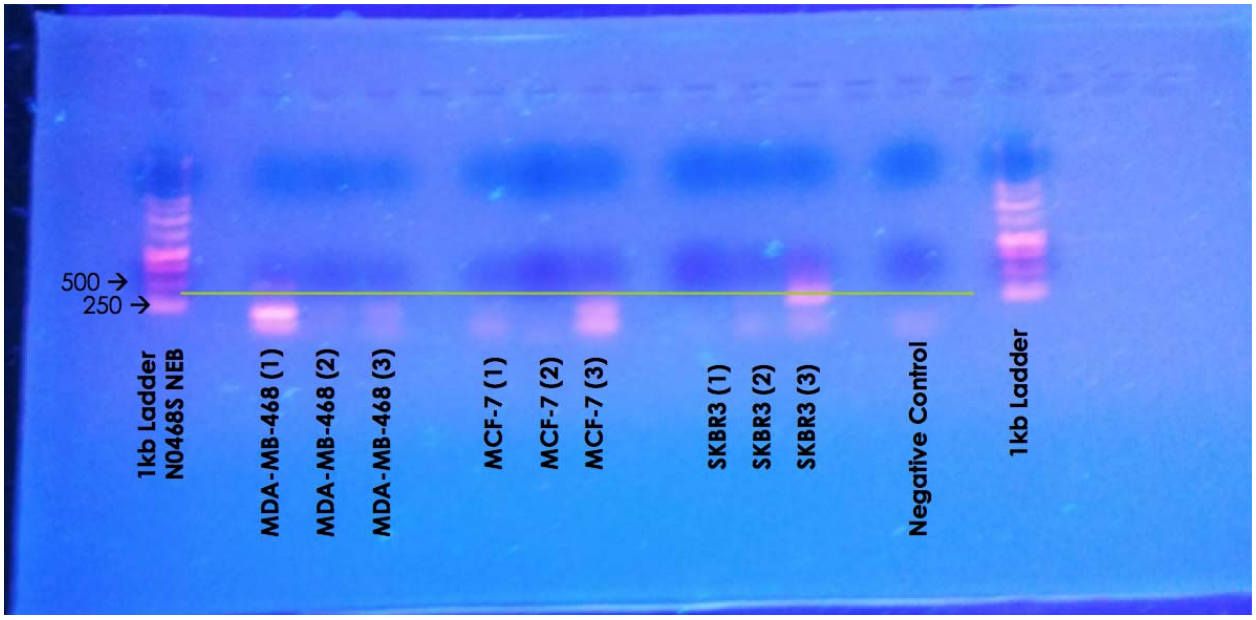

Figure 1- the agarose gel produced for the cDNA products of the RT-PCR reaction using Herstatin primers. A green line indicates a boundary that we expect to see banding above if substantial amounts of herstatin mRNA was successfully copied into cDNA using reverse transcriptase. The image was produced using a Samsung Galaxy S5 camera of the gel on a UV transilluminator. Negative control also shows no banding. The 500 and 250 designate band sizes (in basepairs) as outlined by New England Biolabs for that particular ladder type.

The androgen receptor mRNA transcript was chosen to find X-chromosome inactivation throughout the population of cancer cells. In every cell line and every pseudoreplicate a single distinct and clear band was produced in the ~500 base pair region of the size exclusion gel. Negative control with no template shows no band and any DNA present before RT-PCR was degraded using DNase prior to RT-PCR and gel electrophoresis. While these results are clear, future research using restriction endonuclease digestion and 6% non-denaturing poly acrylamide gel electrophoresis will seek to validate the finding further. The gel produced for the

androgen cDNA is shown below in figure 2. Replicate 3 for MCF-7 shows a fainter single band at ~433 bases.

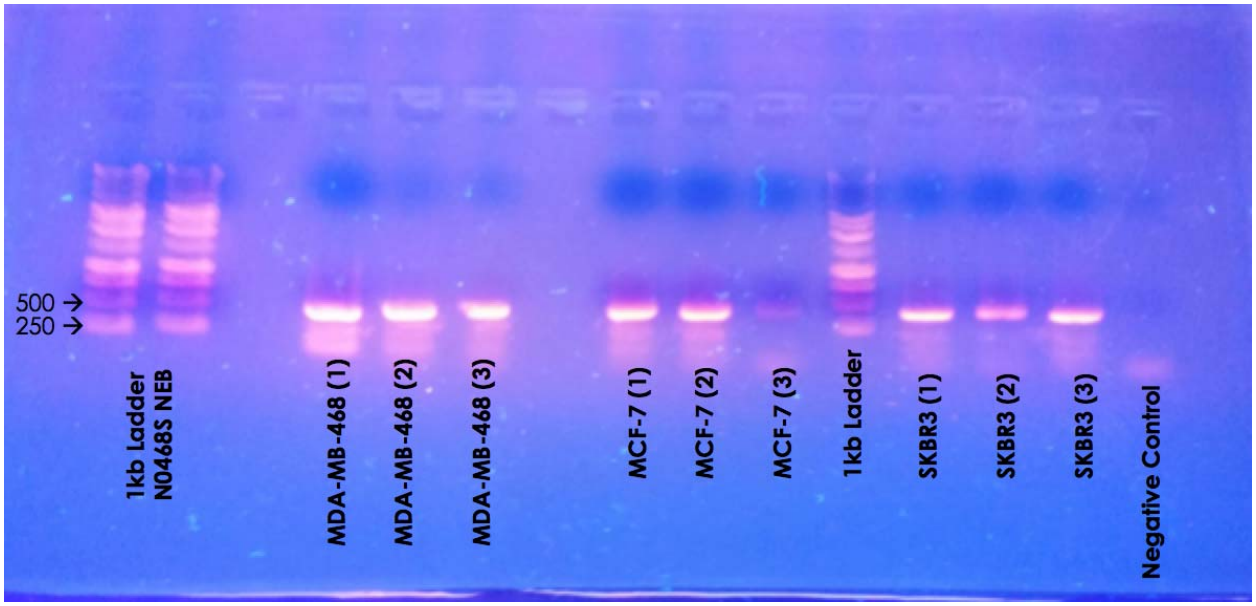

Figure 2- the agarose gel produced for the cDNA products of the RT-PCR reaction using androgen primers. The image was produced using a Samsung Galaxy S5 camera of the gel on a UV trans-illuminator. 500 and 250 designate band sizes (in basepairs) as outlined by New England Biolabs for that particular ladder type. The gel shows distinct single bands for each cDNA compliment to androgen receptor mRNA. For all replicates a distinct single band is present in the size range where androgen receptor cDNA should be located and the negative control shows no banding. The band for replicate 3 of the MCF-7 replicates shows a lower illumination than replicate 1 and replicate 2 for that cell type.

There was a low affinity for androgen primers and CCHCR1 protein mRNA. If that product had been converted to cDNA there would be bands present at 761 base pairs in size. Affinity was determined to be low using NCBI's primer-BLAST software, which calculates affinity for products via local base alignment. For this reason endonuclease digestion becomes a good method of discerning the product present from the RT-PCR process and presents an opportunity for due diligence. The result of the restriction digestion of androgen receptor cDNA by *SmaI* is shown below in figure 3. It is apparent from the gel that the DNA present is cut into ~284 basepair and ~142 basepair bands.

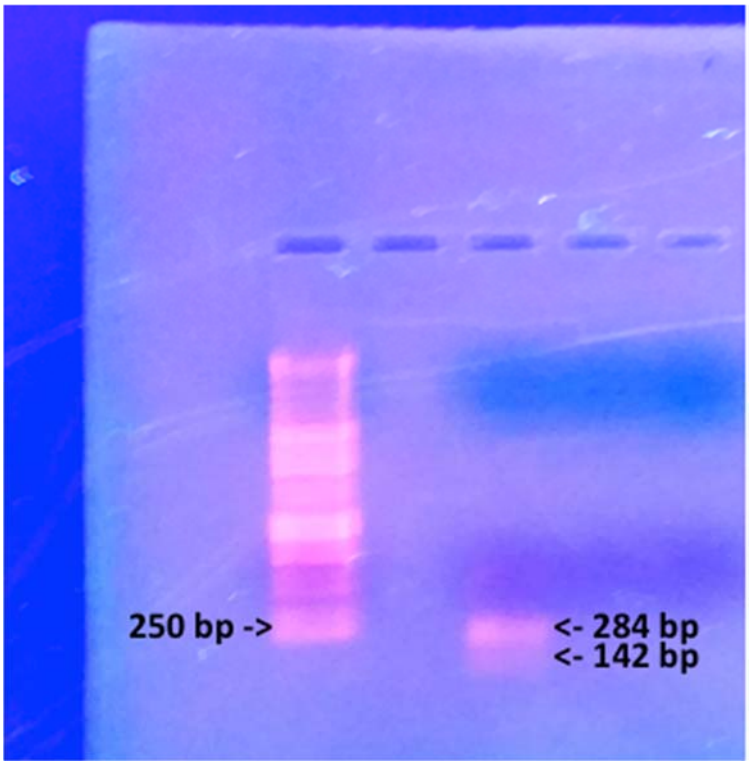

Figure 3- Bands of a size equivalent to ~284 and ~142 basepairs as derived from G571A Promega ladder are visible on this 1% agarose gel. The bands are the result of a *SmaI* digestion of the suspected androgen receptor cDNA.

In order to get a better resolution of the ~500 base pair sized androgen receptor cDNA bands from figure 1 a 6.5% polyacrylamide gel was run and the results of that are shown below in figure 4. Lanes are numbered from left to right and the first three lanes are 1kb ladder purchased from New England Bio Labs (Cat. N0468S). Lanes 4, 5, and 6 are the androgen receptor cDNA isolated from MDA-MB-468 replicates. Lanes 7,8, and 9 are the androgen receptor cDNA isolated from MCF-7. Lanes 10, 11, and 12 are the androgen receptor cDNA isolated from SKBR3 and Lane 13 is a Negative control. With no glutamines present in the receptor protein there would be a piece of cDNA produced equal to 426 base pairs. As can be seen in figure 4 there are 500 base pair polymorphs in lanes 4 and 12; ~480 base pair polymorphs in lanes 4 and 12; ~466 bp bands in lanes 4, 6, 11, and 12; ~450 base

pair polymorphs in lanes 4, 5, 6, 7, 9, and 12; ~433 basepair polymorphs in lanes 4, 5, 6, 7, 8, 9, 11, and 12; and finally an approximately 400 base pair band in every lane. The 433 base pair polymorph was found in all, but one lane that being for the SKBR3 replicate 1 which showed no androgen polymorph. In total then there was found anywhere from 0 to 5 different androgen polymorphic variants in any one cell population (10^6 cells) and there were shared polymorphs within and between different cell lines.

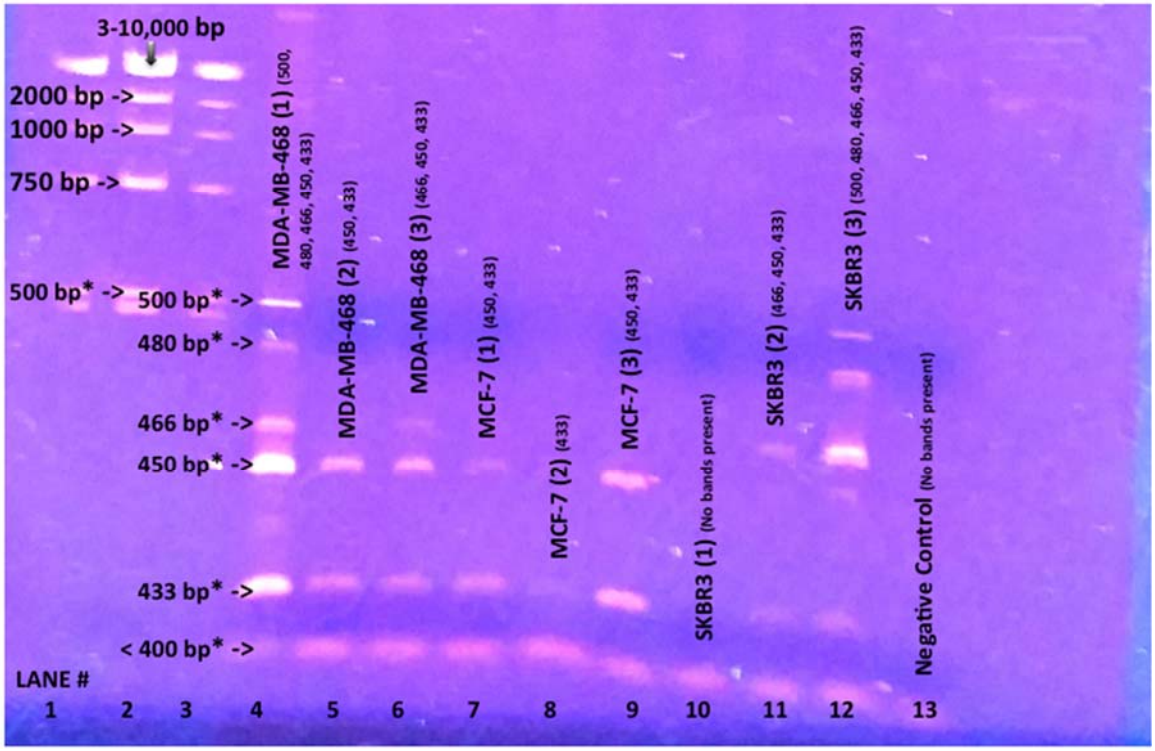

Figure 4- the 6.5% polyacrylamide gel produced for the androgen polymorph cDNA to better resolve the single bands present in figure 2. The largest band can be no larger than 500 as no DNA was present larger than that amount in the 1% agarose gel so that was used to align the 1kb N0468S ladder. The asterisk * is used because the 500 basepair band is split from NO468S 1kb ladder and there is no lower bound from the ladder chosen so the sizes of androgen polymorphs were determined from extrapolation of the molecular weight vs. distance curve created in excel. Band sizes are indicated in the second set of parentheses beside each cancer cell type and its replicate number.

DISCUSSION and CONCLUSIONS

The initial point of the research was to elucidate whether a single polymorphic variant of androgen was used in any cell line population. This would have had implications regarding only a single X chromosome being activated or inactivated throughout the cancer and potentially lead to a target for immunotherapy. However, it is clear that due to the mutator phenotype consistent with cancer (Loeb 1997) that there is not only one X expressed in any population. In fact, in a single population there may be up to 5 different polymorphic variants expressed, indicating 5 different X-chromosome variants meaning the active X-chromosome is unlikely to be a good target for immunotherapy.

Herstatin products from RT-PCR were not consistent with the expected DNA length of 545 base pairs. Instead there was an ~250 basepair band present in many samples but not all suggesting the gene may have been mutated wildly. This could be elucidated using sequencing, but the genetic material garnered from the reaction was used and so this was not accomplished in this work. It is a consideration for the future as it would be interesting to see if the DNA sequence showed any homology to the expected herstatin sequence or not (appendix I).

The *SmaI* restriction digestion of the ~500 basepair products from the RT-PCR reaction to ascertain whether the product was indeed cDNA from androgen mRNA produced bands that were approximately 284 and 142 base pairs in size. This finding is consistent with the restriction site for *SmaI* in the androgen sequence detailed in Appendix I alongside the primer details for this experiment. This provides further evidence and due diligence towards the identification of the 500

base pair bands as being androgen receptor DNA.

Interestingly, borne from this research is the finding that several androgen polymorphs are used within and between cancer cell types MCF-7, MDA-MB-468. Since an androgen gene variant with no glutamine codons would be 426 base pairs in size the number of glutamine codons can be calculated for each polymorph present. The two most prevalent polymorphs are the 433 base pair variant, and the 450 base pair variant. The 433 base pair variant was found in 8 of the 9 cell lines and is consistent with an androgen that has two glutamine codons in its polymorphic region. The 450 base pair polymorph was found in 7 of the 9 cancer cell lines and is consistent with an androgen variant that has 8 glutamine condons in its polymorphic poly-Q region. Given the diminishing quantity of cDNA and its exposure to many freeze thaw cycles it behooves this researcher to replicate the findings with an aim at elucidating polymorphic androgen variants in the approximately 500 base pair region. A better ladder should be used and better care should be taken to avoid the running off of the 250-253 base pair fragment from that ladder. Both of these considerations during replication of these results would likely work to achieve a better result. A gel run time of 35 minutes at 150 V should be sufficient to achieve this aim with the 6.5% poly acrylamide gel.

The finding that there is a potential selective pressure on the cancer cell lines causing advantage from certain variants, suggests a future avenue of research. The finding that certain polymorphs are favored within and between metastatic breast cancer lines suggests that there may be some reason to believe that androgen ablation therapy may have an impact on breast cancers. Presently androgen

ablation therapy is used in the treatment of prostate cancers, but there is only a tenuous link to better patient outcomes with its use as a neoadjuvant therapy after 5 years when used in combination with surgery (Soloway 2002). It is unwise to extend the findings too far, but an opportunity exists for research drawing out this finding further.

REFERENCES

Begley J, Ribas A. 2008. Targeted Therapies to Improve Tumor Immunotherapy. *Clinical Cancer Research*. 14(14): 4385-4391.

Belsches-Jablonski AP, Biscardi JS, Peavy DR, Tice DA, Romney DA, Parsons SJ. 2001. Src family kinases and HER2 interactions in human breast cancer cell growth and survival. *Oncology* 20(12): 1465-1475.

Bukur J, Herrmann F, Handke D, Recktenwald C, Seliger B. 2010. Identification of E2F1 as an Important Transcription Factor for the Regulation of Tapasin Expression. *Journal of Biological Chemistry*. 285: 30419-30426.

DeLeo AB, Jay G, Appella E, Dubois GC, Law LW, Old LJ. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proceedings of the National Academy of Sciences USA.* 76(5): 2420-2434.

Loeb L. 1997. Cancer cells exhibit a mutator phenotype. *Advances in Cancer Research.* 72: 25-56.

Rosenberg SA, Yang JC, Restifo NP. 2004. Cancer immunotherapy: moving beyond current vaccines. *National Library of Medicine* 10: 909-915.

Thermo Fisher Scientific. Protocols. Protocol for Thawing Frozen Cells. [Internet]. Life Technologies. [cited March 11, 2014]. Available from: http://www.lifetechnologies.com/ca/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/thawing-cells.html.

Soloway MS, Pareek K, Sharifi R, Wajsman Z *et al.* 2002. Neoadjuvant androgen ablation before radical prostatectomy in cT2bNxMo prostate cancer: 5-year results. *Journal of Clinical Urology.* 167(1): 112-116.

APPENDIX I PRIMER DETAILS

The primers I chose are highlighted in the sequences.

Human androgen-receptor mRNA, complete cds

GenBank: M34233.1

FASTA Graphics

Go to:

LOCUS HUMARX 3231 bp mRNA linear PRI 31-OCT-1994 DEFINITION Human androgen-receptor mRNA, complete cds. ACCESSION M34233 VERSION M34233.1 GI:179033 KEYWORDS androgen receptor. SOURCE Homo sapiens (human) ORGANISM Homo sapiens Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo. REFERENCE 1 (bases 1 to 3231) AUTHORS Govindan,M.V. TITLE Specific region in hormone binding domain is essential for hormone binding and trans-activation by human androgen receptor JOURNAL Mol. Endocrinol. 4 (3), 417-427 (1990) PUBMED 2342476 COMMENT Original source text: Human testis and prostate cancer cell line LNCaP, cDNA to mRNA. FEATURES Location/Qualifiers source 1..3231 /organism="Homo sapiens" /mol type="mRNA" /db_xref="taxon:9606" /map="Xq11.2-q12" gene 1..3231 /gene="AR" CDS 109..2829 /gene="AR" /note="androgen-receptor" /codon start=1 /protein id="AAA51780.1" /db xref="GI:179034" /db xref="GDB:G00-120-556" /translation="MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREVIQNPGPRHPEA
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FTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRMRHLSQEFGWLQITPQEFLCMKAML

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androgen receptor, ligand activated transcription
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2407..2409,2428..2430,2686..2688,2698..2700)
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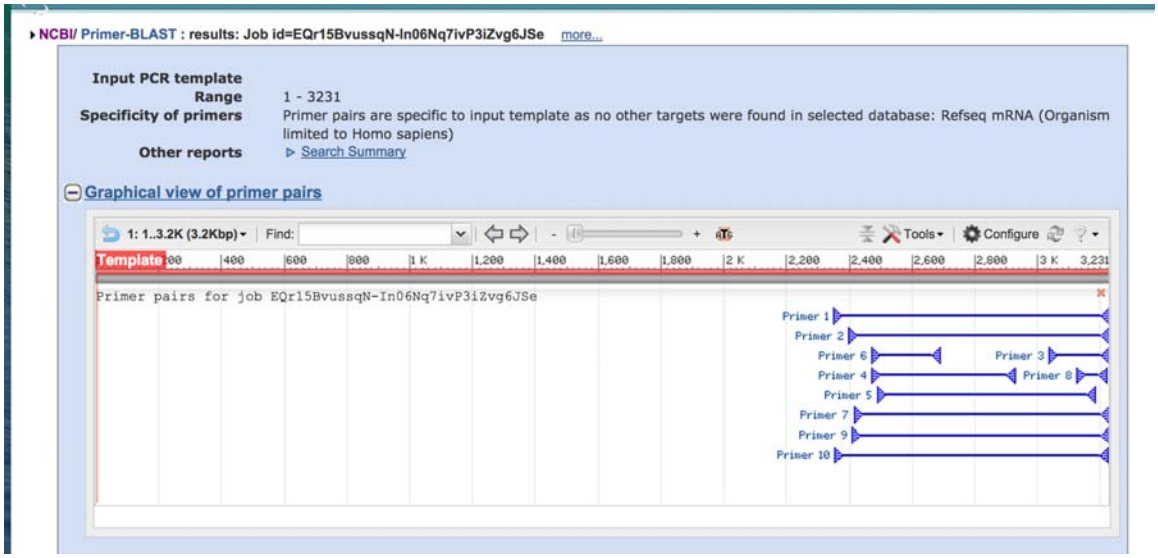

Database derived primers were not useful for the research proposed as they did not include the polymorphic region which is at the crux of deriving X-chromosome inactivation so we used.
Green highlighting above indicates primers and the yellow highlighting indicates the polymorphic region.

Forward 75-95: 5'-GGG AAG TAG GTG GAA GAT TC -3'
Reverse 500-481: 5'-GCG GCT CCA GGC TCT GGG AC-3'
Total Length: 433 Base pairs making it easy to discern its size as compared to the CCHRCR1 transcript variant at ~760 base pairs long. All other complimentary regions in the human genome produce strands that are very small (<100 bp) or very large (>1000 base pairs.)

Primer-BLAST *Primer-Blast results*

NCBI/ Primer-BLAST : results: Job id=BB_--A3ypNab-5_3_tmtieX0n5j264Kd more...

Input PCR template none
Specificity of primers Target templates were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports ▷ Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGGAAGTAGGTGGAAGATTC	20	54.84	50.00	4.00	4.00
Reverse primer	GCGGCTCCAGGCTCTGGGAC	20	67.44	75.00	6.00	4.00

Products on target templates

>NM_000044.3 Homo sapiens androgen receptor (AR), transcript variant 1, mRNA

```
product length = 447
Forward primer  1     GGGAAGTAGGTGGAAGATTC  20
Template        1082  ....................  1101

Reverse primer  1     GCGGCTCCAGGCTCTGGGAC  20
Template        1528  ....................  1509
```

>XM_006726004.1 PREDICTED: Homo sapiens coiled-coil alpha-helical rod protein 1 (CCHCR1), transcript variant X4, mRNA

```
product length = 761
Reverse primer  1     GCGGCTCCAGGCTCTGGGAC  20
Template        1323  C.T.....T..........T  1304
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Homo sapiens herstatin (HER-2) mRNA, alternatively spliced, complete cds

GenBank: AF177761.2

FASTA Graphics

Go to:

LOCUS AF177761 1316 bp mRNA linear PRI 20-SEP-2000
DEFINITION Homo sapiens herstatin (HER-2) mRNA, alternatively spliced, complete cds.
ACCESSION AF177761
VERSION AF177761.2 GI:10181232
KEYWORDS .
SOURCE Homo sapiens (human)
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 1316)
AUTHORS Doherty,J.K., Bond,C., Jardim,A., Adelman,J.P. and Clinton,G.M.
TITLE The HER-2/neu receptor tyrosine kinase gene encodes a secreted autoinhibitor
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 96 (19), 10869-10874 (1999)
PUBMED 10485918
REFERENCE 2 (bases 1 to 1316)
AUTHORS Doherty,J.K., Clinton,G.M. and Adelman,J.P.
TITLE Direct Submission
JOURNAL Submitted (16-AUG-1999) Biochemistry, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA
REFERENCE 3 (bases 1 to 1316)
AUTHORS Doherty,J.K., Clinton,G.M., Adelman,J.P., Evans,A.J. and Henner,W.D.
TITLE Direct Submission
JOURNAL Submitted (18-SEP-2000) Biochemistry, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201, USA
REMARK Sequence update by submitter
COMMENT On Sep 18, 2000 this sequence version replaced gi:5917723.
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LCYQDTILWKDIFHKNNQLALTLIDTNRSRACHPCSPMCKGSRCWGESSEDCQSLTRT

VCAGGCARCKGPLPTDCCHEQCAAGCTGPKHSDCLACLHFNHSGICELHCPALVTYNT

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ORIGIN

I've Highlighted the primer sequences.

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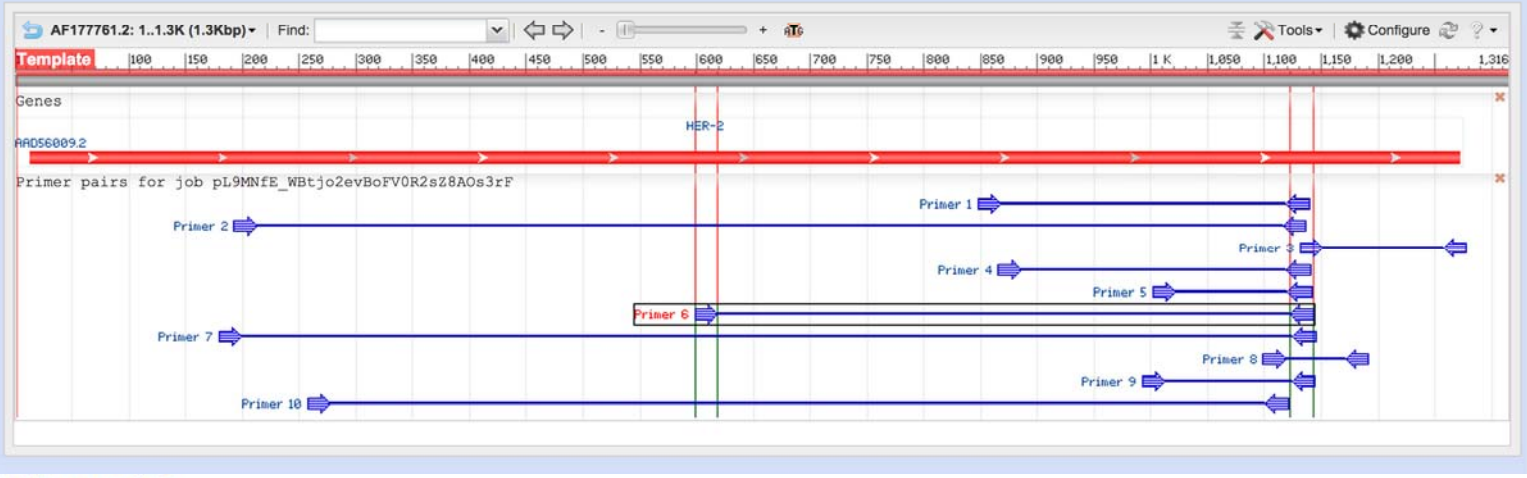
NCBI/ Primer-BLAST : results: Job id=pL9MNfE_WBtjo2evBoFV0R2sZ8AOs3rF more...

Input PCR template AF177761.2 Homo sapiens herstatin (HER-2) mRNA, alternatively spliced, complete cds
Range 1 - 1316
Specificity of primers Primer pairs are specific to input template as no other targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)
Other reports ▷ Search Summary

Graphical view of primer pairs



Primer pair 6

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTCCGATGTGTAAGGGCTCC	Plus	20	599	618	59.89	60.00	3.00	2.00
Reverse primer	GACTAGGTCCCAAGAGGGTCT	Minus	21	1143	1123	59.99	57.14	4.00	3.00
Product length	545								