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Targeting Expression of an Oncogene by Splicing Interference (SPLICEi) in Human Mammary Carcinoma Cell Culture Model

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ABSTRACT

In nuclear pre-mRNA splicing, introns are removed through cooperative interactions of small nuclear RNAs (snRNAs). Many human genes are interrupted by two types of nuclear pre-mRNA introns. Major class or the U2-dependent type introns are spliced by U1, U2, U4, U5 and U6 snRNAs. The minor class or U12-dependent type introns are spliced by U11, U12, U4atac, U5, and U6atac snRNAs. It has been shown that over expression of minor class spliceosomal snRNAs can have an inhibitory effect on the splicing of major class introns. To further test the concept, we targeted HER-2/Neu proto-oncogene, which is over-expressed in 20-30% of human mammary tumors. We constructed a series of mutant human U6atac and U11 snRNAs to target 5' splice site of introns 1, 2, 6, 8, 12 and 13 of HER-2/Neu in order to prevent nuclear pre-mRNA splicing and to down regulate protein synthesis. To determine the efficacy of our approach, we transiently expressed mutant snRNAs in mammary carcinoma cells to activate splicing interference (*SPLICEi*) of HER-2/Neu pre-mRNA. Our data indicates that the modulation of HER-2/Neu pre-mRNA by mutant snRNAs mediated *SPLICEi*

affect the protein expression in cultured cells. Furthermore, albeit modest, we observed inhibition of cancer cell proliferation and activation of apoptosis. Although, our approach is still at experimental stages, it presents another opportunity for targeting of oncogenes at RNA level.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

1.1 Gene Expression

Currently, the most abundant mode of treatment for cancer is chemotherapy, which chemically poison the body tissues in an attempt to destroy the cancerous cells. Chemotherapy is highly toxic yet not specific. Along the same approach is an attempt to treat cancer by a very broad scope is radiation therapy. Radiation therapy uses harmful radiation in the same manner as chemotherapy to treat cancer. The outcome is just as grim with the side effects being very just as catastrophic ranging from nausea and hair loss to mouth sores.

Recent advancement in research has made the possibility of more specific types of cancer therapy using genetic approaches. Now that specific human genome sequence is known, researchers are taking advantage of bioinformatics in attempts to

develop more specific gene targeted therapies. Many studies have been done to understand the molecular and cellular pathways involved in gene expression.

The “central dogma” of molecular biology states that development of a cell is governed by a series of intrinsically orchestrated cellular events in gene expression. Gene expression begins with DNA coded information being transcribed to RNA and RNA in turn is translated to proteins, which affects cellular functions (Zhou, 2010). Genetic transcriptional and translational events are the building blocks of molecular biology and are stringently regulated at several steps (Hartwell et al, 2004). It because of new insight into these steps that enable researcher to develop more direct approaches to treatment of various diseases, including cancer.

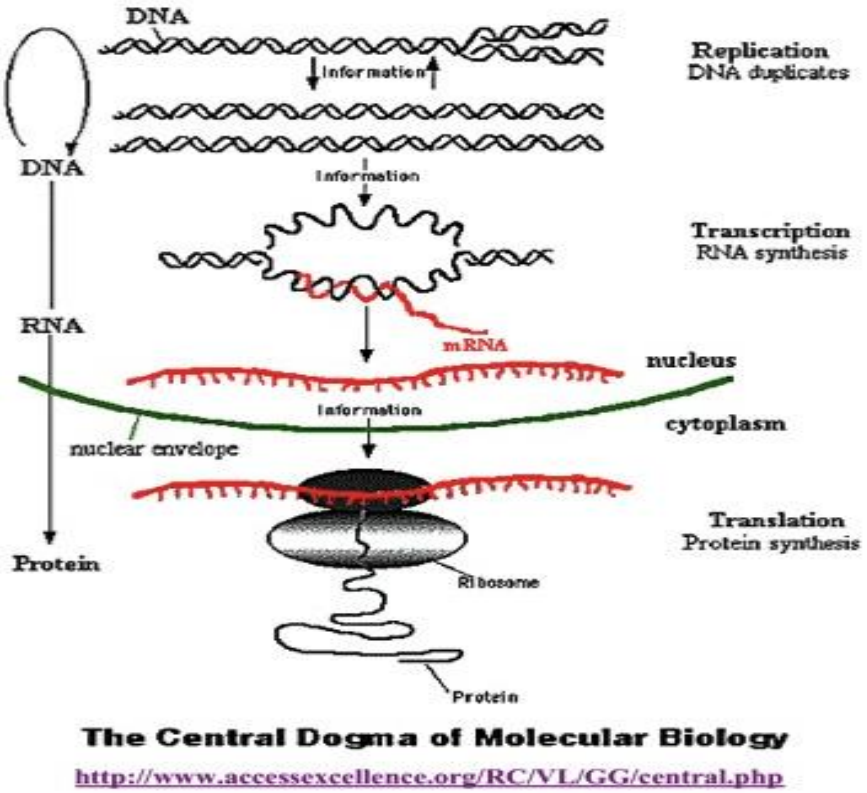


Figure 1: Different Levels of control of gene expression: In the cell, gene expression is regulated by a variety of steps at the DNA, RNA, and protein levels.

During the transcriptional processes from DNA to RNA there are molecules involved to ensure the accurate synthesis of functional RNA. In disease cells the intrinsic machinery to ensure protein synthesis from DNA is perturbed and needs to be targeted to resume proper function. Nucleic acid therapy targets the transcriptional aspect of this machinery in attempts to specifically target the perturbation.

1.2 Strategies For Gene Targeting

Currently there are some therapies for different types of cancer as well as other diseases that are nucleic acid based. These therapies include targeting diseases

using antisense olidonucleotides, small interfering RNA (siRNA), and micro RNA (miRNA).

1.2.1 Antisense oligonucleotides

Antisense oligonucleotides (AO) are short nucleic acids designed to bind to specific sequences of pre-mRNAs in an attempt to manipulate mRNA splicing that can eventually affect protein synthesis. The goal of this type of genetic therapy is to modulate the expression of the specific gene through alternative splicing (Goyenvalle et al. 2011).

Gene therapy can have either translational suppressive capability (anti-sense oligonucleotides [oligos]) or replacement (of inactivated, mutated or deleted suppressor genes such as PTEN) techniques (Huang et al. 2001). Both tumor and normal cells express the same genes that differ in expression levels. Gene therapeutics can be incorporated in the cell proliferation and growth of tumors, but resistance eventually develop because the endogenous biochemical pathways of the cell are complex and highly regulated by many factors that are easily altered. It has been suggested that tumors can change their dependence upon one factor by relying upon others through compensation (Rubenstein et al. 2011).

Progress has been made in the development of drug therapy using antisense oligonucleotides but it still face obstacles including, but not limited to, effectively delivering drugs containing AO, and properly regulating the outcome of this therapy. Splicing is a nuclear event and AO has to be delivered to specific cell types without the pre-mRNA degradation before splicing. The delivery mechanism currently

available is slow and relatively ineffective. Another very substantial problem is the toxicity experienced but the non-targeted cells.

1.2.2 Small Interfering RNA (siRNA)

Small interfering RNA (siRNA) is a double stranded RNA molecule that is involved in mRNA cleavage at specific sequences. Because of this specificity, siRNA is frequently used effectively in RNA interference methods in the down regulation of genes involved in diseases (Gao et al, 2011).

RNA interference was discovered in Caenorhabditis elegans (*C. elegans*) in 1985. RNA interference, a process that requires sequence-specificity, it is post-transcriptional gene silencing directed by short interfering 21–23 nucleotide double-stranded RNA (siRNA) while specifically and dramatically reducing the expression of its targeted mRNA. In siRNA therapeutics, a lot of vectors for siRNA delivery have been reported to achieve perfect results with in vitro applications, but these vectors were mostly inappropriate for use in vivo or are only administered locally. To be administered systemically, targeted siRNA delivery should be designed to improve accumulation of siRNA at three levels; the target tissue, target cell, and intracellular target site of action (Lu et al. 2011)

However, siRNA are highly unstable molecules. Problems hindering effective therapeutic application lie predominantly in their delivery, stability, and off-target effects. Reliable effective delivery systems can provide solutions to many of the challenges facing siRNA therapeutics. Due to some fatal disadvantages of using viral vectors, nonviral carriers are being studied extensively. Aside from liposomes,

nanoparticles and cationic polymer, carriers have exhibited improved in vivo stability, better biocompatibility, and efficiency for gene silencing, but cellular toxicity still remain problematic (Allain et al, 2011).

1.2.3 MicroRNA (miRNA)

MicroRNAs (miRNAs) are small stretches of non-coding sequences that are (does not transcribe for a protein) RNA molecule which are involved in regulating gene expression. Their presence has been shown to have implications to be in numerous cellular processes such as cell proliferation and differentiation. Hundreds of miRNAs have been shown to play a role in diseases including cancer. miRNAs are important regulators of genes, and when their expression levels are abnormal it can lead to disease. Deregulation of key components of the miRNA machinery have been shown in cancer to alter expression of miRNAs and shown to play a major role in pathogenesis (Rupaimoole et al, 2011).

Inhibiting mRNA translation using miRNAs could soon represent a valid option for the treatment of specific patients. A specific case described in detail by Hui Ling et al. (2011) shows the use of miRNAs inhibited translation as a way to treat chronic lymphocytic leukemia (CLL). According to Ling, there would be two advantages to using miRNAs: 1) miRNAs are a natural product produced in human cells (unlike chemotherapeutic agents or antisense oligonucleotides), and 2) miRNAs target multiple genes from the same pathway and therefore the action occurs at multiple levels in the same pathway (for example, miR-16 targets both antiapoptotic genes Bcl-2 and Mcl-1) (Ling et al, 2011).

MicroRNA therapy is an emerging field but there is still much to be established about these non-coding RNAs. Since, microRNAs do not need perfect complimentary to have an effect, off target effects still pose a major problem. A more general problem with microRNA therapeutics is the inadequate delivery system in place to have the desired effect.

1.3 Small Nuclear RNA (snRNA)

The foundation for our study is the targeting the RNA:RNA interactions that take place between pre-mRNA and small nuclear RNAs (snRNA). snRNA is a group of RNA molecules that is crucial to pre-mRNA splicing process (Fischer et al, 2011). Splicing is the removal for introns and the ligation of the exons. Introns are the untranslated noncoding region of DNA, whereas the exon is the translated coded region of RNA. After splicing occurs a mature mRNA transcript is ready to be translated into the corresponding protein.

Small Nuclear Ribonucleoprotein (snRNP) - are complexes formed with small nuclear RNAs and their respective proteins to facilitate splicing. The major class of spliceosome includes U1, U2, U4, U5, and U6 snRNAs. The minor class of spliceosome includes, U11, U12, U4ATAC, U5 (same as in major class), and U6ATAC snRNAs (Shukla et al, 2004).

The four snRNAs that are unique to each of the spliceosomal machineries have been shown to have analogous functions. Therefore, U11 snRNA is the functional analog of U1 snRNA, U12 snRNA is the analog of U2 snRNA, U4atac snRNA is the analog of U4 snRNA, and U6atac snRNA is the analog of U6 snRNA.

U5 snRNA appears to function in both spliceosomes. These analogies are based, in large part, on the similarities in RNA:RNA interactions displayed by the respective snRNAs. (Shukla et al, 2004).

snRNAs DEPICTING THEIR CORRESPONDING FUNCTIONAL ANALOGS

MAJOR CLASS (U2-dependent)	MINOR CLASS (U12-dependent)
U1	U11
U2	U12
U4	U4ATAC
U5	U5
U6	U6ATAC

1.4 Nuclear Precursor mRNA (pre-mRNA) Splicing

Splicing is a dynamic chemical process that occurs through the formation of spliceosomal complex. The spliceosomal complex is a ribonucleoprotein complex consisting of the 5 snRNAs and more than 300 auxillary proteins which aids in splicing effiency. Splicing convert the pre-mRNA to the mRNA through two transesterification reactions that is presumably catalyzes by an RNA (Newman et al, 1995).

The pre-mRNA is made up a 5’ exon followed by an intron which contains; splice site consensus sequence (differs in major and minor class introns) at the exon/intron boundary, a branch site sequence containing a conserved adenine, a polypyrimidine tract (only in major class introns), a 3’ splice site consensus sequence (differs between major and minor class) at the intron/exon boundary, and a 3’ exon (Will et al, 2005).

The first transesterification reaction occurs when the 2’OH from the adenine attacks the phosphodiester bond at the 5’splice site, freeing the exon 1, leaving a lariat shaped intron and the following exon. The second transesterification reaction occurs when the 3’OH from the 5’ exon attacks the phosphodiester bond between the intron and the 3’ exon. The products are the exons spliced together and the intron lariat was removed. Figure 2.

U2 and U12 dependent introns are depicted by short conserved sequences ate the 5’ splice site, 3’ splice and branch site. U12 dependent introns was first recognized by Jackson (1991) and Hall and Padgett (1994) because of the terminal dinucleotides of the U12 dependent contained AT at the 5’ splice site and AC at the 3’ splice site (Burge et al. 1998). This observation in intronic sequence broke the previously observed GT at the 5□ splice site and AG at the 3’ splice site of U2 dependent introns.

U2 dependent class of spliceosomes is responsible for over 99% of all the splicing of pre-mRNA that occurs in eukaryotic cells and is subsequently referred to as major class splieosomes. Consequently, less than 1% of splicing of eukaryotic pre-mRNA is done by minor class spliceosomes (Burge et al. 1998)

Figure 2

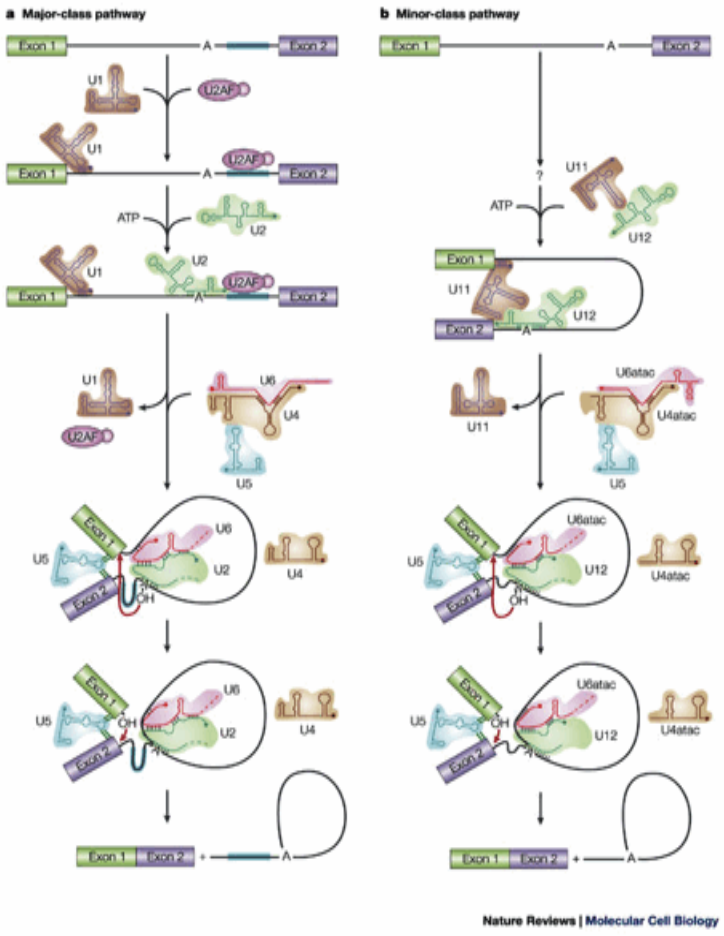

a Major-class pathway
b Minor-class pathway
Exon 1
Exon 2
A
U1
U2AF
ATP
U2
U6
U4
U5
U11
U12
U6atac
U4atac
OH
Nature Reviews | Molecular Cell Biology

Figure 2: Major and minor spliceosomal pathways: (a) Shows the major class splicing pathway containing two exons, conserved "A", and the polypyrimidine tract (blue). First the U1 snRNA binds the 5' splice-site, the U2 binds the A. Secondly, U1 is released as the U4 snRNA and U6 snRNA, di-snRNP complex, along with U5, binds to the intron forming the intron lariat. Then, U4 is released, U5 facilitates the stitching of the two exons, forming the mRNA and the spliced lariat, and the remaining snRNAs are then released. (b) Shows the minor class pathway with the two exons, branch site with the conserved "A", but no polypyrimidine tract. The U11 snRNA and U12 snRNA di-SNRP complex binds to the 5' splice-site and 3' splice-site respectively, bringing the two exons in close proximity, releasing U11. Following, is the U4ATAC snRNA and U6ATAC snRNA di-SNRP complex, along with U5, binding to the intron, and U4ATAC is released. U5 also facilitates the stitching of the two exons, forming the mRNA and the spliced lariat, and the remaining snRNAs are released also in this pathway.

As reported by Shukla et al, (2004), U12 dependent snRNAs are capable of binding to U2 dependent introns, we proposed that mutants of U12-dependent snRNAs, U11 and U6atac, to bind complementarily the 5' splice site of the major class intron, should cause splicing interference (*SPLICEi*) in the gene. To test this model we chose the HER-2/Neu gene, a gene spliced through the U2 dependent pathway, and is over-expressed in specific type of aggressive mammary carcinoma.

Currently, nucleic acid therapeutics mainly target genes post-transcriptionally. Targeting the snRNAs and pre-mRNA binding, is a novel approach in targeting diseases in the pre-mRNA stage. Understanding the RNA:RNA base-pairing that occurs between the pre-mRNA and the snRNAs, as well as the HER-2 gene sequence, we mutated specific nucleotides of the snRNAs (U6ATATC and U11) to forcibly cause the HER-2 gene to bind with the U12-dependent snRNAs in attempts to down-regulate the gene expression, activating *SPLICEi*.

1.5 HER-2/Neu Gene and Mammary Carcinoma

Human epidermal growth receptor- 2 (HER-2/Neu), is a transmembrane receptor tyrosine kinase (Shigematsu et al, 2011). HER-2/Neu protooncogene is over-expressed in nearly 20%–40% of breast carcinomas, and its expression levels has been used an indicator of tumor aggressiveness and poor prognosis (Skalova et al, 2011). HER-2/neu has become an essential part of the clinical evaluation of breast cancer patients. Currently HER-2+ patients are treated with a humanized monoclonal antibody trastuzumab (Herceptin; Genentech, San Francisco, CA, USA). (Kapila et al, 2011)

Trastuzumab shows clinical activity in women with HER2/neu-overexpressing metastatic breast cancer antitumor effects when combined with paclitaxel or anthracyclines, other available cancer treatment drugs, achieving an overall response of 40%–60% of the patients observed (Kapila et al, 2011). In spite of its efficacy of drug cocktail, the response to trastuzumab only is 7%–35%, depending on the level of HER2/neu expression, and the median duration of response is less than 9 months (Costantini et al).

HER-2 gene contains 26 exons and 25 introns and to test our hypothesis we generated U6ATAC and U11 snRNAs containing complementary nucleotides to base-pair with the 5’ splice sites of HER-2/Neu pre-mRNA. The targeted introns are shown in figure 3 (Introns: 1, 2, 6, 8, 12,and 13). Splicing interference or *SPLICEi* was be introduced by liposome based transient transfections of the modified snRNAs

and the consequence of HER-2/Neu SPLICE*i* mediated repression was studied using various cell and molecular techniques.

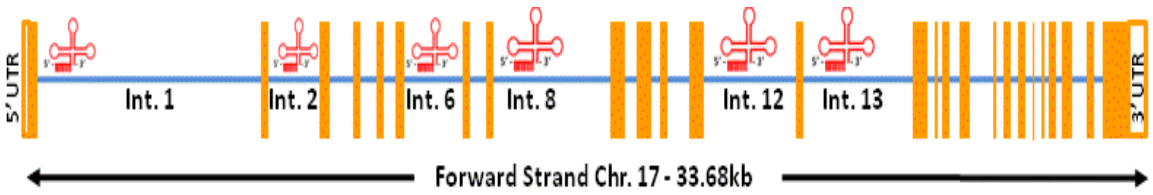


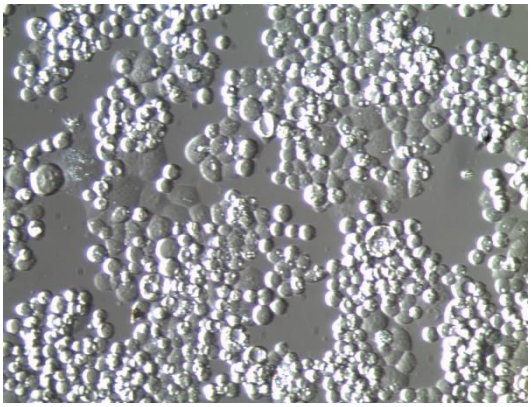
Figure 3: Schematic diagram of the HER-2/Neu gene structure: Orange bars correspond to the exons and blue line, the introns. The red color stem-loop signifies the targeted binding of U6ATAC and U11 to the 5' splice-site of multiple introns.

CHAPTER II

MATERIALS AND METHODS

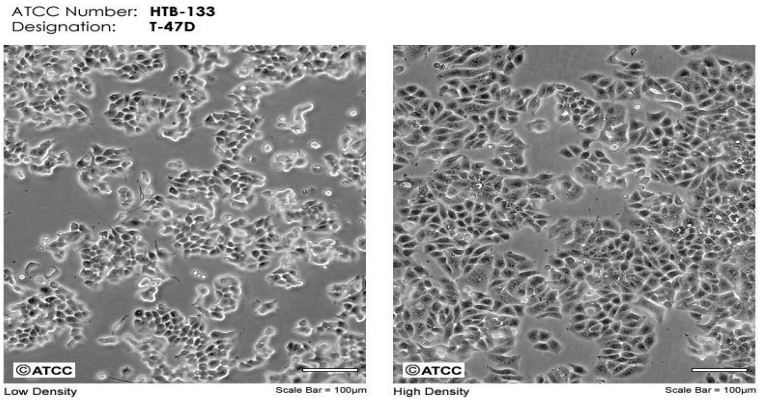
2.1 Cell lines

MDA-MB-453



Adherent epithelial cells derived from human female mammary gland (breast) with metastatic carcinoma. MDA-MB-453 over-express HER-2 gene which is a marker for breast cancer. Chan et al. (1995), was able to shown the overexpression of HER-2/Neu in the MDA-MB-453 cell line.

T-47D



Adherent epithelial cells derived from h uman female mammary (breast) duct with ductal carcinoma. The HER-2 gene is used as a marker in breast cancer, and is overexpressed in T-47D cells. Based on research done by Lyons et al. (2001), T-47D has a threefold increase of the copy of HER-2/Neu gene.

2.2 Mammary Carcinoma Cell Culture

MDA-MB-453 and T-47D cells were obtained from the ATCC to be used . Cells were cultured in Dulbecco Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), 1% Penicillin:Streptomycin (Pen-strep). Both cell-lines were incubated in 5% Carbon dioxide (CO2) in 37°C, on 100 mm culture plates. MDA-MB-453 cells were split maximum 1:4 ratio, and T-47D were split maximum 1:6 ratio.

2.3 Mutagenesis

In order to introduce the sequence mutations desired we did mutagenesis reactions. The mutagenesis reactions were done using Change-IT Multiple Mutation Site Directed Mutagenesis Kit. A 20µl reaction was done using 5ng of DNA template.

Each reaction mixture included 10X buffer, 5μM amp forward, 5μM of mutant primer, and 0.8μl Change-it enzyme. The PCR conditions consisted of a denaturing step (95°C, 2 min) followed by 37 cycles (95°C, 30 sec; 53°C, 30 sec; 68°C, 35 min) and a final extension step (68°C, 20min). The 10μl of reaction was then DPN-1 treated using 1μl of DPN-1 enzyme and incubated at 37°C for 2hrs. After, additional 0.5μl DPN-1 was added and incubated at 37°C for 1hr.

2.4 Transformation

To introduce the mutated DNA into the MDA-MB-453 and T-47D cells, *E. coli* cells were first transformed with the matant DNA sequence using the following procedure. 4μl of the DPN-1 treated reaction from mutagenesis was added to 100μl of DH5α *E.coli* competent cells kept on ice for 30 min then given a 42°C heat shock for 50 sec and placed back on ice for 2 min. 900μl of Luria Bertani (LB) Broth was added to the DH5α cells and spin for 7 min discarding the supernatant. Add 100μl of LB and plate cells on LB/Ampicillin plate and incubate at 37°C for 12-16hrs. Colonies were picked and placed in 4ml of LB containing ampicillin overnight in shaker at 250 rpm at 37°C, after stored 4°C to be used for mini-prep. All the steps were done aseptically.

2.5 Miniprep

To extract the plasmid DNA from the competent cells miniprep was done using the following procedure. Miniprep was done using Zyppy Plasmid Miniprep Kit. In brief, using 600μl of bacterial culture grown in LB medium add 100μl of 7X

lysis buffer, neutralizing with 350µl Neutralization buffer and centrifuged. The supernatant was transferred to the zymo-spin IIN column and centrifuged. Discarding the follow through, the column was washed 2x with zippy wash buffer and centrifuged. The column was placed in clean microcentrifuge tube 30µl of elution buffer was added directly to the column and centrifuged after 1min of standing at room temperature. The plasmid DNA was collected in the clean microcentrifuge tube after centrifugation.

2.6 Maxiprep

To obtain a high yield of the desired plasmid DNA maxiprep was done with the following procedure. Maxi-prep was done using Qiagen Maxi Kit. RNase A solution was added to buffer P1, buffer P3 was pre-chilled to 4°C. In brief, 500µl of overnight LB culture was centriguged at 4°C for 15 min. Pellet was resuspended in buffer P1, buffer P2 was then added and sample was mixed thoroughly and incubated at room temperature for 5 min. Buffer P3 was added and sample was centrifuged at 4°C for 30 min. The supernantant was re-centrifuged at 4°C for 15 min. Qiagen-tip was equilibriated with 10ml of buffer QBT and column was allowed to empty by gravity flow. DNA was eluted with buffer QF in a clean vessel. Isopropanol was added to precipitate DNA and kept overnight at -20°C. After centrifugation the DNA pellet was washed with 70% ethanol. Pellet was allowed to air-dry and re-dissolved in 100µl of TE buffer.

2.7 Transfection

To introduce the mutations into the mammary carcinoma cell lines they needed to be transfected. Transfection was done using Roche Fugene 6 tranfection reagent. Transfection was done in 3:2 ratio (3µl of Fugene 6 with 2µg of mutant DNA constructs). Transfections were done in 6-well plates. In brief, cells were plated in 6-well plates in antibiotic-free media overnight. 24hrs after, 3µl fugene-6 reagent and 2µg DNA were added to 100µl of antibiotic-free serum-free media per well. The media containing fugene-6 and DNA was added directly to plated cells and incubated for 48hrs at 37°C 5% CO2.

2.8 RNA Extraction Using TRIzol Reagent

In order to extract RNA from the transfected cells TRIzol reagent was used according to the following procedure. In brief, media was aspirated and 1ml TRIzol was added directly to cells. The lysate was passed through the pipette several times and collected in microcentrifuge tubes. 200µl of chloroform was added to each tube shaking vigorously. Samples were centrifuged and the aqueous phase was collected in fresh microcentrifuge tube. 500µl of isopropanol and centrifuged at 4°C for 10 min. Pellet was wash with 75% ethanol vortexed and centrifuged at 4°C for 10 min. RNA pellet was air-dried then dissolved in 30µl RNase-free water. Sample was incubated at 55°C for 10 min to resolubilize the RNA and stored at -80°C for future use.

2.9 Removal of Nuclear DNA Contamination from Total RNA

The total RNA was purified of nuclear DNA contamination using Promega Kit. In brief, to each sample 10µl RQ1 10X buffer + 10µl RQ1 Dnase enzyme was added and incubated at 37°C for 20 min. Additional 5µl of DNase enzyme was added to each sample and incubated for at 37°C for 20 min then centrifuged. 100µl phenol chloroform was added vortexed then centrifuges for 5 min. Upper phase was collected in fresh microcentrifuge tube. 35µl chloroform was added, sample was vortexed, and centrifuged. The upper aqueous phase was collected in fresh microcentrifuge tube, sodium acetate $1/10^{th}$ the collected volume was added + 1µl glycogen and vortexed. 2 ½ total volume 100% ethanol was added, mixed, and stored at -80°C overnight. Samples were centrifuged at 4°C for 30 min, the RNA pellet washed with 250µl 75% ethanol/DEPC treated water and re-centrifuged. RNA Pellet was air-dried and re-suspended in 20µl DEPC treated water. Samples were heated at 50°C for 15 min to resolubilzes RNA and placed at -80°C for future use.

2.10 cDNA Synthesis

In brief, using Promega- Improm-II Reverse Transcription System 0.5µg RNA was incubated with 10µM reverse transcription primer at 70°C for 5 min. Using 25mM magnesium chloride and 0.5mM, 0.5µl recombinant RNasin ribonuclease inhibitor, and 1µl Improm-II reverse transcriptase. The reaction mixture was placed in thermal cycler at 25°C, 5 min; 42°C, 60 min; 70°C, 15min.

2.11 Polymerase Chain Reaction

DNA synthesized was amplified using Promega - GoTaq PCR core systems a reaction containing 2mM magnesium chloride, 5nM gene specific forward primer, 5nM gene specific reverse primer, 0.125µl Go Taq DNA polymerase, and 1µl cDNA template. The PCR conditions consisted of a denaturing step (94°C, 3 min) followed by 24 cycles (94°C, 1 min; 55°C, 1.05 min), and extension step (70°C, 1 min).

2.12 Quantitative Real Time – Polymerase Chain Reaction (qRT-PCR)

Using SYBR GreenER qPCR super mix 20µl reactions were done with 0.1nM forward and reverse primers, and 1µl cDNA template. The PCR conditions consisted of pre-cycling stage (50°C, 2 min), followed by denaturation step (95°C, 10 min), then 40 cycles (95°C, 15 sec; 60°C, 1 min). Melt curve was obtained with 100% ramp (95°C, 15 sec; 60°C, 1 min), +0.3 (60°C, 1 min; 95°C, 15 sec). In these experiments Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control to compare gene expression from the HER-2/Neu data obtained.

2.13 Total Cell Lysate

To obtain total cell lysate from transfected cells, they were lysed using lysis buffer containing 98% M-PER reagent, 1% phosphatase inhibitor cocktail 2 (Sigma), and 1% protease inhibitor cocktail. 70µl of lysis buffer was added to each sample and placed in shaker at 900 rpm, 4°C, for 40 min. The supernatant was collected for future experiments. Bradford assay was done to determine the protein concentration.

2.14 Western Blot Analysis

To observe the protein product of the HER-2/Neu gene total cell lysate was analyzed by electrophoresis. Using NuPAGE 4-12% Bis-Tris Gel 1.5mm x 15well precast gel (Invitrogen) electrphoresis was done using 1X NuPAGE MOPS SDS running buffer. 5μg of proteins were loaded with loading buffer (90% NuPAGE LDS Sample Buffer (4X) (Invitrogen)
10% β-Mercaptoethanol) and run at 140V, 1 ½ hrs. The gel was then carefully transferred to nitrocellulose paper (NCP) in apparatus containing 1liter transfer buffer (3.02g Tris, 14.4g glycine, 200ml methanol).

When transfer was complete the NCP was placed in blocking solution (5% dry milk, 50ml TBS-T) for 1hr then washed (5min, 8x) with TBS-T. Primary antibodies for HER-2/Neu (ratio 1:1500), and β-actin (ratio 1:20,000) were added to blocking solution and Blot was incubated shaking overnight at 4°C in solution. Next day the primary antibodies were washed off blot using TBS-T (5 min, 8x). Secondary antibody (ratio 1:10,000) was added to blocking solution and added to blot and incubated at room temperature for 2hrs. Blot was washed with TBS-T (5min, 8x), bands were visualized using ECL Western Blot Detection Kit (GE Healthcare) and Typhoon scanner.

2.15 Cell Viability Assay

To analyze the cells health after transfecting the cell viability assay was done according to the following procedure. Cells were plated using 96-well plate,

transfected as stated previously, and harvested at different time points (2, 4, and 6 days). At time of harvest Cell Titer-Glo Luminescent Cell Viability Assay substrate was thawed and equilibriate at room temperature (~1-2hrs). The cell culture plate and its content was allowed to equilibrate at room temperature 30 min. 100µl of substrate was added to each well and the plate was scanned in the luminometer (Shake 15min, incubate 20 min, and reading taken at 1 sec intervals.

2.16 Apoptosis Assay

To determine whether the transfection of the mutation snRNAs transfect had an impact on the life of the cells an apoptosis assay was done according to the following procedure. The cells were transfected as previously outlined. Apoptosis assay was done using FITC Annexin V. In brief, transfected cells were washed using PBS and centrifuged to collect the pelleted cells. 100µl of binding buffer was added and cells were gently re-suspended in buffer. 5µl FITC Annexin V + 5µl Propidium Iodide (PI) was added to each sample in dim lighting and incubated in dark at room temperature for 15 min. An additional 400µl of 1X binding buffer was added to each tube and samples were analyzed via flow cytometry using the Fluorescence-activating cell sorting (FACS) machine. Cells only sample to calibrate the FACS machine.

CHAPTER III

RESULTS and DISCUSSION

3.1 Construction of Sequence Mutations

To study the effects the mutated snRNAs U6ATAC and U11 (Appendix 3) have on the splicing of HER-2/Neu pre-mRNA the first aspect was to generate the mutant to be transiently transfected into the cells. Using HER-2/Neu gene as a template the mutants snRNAs were generated to be complementary to the HER-2 pre-mRNA 5' splice site for introns 1, 2, 6, 8, 12, and 13 (Figure 3). The introns were primarily chosen because they are located at the 5' end of the HER-2 gene transcript and are larger than the remaining HER-2 introns.

Cells were transiently transfected using liposome-based delivery system. Liposome-based non-viral delivery system is not as effective as viral systems, but it is a comparable alternative when the overall cell safety is factored (Madeira et al., 2010).

Using Fugene 6 reagent transfection system cells were transfected with 3μl of Fugene and 2μg of total mutant DNA per well of six well plates along with the appropriate controls, incubated for 48-72 hrs, then harvested. After experiment and treatment, different cell assays were done on MDA-MB-453 and T-47D cells to analyze both the HER-2/Neu mRNA and protein expressions.

It is expected that the mutant U11 snRNAs would forcibly bind to the 5' splice site of the HER-2 gene, which would compete and inhibit the binding of U1 (the endogenous snRNA for HER-2 introns). U6ATAC

MUTANT SEQUENCE FOR TARGETED INTRONS

snRNA	**5'-3' INTRONIC MUTANT SEQUENCE**
U6ATAC I1	GTG TTG TAT GAG ACC CGA GAA GGT T
U6ATAC I2	GTG TTG TAT GGG TCT CGA GAA GGT T
U6ATAC I6	GTG TTG TAT GGC ACA TGA GAA GGT T
U6ATAC I8	GTG TTG TAT GAC TCT TGA GAA GGT T
U6ATAC I12	GTG TTG TAT GTG TCT TGA GAA GGT T
U6ATAC I13	GTG TTG TAT GCC TCA TGA GAA GGT T
U11 I1	CGA AGA TCT CAA AGA CCC CTT CTG TCG T
U11 I2	CGA AGA TCT CAA GGT CTC CTT CTG TCG T
U11 I6	CGA AGA TCT CAA GCA CAT CTT CTG TCG T
U11 I8	CGA AGA TCT CAA ACT CTT CTT CTG TCG T
U11 I12	CGA AGA TCT CAA TGT CTT CTT CTG TCG T
U11 I13	CGA AGA TCT CAA CCT CAT CTT CTG TCG T

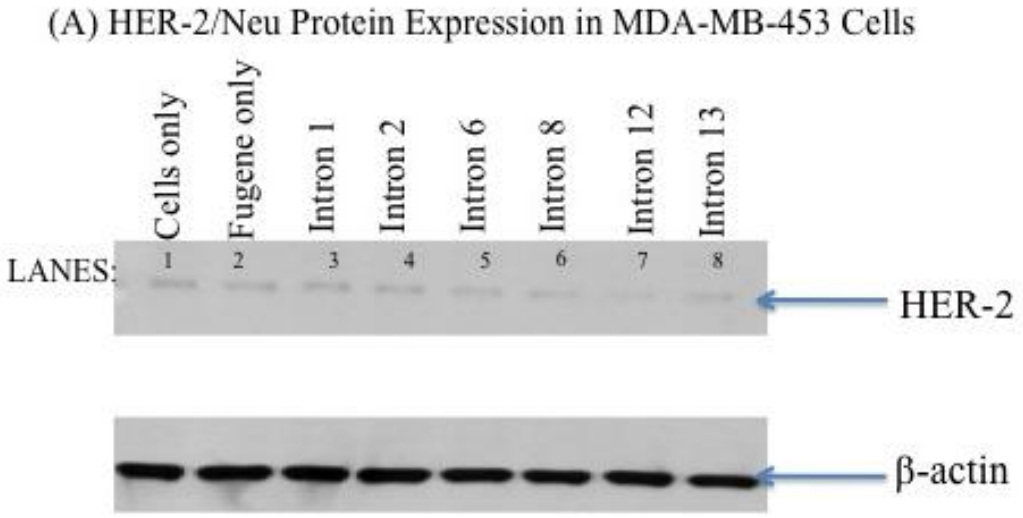
Table 1: Sequence of snRNAs mutations: The sequences for snRNAs U6ATAC and U11 that mutated to bind complementary to the HER-2/Neu gene in the 5' splice-site of the corresponding introns (I). The numbers correspond to the targeted intron.

3.2 Inhibitory Effect of snRNA Mutants on MDA-MB-453 and T-47D HER-2 Protein Expression

To quantify and compare protein expression in both MAD-MB-453 and T-47D cell line western blot assay was performed using total protein lysate as previously outlined in 2.14. 48hrs after transfection, cells were harvested, lysed, and the protein concentration was obtained using the Bradford assay system. To optimize the western assay, concentrations of proteins: 5μg, 10μg, 15μg were ran on the gel and observed. It was determined that 5μg concentration was sufficient to be able to quantify protein expression in both cell lines.

Figure 4 and 5 shows the result of the western blot analysis. Figure 4 shows the data retrieved from HER-2 protein expression in the MDA-MB-453 cell line, and Figure 5 shows the HER-2 protein expression in T-47D cell line. It is apparent that in both cell lines there is a variable affect on the protein expression with the transiently transfected snRNA mutants. In figure 4 cells only were the untreated cells, and fugene only was simply treated with transfection reagent to compare the data obtained for the snRNA mutants to. The U6ATAC and U11 snRNA double transfected mutations for intron 1 show no significant difference in protein expression levels as compared to the controls. However, in the U6ATAC and U11 snRNA mutations for introns: 2, 6, 8, 12, and 13, shows significant repression in protein expression. According to this data we can attribute the repression of protein expression, does

activate SPLICE*i* as a direct consequence of transfection of these mutants in MDA-MB-453 cell line.



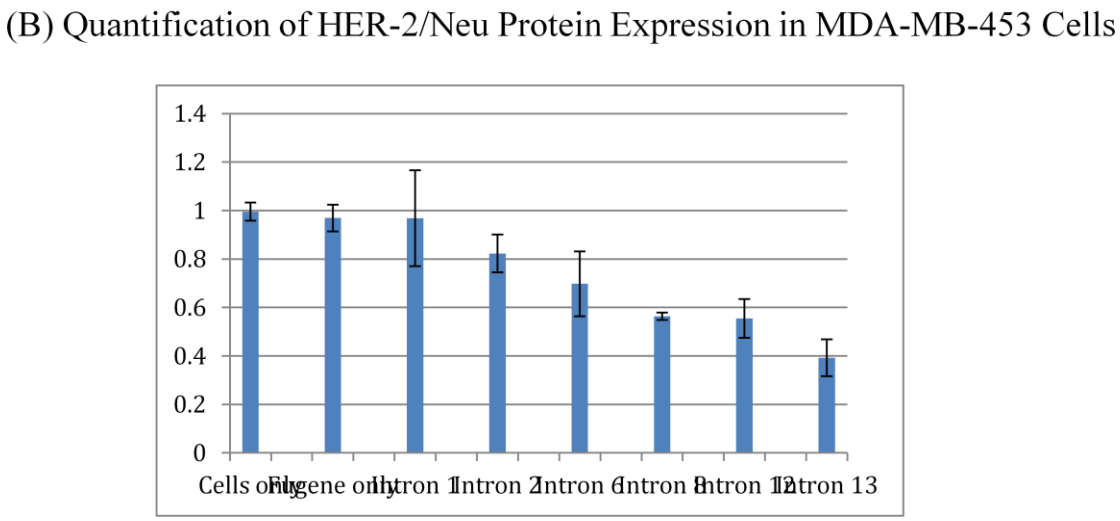
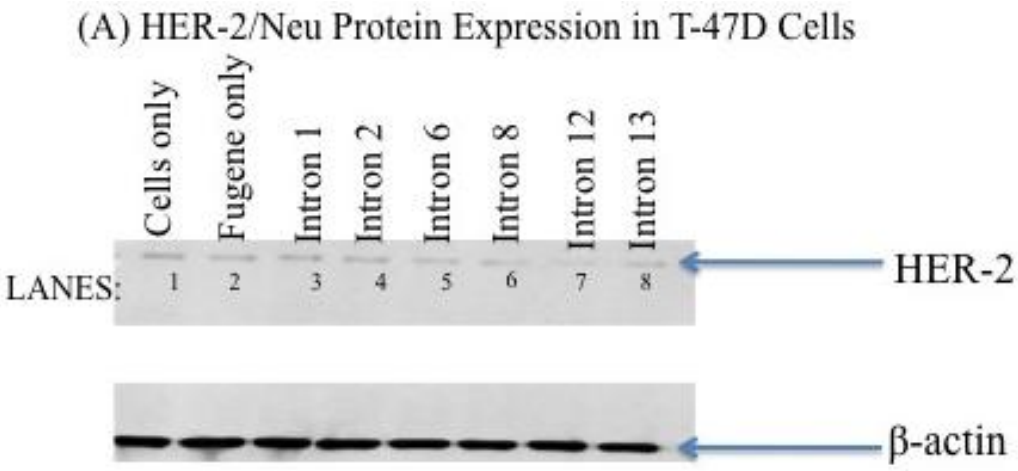


Figure 4: Western Blot showing HER-2/Neu protein expression in MDA-MB-453 mammary carcinoma cells. The upper panel shows HER-2/Neu expression and the lower panel shows β-actin expression as an endogenous control. (A) Actual western blot, lane 1: Cell only (untreated), lane 2: Fugene only (treated with 3µl transfection reagent only), lanes 3-8: cells treated with mutant snRNAs targeting the various introns. (B) The quantification of the western blot shown in (A) showing the HER-2 protein expression.



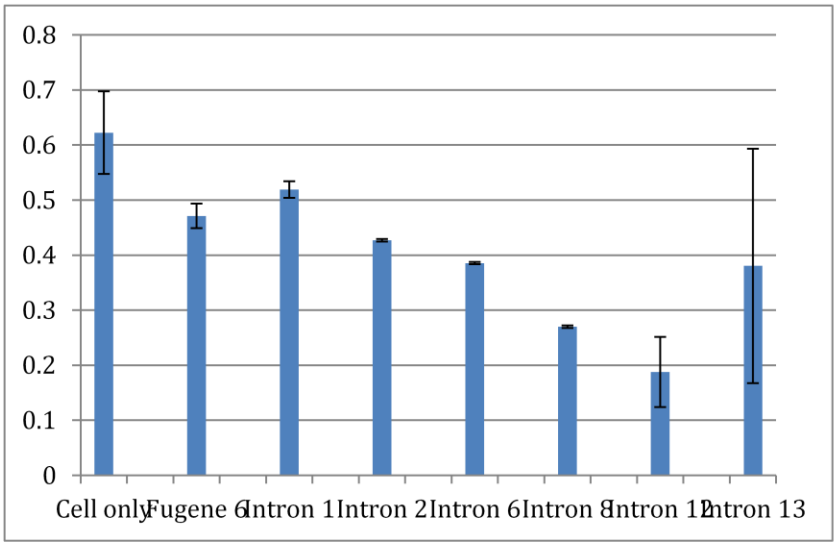


Figure 5: Western Blot showing HER-2/Neu protein expression in T-47D mammary carcinoma cells. The upper panel shows HER-2/Neu expression and the lower panel shows β-actin expression as an endogenous control. (A) Actual western blot, lane 1: Cell only (untreated), lane 2: Fugene only (treated with 3μl transfection reagent only), lanes 3-8: cells treated with mutant snRNAs targeting the various introns. (B) The quantification of the western blot shown in (A) showing the HER-2 protein expression.

Figure 5 shows the data for T-47D cell line under the same conditions as outlined in 2.14. According to the data from figure 5, there is no significant difference in protein expression due to targeted snRNA mutations for intron 1, similar to that shown in figure 4 for the MDA-MB-453 cell line. Again similar to the MDA-MB-453 cell line, figure 5 shows that in the T-47D cell line there is significant difference in the data for the targeted intron 2, 6, 8, and 12. For intron 13 mutations in the T-47D cell line there was no significant difference in HER-2 protein expression observed.

There appear to be an overall trend within both cell lines, as the targeted intron number increases the less is the HER-2 protein expression. Perhaps the further away from the 5' end of the gene, or the closer to the 3' end of the gene the greater the mutant snRNAs U11 and U6ATAC have on down regulating HER-2 protein expression.

Because of the western blot data for both cell line, it can be said with a degree of certainty that the transfection of the mutated snRNA chosen to forcibly bind to the HER-2/Neu gene at the 5' splice-site, does cause varying degrees of splicing interference in the pre-mRNA possessing that is reflected in the repression of protein synthesis for some of the targeted introns of the HER-2 pre-mRNA in mammary carcinoma cells.

3.3 HER-2/Neu mRNA Expression Analyzed Via Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

To observe the level of HER-2/Neu gene expression during "real-time" qRT-PCR was performed as outlined in 2.12. After 48hr incubation of the transfection RNA was harvested from MDA-MB-453 and T-47D as outlined in 2.8. With the extracted RNA cDNA was synthesized for both cell lines, MDA-MB-453 and T-47D, according to 2.10. Using the synthesized cDNA qRT-PCR was performed.

The qRT-PCR analysis was done in triplicates and figure 6 and 8 shows the HER-2 mRNA expression for both MDA-MB-453 and T-47D cell lines. Figure 7 A, B, and C, shows the internal validity of the qRT-PCR procedure with HER-2 standard curve r2=0.986 and GAPDH r2=0.991. GAPDH was the chosen endogenous control because it is a housekeeping gene that is present in all cell types. Using the appropriate controls it is with a certain degree of certainty that the data obtained in the assay is reliable.

The data in figure 6 and 8 shows the level of HER-2/Neu gene expression as obtains from the qRT-PCR. Figure 6 shows that targeting intron 8 and 12 had significant effect on gene expression in MDA-MB-453 cell line. The remaining targeted introns for MDA-MB-453 cell line seem to have no significant effect on gene expression.

QUANTIFICATION OF qRT PCR ANALYSIS
MDA-MB-453

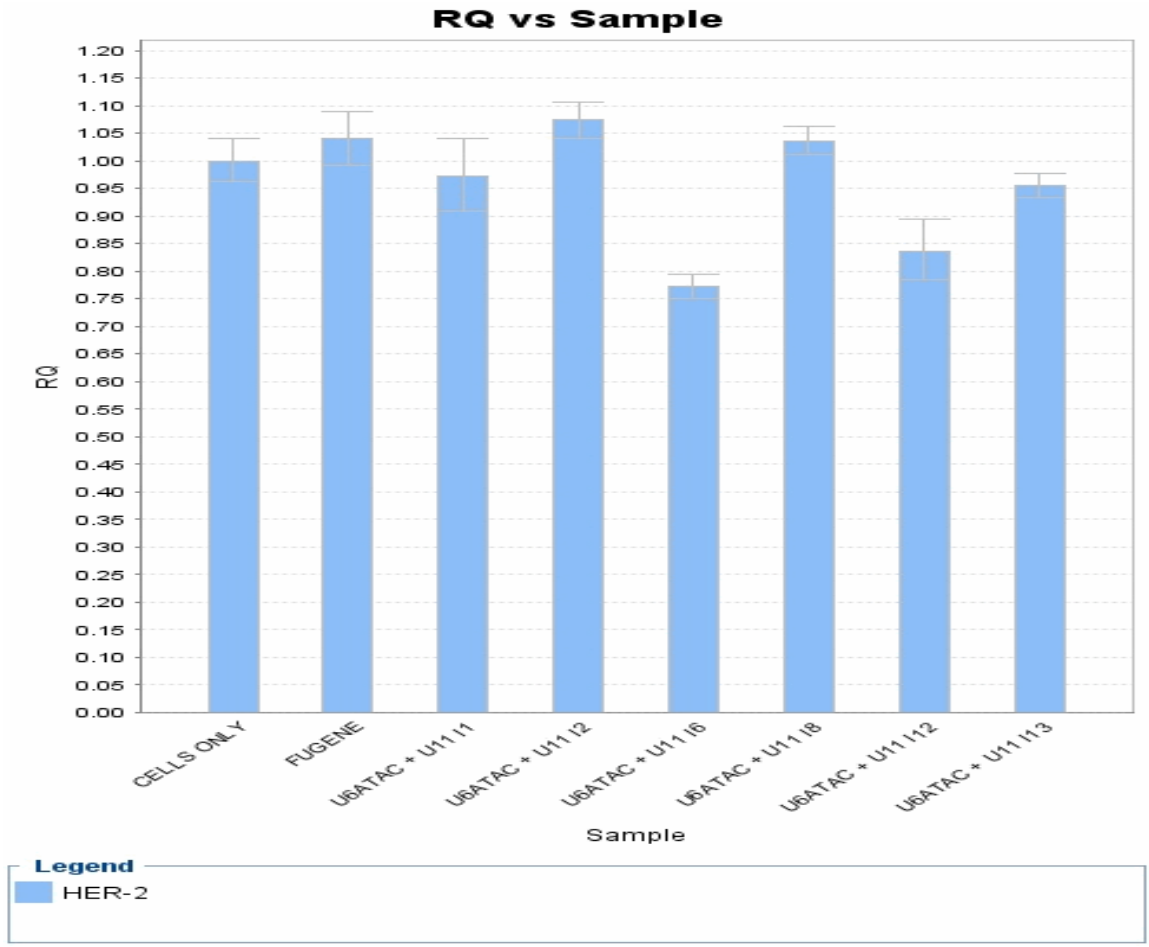


Figure 6: Quantification of qRT PCR analysis for MDA-MB-453 cells: The HER-2 gene expression with the cDNA synthesized from RNA extracted from MDA-MB-453 transfected cells. cDNA was synthesized with gene specific primers. Cells only lane is gene expression data for MDA-MB-453 cells untreated (No transfection reagent, no mutant DNA). Fugene lane data is HER-2/Neu gene expression data from MDA-MB-453 cells treated with 3µl of fugene 6 transfection reagent only. The remaining six lanes show HER-2/Neu gene expression for MDA-MB-453 cells transfected with 3µl of fugene 6 transfection reagent and 2µg total of the corresponding double mutants (U6ATAC and U11) DNA for the depicted intron.

STANDARD CURVES FOR MDA-MB-453 qRT-PCR

(A) HER-2

(B) GAPDH

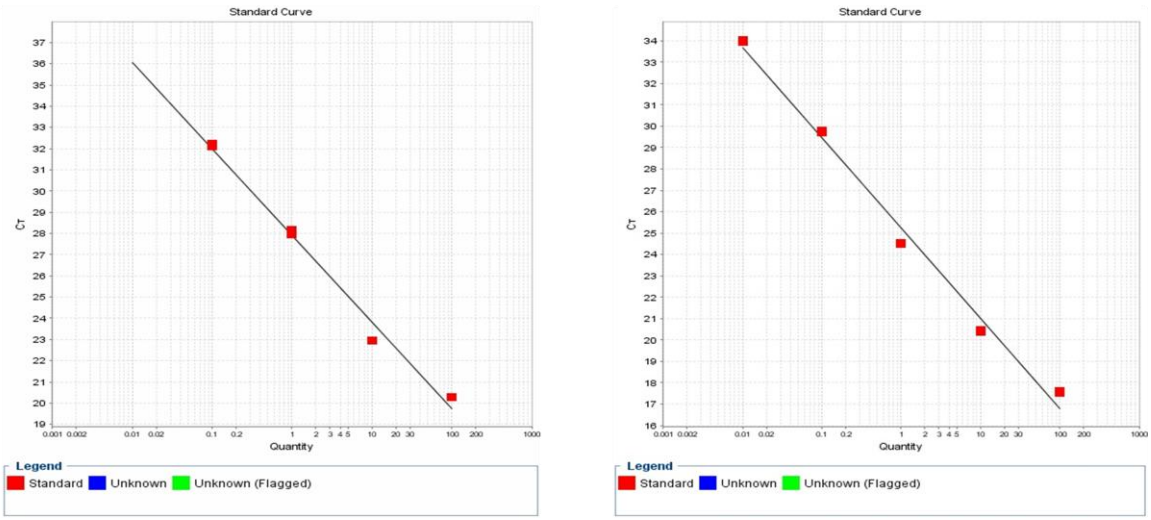
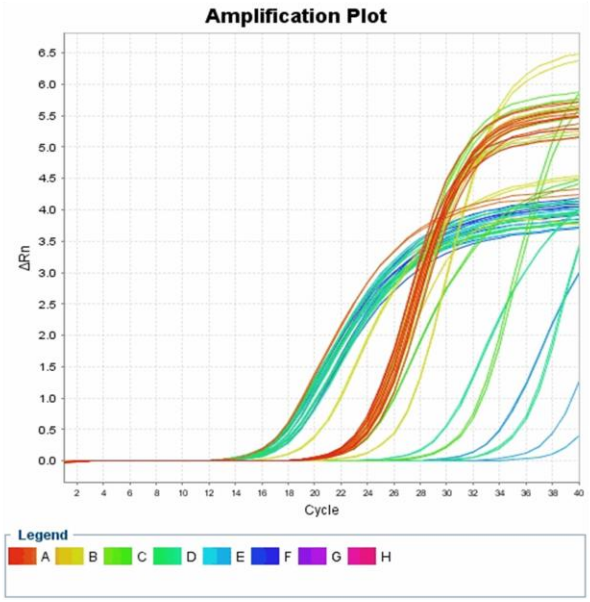


Figure 7: Data to support the validity of qRT-PCR: A) Standard curve for MDA-MB-453 qRT-PCR with HER-2 gene specific primers, r2=0.986.B) Standar curve for MDA-MB-453 qRT-PCR with endogenous controlGlyceraldehyde-3-phosphate dehydrogenase (GAPDH), r2=0.991. C) Amplification plot for the same qRT-PCR

(C)



The data obtained from qRT- PCR for T-47D cell line, as shown in figure 8, shows that in comparison to the appropriate controls there is no significant difference in HER-2/Neu gene expression among and of the targeted introns.

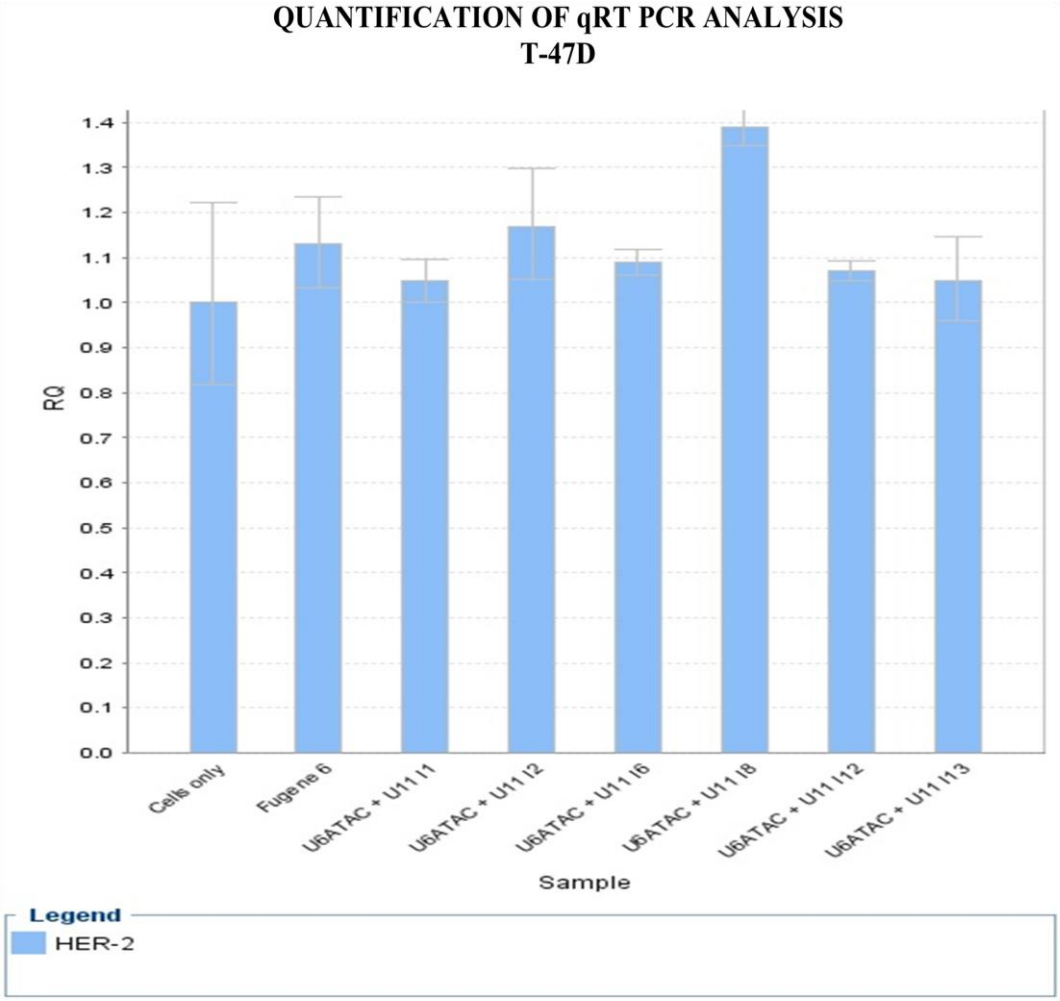


Figure 8:Quantification of qRT PCR analysis of T-47D cells): HER-2 gene expression with cDNA synthesized from RNA extracted from T-47D transfect cells. cDNA was synthesized with gene specific primers. Cells only lane shows HER-2 gen expression for T-47D cells untreated (No transfection reagent, no mutant DNA). Fugene lane shows HER-2/Neu gene expression from cells treated with 3µl of fugene 6 transfection reagent and 2µg total of the corresponding double mutants (1µg U6ATAC and 1µg U11) DNA for the depicted intron.

Figure 9 A, B, and C shows the qRT-PCR standard curves for T-47D cell line as well as the amplification plot. HER-2 standard curve r2 = 0.91 and GAPDH r2 =

0.991.

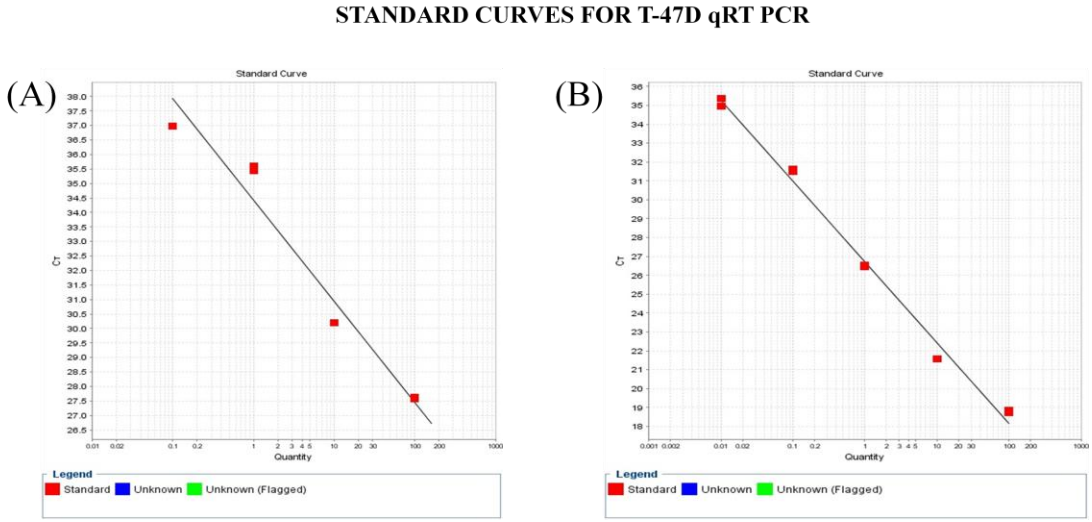
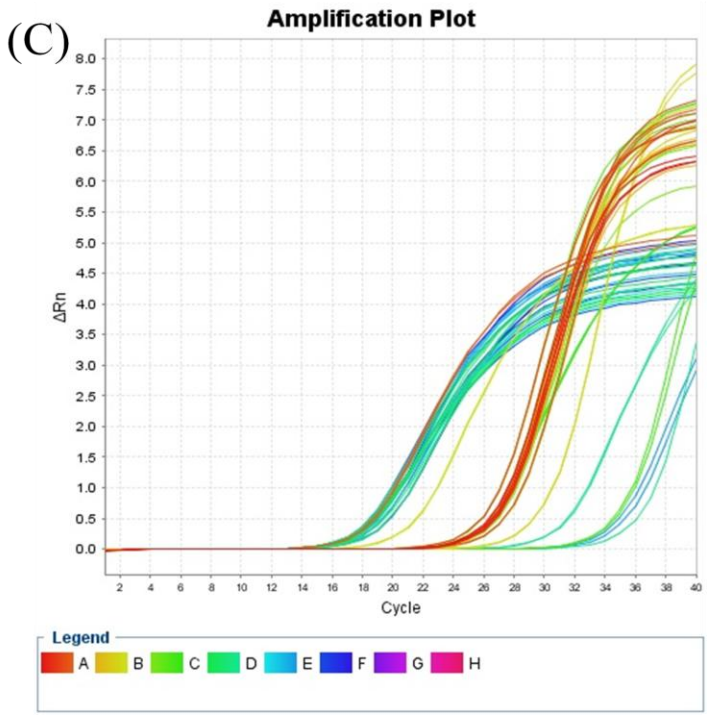


Figure 9: Data to support the validity of qRT-PCR: A) Standard curve for T-47D qRT-PCR with HER-2 gene specific primers, r2=0.91.B) Standar curve for T-47D qRT-PCR withn endogenous controlGlyceraldehyde-3-phosphate dehydrogenase (GAPDH), r2=0.991. C) Amplification plot for the same qRT-PCR



The data in figure 9 shows the reliability of the qRT-PCR for T-47D and it can be said with a degree of certainty that the data obtained is reliable.

It can be said that there is some splicing interference taking place in the MDA-MB-453 cell line but none that is apparent in the T-47D. Whatever effect is taking place as a consequence of the mutant snRNA transfected is not reflected in the overall mRNA expression for both cell lines.

3.4 Mammary Cell Viability After Transfection

Another step in attempting to determine the effect the transfection of the mutated snRNA may have on mammary carcinoma cells is assessing possible phenotypically changes that may occur. One way to do this was to determine the cells' health after transfection by means of the cell viability assay.

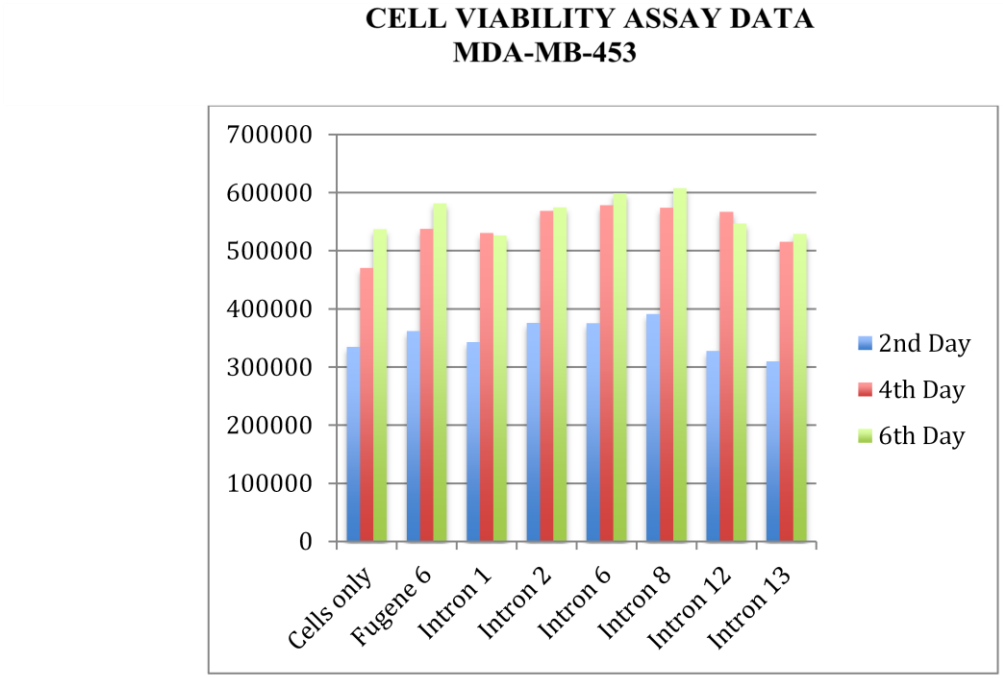


Figure 10: Viability assay for MDA-MB-453 cells: MDA-MB-453 cells were harvested on 2nd, 4th, and 6th day and the viability of the transfected cells and controls were assessed.

The cell viability assay was done according to the procedure outlined in 2.15 for both MDA-MB-453 and T-47D cell lines. Figure 10 is a graphical depiction of data obtained from cell viability assay for MDA-MB-453, and figure 11 data collected for T-47D cell line.

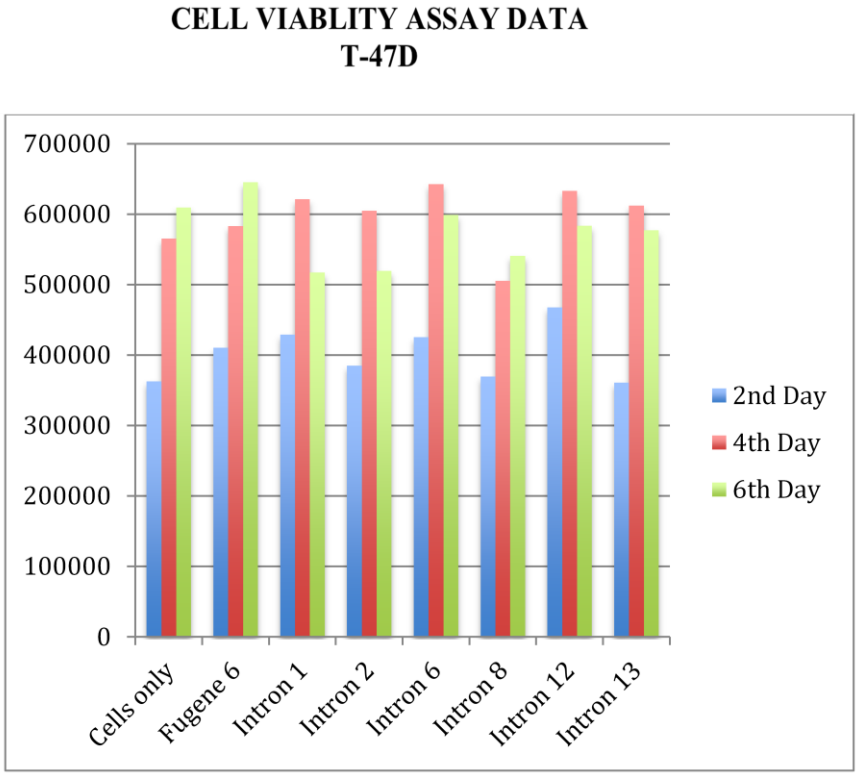


Figure 11: Viability assay for T-47D cells: Transfected with mutant snRNA T-47D cells were harvested on 2nd, 4th, and 6th day and the viability of the transfected cells and controls were assessed.

According to data shown in figure 10 and 11, cell only lanes and fugene 6 lanes in both MDA-MB-453 and T-47D shows steady cell growth on the 2nd, 4th, and 6th days. MDA-MB-453 cells treated with mutant snRNAs (U11 and U6ATAC) targeting intron 1 and 12 show steady growth in cells on 2nd and 4th day, but on 6th

day the cells show slight decrease in the number of cells, indicating cells growth stopped and some cell death may have occurred. T-47D cells with mutant snRNAs targeting introns 1, 2, 6, 12, and 13 show decrease in cell number, indicating the mutants may cause cell death possibly due to intrinsic cellular apoptotic mechanisms or consequence of some extrinsic factors. Further testing, such as longer growing period before harvesting, would need to be done to determine the validity of this emerging trend and apoptosis assay to confirm it is due to intrinsic apoptosis.

3.5 Apoptosis in MDA-MB-453 mammary cells

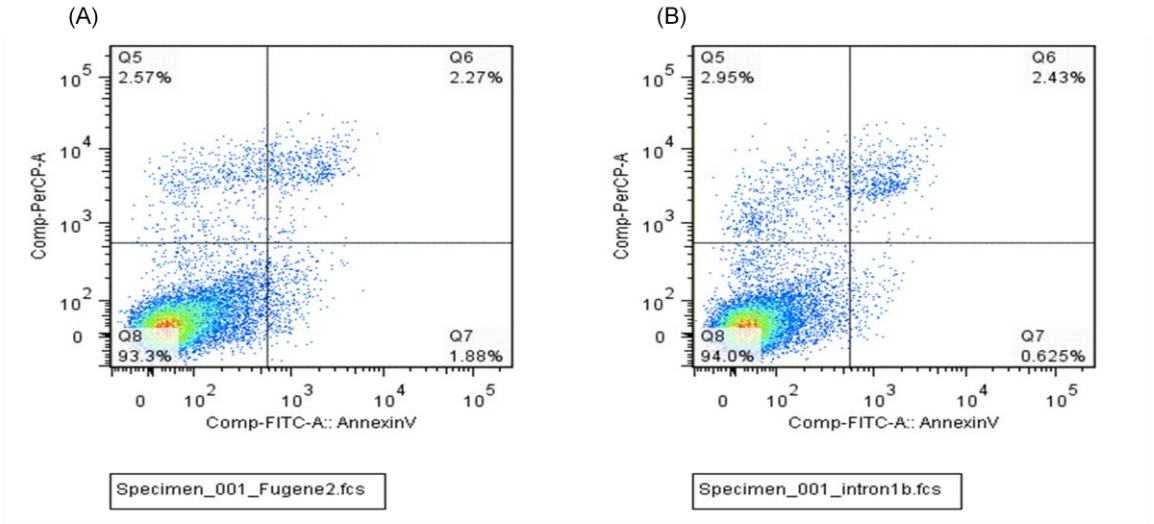
Another assay done with the MDA-MB-453 cell line to determine if the transfections of mutated snRNAs for introns 1, 2, 6, 8, 12, and 13 of the HER-2/Neu gene have an effect on the overall viability of the cell lines. To determine if the increase in cell death observed in cells ttransfected with mutant snRNAs is due to intrinsic apoptosis pathway an apoptosis assay was done and analyzed via flow cytometry using Fluorescence-activated Cell Sorting (FACS) machine. To assess the reason for cell death observed apoptosis assay was performed according to the procedure outlined in 2.16.

Figure 12 shows data obtained from the Fluorescence-activated Cell Sorting (FACS) machine. The FACS machine observed 20,000 cells of MDA-MB-453 cell line per treatment. According to these data, in comparison to fugene 6 treated cells (negative control) (2.27% apoptosis, ~454 cells) there appear to be more cells undergoing apoptosis in cells transfected with mutant snRNAs. Based on the result of the apoptosis assay obtained from FACS analysis, show the increase in cell death

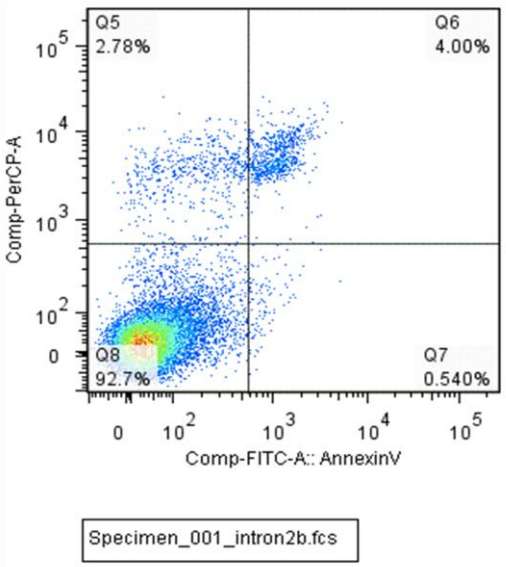
observed in the transfected cells was most likely due to intrinsic apoptotic pathway. (Figure 12)

Figure 12 show there is a slight increase in apoptosis with the cells containing mutations for targeted intron 1 (2.43% = ~486 cells) as compared to the fugene 6 treated cells. Targeting introns 2, 6, 8, 12 and 13 with mutant snRNAs appear to show more significant increase in apoptosis. Transfected cells targeting intron 2 had apoptosis at 4% (~800 cells), intron 6 had apoptosis of 4.10% (~820 cells), intron 8 had apoptosis of 3.99% (~800 cells), intron 12 showed apoptosis of 3.43% (~686 cells), and intron 13 showed apoptosis of 5.24% (~1048 cells).

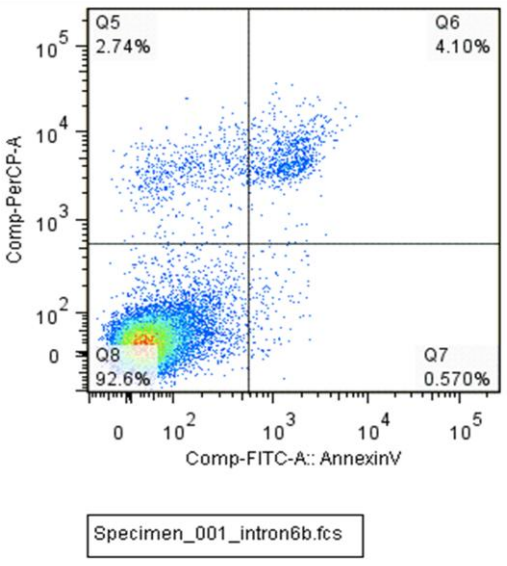
QUANTIFICATION OF APOPTOSIS IN MDA-MB-453 MAMMARY CARCINOMA CELLS



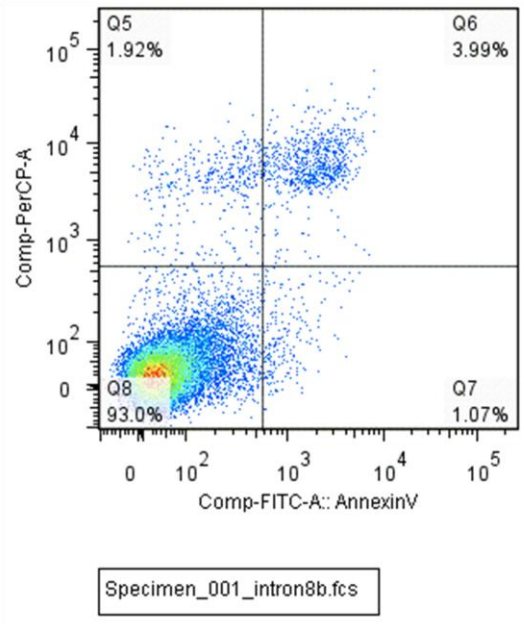
(C)


Q5
2.78%
Q6
4.00%
Q8
92.7%
Q7
0.540%
Comp-PerCP-A
Comp-FITC-A:: AnnexinV
Specimen_001_intron2b.fcs

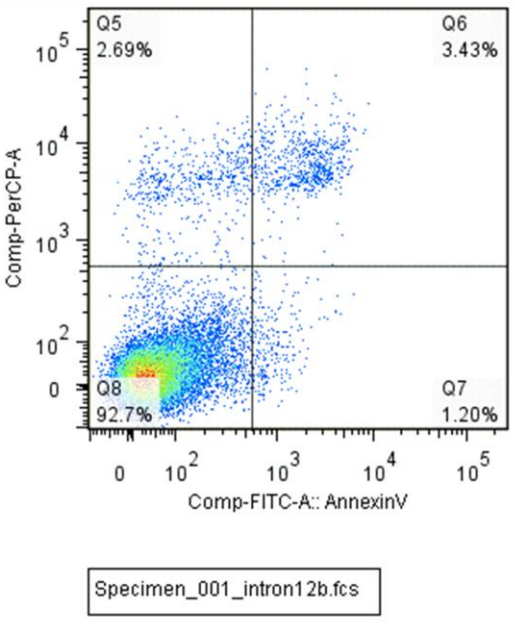
(D)


Q5
2.74%
Q6
4.10%
Q8
92.6%
Q7
0.570%
Comp-PerCP-A
Comp-FITC-A:: AnnexinV
Specimen_001_intron6b.fcs

(E)


Q5
1.92%
Q6
3.99%
Q8
93.0%
Q7
1.07%
Comp-PerCP-A
Comp-FITC-A:: AnnexinV
Specimen_001_intron8b.fcs

(F)


Q5
2.69%
Q6
3.43%
Q8
92.7%
Q7
1.20%
Comp-PerCP-A
Comp-FITC-A:: AnnexinV
Specimen_001_intron12b.fcs

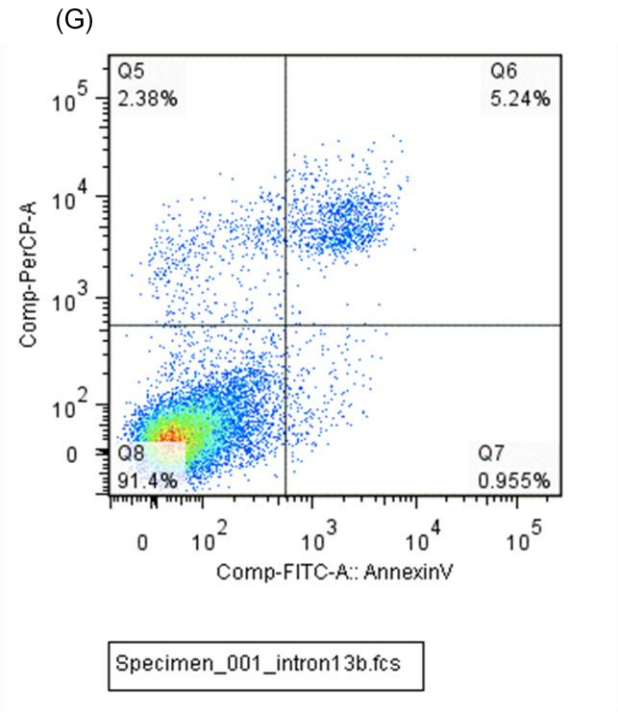


Figure 12: Fluorescence-activated cell sorting analysis of apoptosis assay done on MDA-MB-453 for 20,000 events: Q6 (Quadrant 6) shows the cells undergoing late stages of apoptosis (A) Apoptosis in fugene 6 only treated cells (negative control) = 2.27% (~454 cells) (B) Apoptosis in cells treated with mutant snRNAs targeting intron 1= 2.43% (~486 cells). (C) Apoptosis in cells treated with mutant snRNAs targeting intron 2 = 4.0% (~800 cells). (D) Apoptosis in cells treated with mutant snRNAs targeting intron 6 = 4.10% (~820 cells). (E) Apoptosis in cells treated with mutant snRNAs targeting intron 8 = 3.99% (~800 cells). (F) Apoptosis in cells treated with mutant snRNAs targeting intron 12 = 3.43% (~686 cells). (G) Apoptosis in cells treated with mutant snRNAs targeting intron 13 = 5.24% (~1048 cells).

According to the apoptosis data obtained or MDA-MB-453 cell line it appear that the transfected snRNA mutations does have some effect of the number of cells undergoing apoptosis (Figure 12). The effect is consistently more that the negative control. Across all samples snRNAs, the negative control fugene 6 only treated cells have the least number of cells undergoing apoptosis (~454 cells) and greatest number of cells undergoing apoptosis was seen when intron 13 was targeted (~1048 cells)

(Figure 12). This apoptosis data is consistent with the trend observed with the HER-2 protein expression, where there was a decrease in the protein expression. The apoptosis assay data was also consistent with the data obtained from the cell viability assay, which showed a decrease in the number of viable cells in the transfected population as compared to the cells not transfected with the mutant snRNAs.

CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

The novel experiment of mutating U12-dependent (minor class) snRNA U11 and U6ATAC to be over-expressed in the U2-depedent (major class) introns as a method of activating splicing interference as an attempt to down regulate HER-2/Neu mammary carcinoma gene show very promising results.

After analyzing the HER-2 protein expression a down regulation trend was evident as the introns number targeted increased, got further away from the 5' end of the HER-2 pre-mRNA, and closer to the 3' end, the less HER-2 protein was expressed. Intron 1 is the largest of the targeted introns but repression of the HER-2 protein was the least when targeting intron 1 in both MDA-MB-453 and T-47D cells. Therefore, based on the HER-2 protein expression data, size does not appear to be a determining factor in the level of protein down regulation.

Perhaps there is some intrinsic mechanism that corrected the splicing interference that may be housed in the larger intron, and the size of the intron may also play a role by allowing surface area to facilitate any possible corrections the cells may have in place.

The qRT-PCR data for both MDA-MB-453 and T-47D showed little down regulation in HER-2/Neu mRNA expression. Only in MDA-MB-453 cell line with mutant snRNA targeting introns 6 and 12 showed some down regulation in mRNA expression, but little to no down regulation in the remaining targeted introns. Whatever effect the mutant snRNA may have on the mRNA level it appears to be very minimal.

For the cells viability assay cell were transfected and harvest after 2, 4, and 6 days in order to assess the growth of viable cells after being transfected with the mutant snRNAs. The cell viability data show some activation of *SPLICEi* that lead to less number of viable cells particularly affect the 6^{th} day of incubation with the mutants. The decrease in the number of viable cells was clearly observed in T-47D mammary carcinoma cell line. Further testing needs to be done in order to better observe the cells' viability perhaps with longer incubation periods an amplification of the effect the mutant snRNAs are having on cells viability can be clearly observed.

To further observe the effect the mutant snRNAs are having on the observed decrease in the number of viable cells an apoptosis assay was done on mutant MDA-MB-453 cells. MDA-MB-453 mammary carcinoma cells were incubated for 72 hrs, dyed and analyzed by the fluorescence activated cell sorting (FACS) machine. The data showed an increase in the number of cells undergoing apoptosis was greater in

the mutant snRNAs transfected cells than the negative control (fugene 6 only) cells. It is possible that longer incubation period would enhance, and clearly show whether there is a definite effect on apoptosis on these mammary carcinoma cells. To further observe the effect the mutant snRNAs are having on the mammary carcinoma cells the apoptosis assay would also be done on the T-47D mammary carcinoma cells.

More work need to be done in order to make a definitive conclusion about the magnitude of the *SPLICEi* activity. Apart from longer incubation periods before harvesting for HER-2 protein and RNA with the already targeted introns, perhaps attempting combinatory transfection targeting multiple introns at once would give an amplified effect in order to have a more definitive conclusion.

As a direct result of the emerging trend observed in the HER-2 protein expression, new introns should be targeted at the 3' end of the HER-2 pre-mRNA, individually and in combination with other introns. The newly targeted introns may show more down regulation in protein expression and may have a greater effect that could also be better observed at the mRNA expression level.

Only 2μg of mutant DNA was used in the transfections, an increase in the concentration of mutant U11 and U6ATAC transfected may also show an amplification in the down regulation of the HER-2 protein and mRNA expression. Exploring methods of transfection can increase transfection efficiency and possibly amplify the initial results of down regulation HER-2 expression observed.

BIBLIOGRAPHY

1. Allain Vanessa, Bourgaux Claudie, and Couvreur Patrick. Self-assembled Nucleolipids: from Supramolecular Structure to Soft Nucleic Acid and Drug Delivery Devices. 2011

2. Burge Christopher B. Evolutionary fates and origins of U12-type introns. 1998

3. Carney W. P., Leitzel K., Ali S., Neumann R., Lipton A. HER-2/Neu diagnostics in breast cancer. Breast Cancer Res. 2007;9(3):207

4. Chan Samuel D. H., Antoniucci Diana M., Fok Katherine S., Alajoki Liisa, Harkins Richard N., Thompson Stuart A., and Wada Garrett H. Heregulin Activation of Extracellular Acidification in Mammary Carcinoma Cells Is Associated with Expression of HER2 and HER3. 1995

5. Costantini Danny L., Chan Conrad, Cai Zhongli, Vallis Katherine A., and Reilly Raymond M. In-Labeled Trastuzumab (Herceptin) Modified with Nuclear Localization Sequences (NLS): An Auger Electron-Emitting Radiotherapeutic Agent for HER2/neu-Amplified Breast Cancer. 2007

6. Fischer Utz, Englbrecht Clemens, and Chari Ashwin. Biogenesis of spliceosomal small nuclear ribonucleoproteins. 2011 John Wiley & Sons, Ltd. WIREs RNA 2011 2 718–731 DOI: 10.1002/wrna.87

7. Goyenvalle Aurelie, Davies Kay E. Challenges to oligonucleotides-based therapeutics for Duchenne muscular dystrophy. 2011

8. Hartwell, L.H., Hood, L., Goldberg, M.L., Reynolds, A.E., Silver, L.M., Veres, R.C. Genetics: From genes to genomes, 5th Edition. 2004

9. Hollywood D. P., Hurst H. C. A novel transcription factor, OB2-1, is required for overexpression of proto-oncogene c-erbB-2 in mammary tumour lines. EMBO J. 1993 Jun;12(6):2369-75

10. Huang Haojie, Cheville John C. Pan Yungian, Roche Patrick C., Schmidt Lucy J., and Tindall Donald J. PTEN Induces Chemosensitivity in PTEN-mutated Prostate Cancer Cells by Suppression of Bcl-2 Expression. 2001

11. Kapila Kusum, Al-Awadhi S., Francis I. M. Her-2 neu (Cerb-B2) Expression in Fine Needle Aspiration Samples of Breast Carcinoma: A Pilot Study Comparing FISH, CISH, and Immunocytochemistry. 2011

12. Lang Hui, Zhang Wei, and Carlin George A. Principles of microTNA Involvement in Human Cancers. 2011

13. Levin Aaron, and Durbin Richard. A computational scan for U12 dependent introns in the human genome sequence.
14. Lu Zong-Xia, Liu Li-Ting, Qi Xian-rong. Development of small interfering rNA delivery system using PeI-Peg-APrPg polymer for antiangiogenic vascular endothelial growth factor tumor-targeted therapy. 2011
15. Lyon Elaine, Millson Alison, Lowery Mary C., Woods Rachel, and Wittwer T. Quantification of HER2/*neu* Gene Amplification by Competitive PCR Using Fluorescent Melting Curve Analysis. Clinical Chemistry 47:5 844-851. 2001
16. Newman A. J., Teigelkamp S., and Beggs J. D. snRNA interactions at 5' and 3' splice sites monitored by photoactivated crosslinking in yeast spliceosomes. *RNA*; 1: 968-980. 1995
17. Rupaimoole Rajesha, Han Hee-Dong, Lopez-Berenstein Gabriel, and Sood Anil K. MicroRNA therapeutics: principles, expectations, and challenges. 2011
18. Shigematsu Hideo, Kadoya Takayuki, Kobayashi Yoshie, Kajitani Keiko, Sasada Tatsunari, Emi Akiko, Masumoto Norio, Haruta Rumi, Kataoka Tsuyoshi, Oda Miyo, Arihiro Kouji, and Okada Morihito. A case of HER-2-positive recurrent breast cancer showing a clinically complete response to

trastuzumab-containing chemotherapy after primary treatment of triple-negative breast cancer. World Journal of Surgical Oncology; 9:146. 2011

19. Shiu Robert P. C., and Paterson Jean A. Alteration of Cell Shape, Adhesion, and Lipid Accumulation in Human Breast Cancer Cells (T-47D) by Human Prolactin and Growth Hormone. *Cancer Res*;44:1178-1186. 1984

20. Shukla Girish C., and Padgett Richard A. U4 small nuclear RNA can function in both the major and minor spliceosomes. 2004

21. Shukla GC, Padgett RA. The intermolecular stem-loop structure of U6 snRNA can functionally replace the U6ATAC snRNA stem-loop. RNA; 7(1):94-105. 2001

22. Skalova H., Dundr P., Povysil C. Velenska Z., Petruzelka L., and Tvrdih D. Study of the Effect of Neoadjuvant Chemotherapy on the Status of Her2/neu (breast cancer / neoadjuvant chemotherapy / Her2/neu / IHC / FISH / real-time PCR). Institute of Pathology, Department of Oncology, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic. 2011

23. Sleeman J. P., Cremers N. New concepts in breast cancer metastasis: tumor initiating cells and the microenvironment. Clin Exp Metastasis;24(8):707-15. 2007

24. Warf Bryan M., and Berglund Andrew J. The role of RNA structure in regulating pre-mRNA splicing. Institute of Molecular Biology and Department of Chemistry, University of Oregon. Trends Biochem Sci. March ; 35(3): 169–178. doi:10.1016/j.tibs.2009.10.004. 2010

25. Will Cindy L., and Luhrmann Reinhard. Splicing of rare class of introns by U12-dependent spliceosome. 2005

26. Zhou Alice Qinhua. Exploration of the Central Dogma at the Interface of Chemistry and Biology Yale Chemical Biology Symposium
Department of Molecular Biophysics and Biochemistry, Yale University. 2010

APPENDICES

APPENDIX

Appendix 1: Sequence of Primers: End PCR

1. U6ATAC WT - 5’ – GTG TTG TAT GAA AGG AGA GA – 3’
2. U6ATAC I1 - 5’ – GTG TTG TAT GAG ACC C – 3’
3. U6ATAC I2 - 5’ – GTG TTG TAT GGG TCT C – 3’
4. U6ATAC I6 - 5’ – GTG TTG TAT GGC ACA T – 3’
5. U6ATAC I8 - 5’ – GTG TTG TAT GAC TCT T – 3’
6. U6ATAC I12 - 5’ – GTG TTG TAT GTG TCT T – 3’
7. U6ATAC I13 - 5’ – GTG TTG TAT GCC TCA T – 3’
8. U11 WT - 5’ – AAA AAG GGC TTC TGT CGT G – 3’
9. U11 I1 - 5’ – AAA GAC CCC TTC TGT CGT G – 3’
10. U11 I2 - 5’ – AAG GTC TCC TTC TGT CGT G – 3’
11. U11 I6 - 5’ – AAG CAC ATC TTC TGT CGT G – 3’
12. U11 I8 - 5’ – AAA CTC TTC TTC TGT CGT G – 3’
13. U11 I12 - 5’ – AAT GTC TTC TTC TGT CGT G – 3’
14. U11 I13 - 5’ – AAC CTC ATC TTC TGT CGT G – 3’
15. U6ATAC REV - 5’ – AGT AGG TGG CAA TGC CT – 3’
16. U11 REV - 5’ – CAC CAG CTG CCC AAA TAC – 3’
17. U6ATAC RT - 5’ – ACG ATG GTT AGA TGC CAC G – 3’
18. U11 RT - 5’ – AGG GCG CCG GGA CCA ACG– 3’

Appendix 2: Sequence of qRT PCR Primers: All primers are in 5’ to 3’ direction

1. U6ATAC Intron 1- GTG TTG TAT GAG ACC CGA GAA GGT T
2. U6ATAC Intron 2- GTG TTG TAT GGG TCT CGA GAA GGT T
3. U6ATAC Intron 6 - GTG TTG TAT GGC ACA TGA GAA GGT T
4. U6ATAC Intron 8 - GTG TTG TAT GAC TCT TGA GAA GGT T
5. U6ATAC Intron 12 - GTG TTG TAT GTG TCT TGA GAA GGT T
6. U6ATAC Intron 13 - GTG TTG TAT GCC TCA TGA GAA GGT T
7. U11 Intron 1 - CGA AGA TCT CAA AGA CCC CTT CTG TCG T
8. U11 Intron 2 - CGA AGA TCT CAA GGT CTC CTT CTG TCG T
9. U11 Intron 6 - CGA AGA TCT CAA GCA CAT CTT CTG TCG T
10. U11Intron 8 - CGA AGA TCT CAA ACT CTT CTT CTG TCG T
11. U11 Intron 12 - CGA AGA TCT CAA TGT CTT CTT CTG TCG T
12. U11 Intron 13 - CGA AGA TCT CAA CCT CAT CTT CTG TCG T

Appendix 3: Mutations of Specific snRNA

INTRON	WT 5' SS HER-2	U11 WT 5'-3'	U11 MUT 5'-3'	U6atac WT 5'-3'	U6atac MUT 5'-3'	Binding
1	GGGTCT	AAAGGG	AGACCC	AAAGGA	AGACCC	U11 3' C-C-C-A-G-A HER2 5' G-G-G-T-C-T U6atac 3' C-C-C-A-G-A
2	GAGACC	AAAGGG	GGTCTC	AAAGGA	GGTCTC	U11 3' C-T-C-T-G-G HER2 5' G-A-G-A-C-C U6atac 3' C-T-C- T- G-G
6	ATGTGC	AAAGGG	GCACAT	AAAGGA	GCACAT	U11 3' T-A-C-A-C-G HER2 5' A-T-G-T-G-C U6atac 3' T-A-C-A-C-G
8	AAGAGT	AAAGGG	ACTCTT	AAAGGA	ACTCTT	U11 3' T-T-C-T- C- A HER2 5' A-A-G-A-G-T U6atac 3' T-T- C-T- C-A
12	AAGACA	AAAGGG	TGTCTT	AAAGGA	TGTCTT	U11 3' T-T-C-T- G-T HER2 5' A-A-G-A-C-A U6atac 3' T- T- C-T-G-T
13	ATGAGG	AAAGGG	CCTCAT	AAAGGA	CCTCAT	U11 3' T-A-C-T-C-C HER2 5' A-T-G-A-G-G U6atac 3' T-A-C-T- C-C