THE ROLE OF MICRORNA-449 IN HUMAN BREAST CANCER

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ABSTRACT

According to the Canadian Cancer Society, an estimated 25 220 cases of breast cancer were diagnosed in Canada and 5 060 of these cases were expected to be fatal in 2015. Current treatment options are often very invasive, harmful, and ineffective. The purpose of this experiment was to mitigate tumour growth by manipulation of microRNA (mRNA) levels. The mRNAs are small molecules that code for proteins, and levels of specific mRNAs are deregulated in cancer cells. In this experiment, levels of micro-RNA 449, which is deregulated in breast cancer cell lines, were returned to their baseline levels. Numerous tests were then conducted to test the viability of the resulting cells. Replicable experiments showed that the strength, motility and invasiveness of the breast cancer cells was greatly diminished after mRNA-449 levels returned to baseline levels. Furthermore, research indicated that 4 potential genes (CRIP2, XBP1, TAF4B, and SFXN2) can be manipulated in future experiments to further diminish the viability of the breast cancer tumours.

INTRODUCTION

Breast cancer is currently one of the most prevalent forms of cancer, comprising up to 26% of newly diagnosed cancer cases (Canadian Cancer Society, 2016). Treatment options include chemotherapy, radiation therapy and invasive surgery (Carlson et al., 2009). None of these treatment options are successful 100% of the time, and many alternative therapies are currently being researched by scientists (Carlson et al., 2009).

MicroRNAs (miRNAs), are small RNAs that are around 20–22 nucleotides long, and have been found to contribute to a number of cellular processes including stem cell self-renewal and differentiation of embryonic stem cells (Liu & Tang, 2011). In a previous miRNA profiling study using a set of lymph node negative (LNN) breast cancer samples, several miRNAs, including miR-449a, were found to be deregulated (Foekens et al., 2008).

The miR-449 family (a, b and c) has been shown to be a potent inducer of cell death, cell cycle arrest, and/or cell differentiation by several studies done in the past (Lizé et al., 2011). In the current study the role of miR-449a in breast cancer cells will be tested. We hypothesized that the upregulation or downregulation of miR-449a back to baseline levels would decrease cancer cell viability.

MATERIALS AND METHODS

The methods used in this experiment were designed to measure levels of microRNA-449 in each cell line, then return it to baseline levels. After doing so, multiple Invasion/Migration assays were conducted to measure cell viability and motility. Finally, a PCR was conducted to identify possible gene targets for future research.
Quantitative Reverse Transcriptase Real-time Polymerase Chain Reaction (qRT-PCR) to identify levels of microRNA-449 in breast cancer cell lines

MicroRNA expression was assessed by qRT-PCR analysis using TaqMan® microRNA Assays (Applied Biosystems, CA, USA). This assay includes a reverse transcription (RT) step using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) wherein a stem-loop RT primer specifically hybridizes to a miRNA molecule, reverse transcribed with a MultiScribe reverse transcriptase, and then analyzed using the Applied Biosystems 7900 HT Real-Time PCR system.

MiR-449 Knock-Down and Up-Regulation

T47D cells were transfected using a 50nM concentration of anti-miR-449 (Applied Biosystems) and Lipofectamine™ RNAiMax transfection reagent (Life Technologies) as per manufacturers’ protocols. MDA-MB-468 cells were similarly transfected with pre-miR-449 (Applied Biosystems). The anti-miR was designed to lower miR-449 to baseline levels in cell lines that had upregulated levels. Similarly, the pre-miR intended to upregulate miR-449 in cell lines that had the molecule underexpressed.

MTS Assay

The viability of T47D and MDA-MB-468 were examined using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (MTS), according to the manufacturer’s protocol (Promega).

Cell Migration and Invasion Assay

The movement of cells from one area to another and the ability of malignant tumor cells to invade normal surrounding tissue were measured using the CytoSelect™ 24-Well Cell Migration and Invasion Assay (8 µm, Fluorometric Format) following the manufacturer’s protocol (Cell Biolabs).

Quantitative Reverse Transcriptase Real-time PCR (qRT-PCR) to identify genes correlated with microRNA-449

Another qRT-PCR and reverse transcription (RT) analysis was performed using TaqMan® microRNA Assay (Applied Biosystems, CA, USA) and TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) respectively. These were to assess various gene expression levels in breast cancer cells.

RESULTS

The experimental results showed that after miR-449 levels were returned to baseline, the viability and motility of breast cancer cells are decreased.

qRT-PCR showed deregulated miR-449 levels in cancer cells

A qRT-PCR showed that miR-449 was up-regulated in patients with tumours (p = 0.0042), specifically recurrent tumours (p = 0.003). This could indicate that the deregulation of miR-449 contributes to the aggressiveness and invasiveness of the breast cancer tumour cells.

MiR-449 is non-predictably regulated in cancer cells

In comparison to a healthy breast cell line (MCF-10A), a qRT-PCR showed that miR-449 can be severely underexpressed or overexpressed, indicating that it could potentially be a trigger for oncogenesis (Figure 1).

Restoring miR-449 to baseline levels decreases invasiveness

After the cell lines were transfected with the anti-miR, Migration/Invasion assays were conducted on two of the treated cancer lines (T47D, MDA-MB-231B), and compared to an untreated group of cells from the same line. The cells treated with anti-miR-449 were shown to have reduced migration and invasion abilities, indicating that this treatment could reduce the aggressiveness of these tumours (Figure 2, Figure 3). Specifically, the MDA-MB-231B cell line’s invasive properties were severely inhibited by anti-miR treatment (Figure 3).

Several genes were identified as targets of miR-449a

An in silico analysis was carried out with the predicted targets of the microRNA, correlated with data from in vitro studies in the Gene Expression Omnibus database. This analysis identified four genes (SFXN2, TAF4B, XBP1, CRIP2) that were predicted targets, correlated in breast cancer, and downregulated by miR-449a (Figure 4).

PCR analysis of identified genes in breast cancer cell lines

Each of the four genes were found to have a role in cell development and a qRT-PCR was conducted to compare baseline levels (in MCF-10A) to levels in the other cancer cell lines. For the most part, it was found that the genes were severely upregulated in the breast cancer lines (Figure 5).
DISCUSSION
Recent studies show that miR-449a, b and c can induce cell death, cell cycle arrest and cell differentiation (Lizé et al., 2011). It plays an important role in cell function, but also to avoid cancer (Lizé et al., 2011). The experiments in this project were specifically designed to test whether returning miR-449 levels back to normal (either upregulation or downregulation, depending on how it was deregulated in each cell line) would affect the oncogenic cells. It was hypothesized that returning to baseline levels would cause a decrease in cell viability.

It appears that treatment of the cells lines with anti-miR or pre-miR does in fact appear to decrease the viability of the cell lines. Anti-miR interferes with microRNA by physically blocking or cleaving the molecules, while Pre-miR increases miR function by providing precursor molecules that are taken up into cells and modified (Stenvang et al., 2012). It was hypothesized that by allowing microRNA-449 to function as usual, the molecule may be able to trigger cell death or cell cycle arrest through its signaling pathways.

The results showed that regulating miR-449 primarily affects the motility and invasiveness of the cancer cells, especially when compared to an untreated group of the same cell line. Computed cell counting showed that the anti-miR treatment was significant in inhibiting invasiveness in two of the cancer cell groups.

By searching through pre-existing databases (primarily the Gene Expression Omnibus Database), it was found that 4 genes are anti-correlated with the miR-449a levels in oncogenic cells. The target genes, SFXN2, TAF4B, XBP1 and CRIP2 could all play some role in breast cancer, and several have potentially oncogenic properties.

Pre-existing literature indicated that XBP1 is one of two genes that are often co-expressed in human breast carcinomas (Dery et al). TAF4B is reported to be overexpressed in stem cells, but levels decrease after cells differentiate - thus denoting a correlation between the gene and cell differentiation (Baha et al). CRIP2 is found to have apoptosis promoting effects in esophageal cancer cells (Lo et al). The genes were all upregulated in the cancer cell lines, and may have oncogenic properties that identify them as future targets for genetic treatment. Further tests should be done to identify other potential targets of miR-449.

The primary limitation of this research would be the difficulty in translating these results to practical treatments. Although this treatment has shown positive in vitro results, implementing it in human or animal species would require extensive research. However, the field of microRNA is still relatively nascent, and showing a tangible link between these molecules and cancer cells is significant.

CONCLUSION
The regulation of miR-449 has shown promising results with a number of cell lines. In the future, further understanding of the effects of this molecule and correlated genes may be a step toward finding an efficient breast cancer treatment. First of all, treatment with anti-miR-449 had a very significant impact on cell mobility (through the Invasion assay), moreso than cell senescence or cell death. Continued microRNA research could reveal how to make tumours fully immotile. The four gene targets identified are a major area for future research; the next logical step would be to learn more about their pathways, and how they may directly influence breast tumour viability.

ABBREVIATIONS
BC – Breast Cancer
miR – microRNA
PCR – Polymerase Chain Reaction

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REFERENCES


APPENDIX

Figure 1. MicroRNA expression levels in breast cancer cell lines. Expression levels of four different miRNA (miR-449, miR-424, miR-486, miR-181d) in four breast cancer cell lines (MDA-MB-468, MCF-7, T47D, and MDA-MB-231B) normalized against MCF-10A cells, obtained through a qRT-pCR analysis. MiR-449 levels vary greatly among the cancerous cell lines, and it was thought to potentially be a trigger for oncogenesis.
Figure 2 (Left). T47D breast cancer cells transfected with anti-miR-449 showed a decreased ability to migrate and invade. This figure shows an image of an untreated group of cells next to an image of treated cells after a migration assay then after an invasion assay.

Figure 3 (Right). The treatment of MDA-MB-231B cells with anti-miR-449 decreased the number of migrating and invading cells. This figure shows an image of an untreated group of cells next to an image of treated cells after a migration and invasion assay.

Figure 4. In silico and experimental genomic analysis to identify gene targets of miR-449a. Venn diagram showing number of genes that are in silico predicted targets of miR-449a (miRWalk – http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk), anti-correlated with miR-449a, and/or down-regulated by miR-449a. The correlation data are from in vitro studies collected under the Gene Expression Omnibus database (Enerly et al., 2011) and down-regulated targets were found in vitro through qRT-PCR analysis.

Table 1. The four genes that are predicted targets of, anti-correlated in breast cancer with, and down regulated by miR-449a.

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<th>Gene</th>
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<td>SFXN2</td>
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Figure 5. Expression levels of gene targets in cancer cell lines. The basal expression levels of miR-449 and three potential targets (TAF4B, XBP1, and SFXN2) in four breast cancer cell lines (T47D, MDA-MB-231B, MDA-MB-468, and MCF-7) and breast cell line MCF-10A, obtained through a qRT-PCR analysis.