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Cancer Associated MicroRNAs are Differentially Expressed in Triple Negative Breast Cancer and Normal Breast Cells

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Abstract: ***Breast cancer is a heterogeneous disease that is sub-categorized based on the expression of certain protein markers including estrogen and progesterone. Triple-negative breast cancer (TNBC) is a subtype of breast cancer that is characterized by its lack of the estrogen, progesterone and HER2/Neu protein receptors, and it has no known biomarkers for diagnosis and drug targeting. TNBC has a high mitotic index and primarily affects women of African origin. MicroRNAs are small non-coding RNA molecules that regulate oncogenic and tumor suppressive pathways in the cel1, and may potentially be used as biomarkers for the diagnosis and prognosis of TNBC. In this study, we compared the expression of microRNAs that play roles in cancer in TNBC cell lines (HCC1806, HCC70, and MDA-MB-157) and normal breast cells (AG11132). Microarray analysis revealed that microRNAs 21, 34a, 103, 141, and let-7a were shown to be differentially expressed in TNBC cells vs. the normal breast tissue cell lines. Quantitative-PCR confirmed the differential expression of the microRNAs in the TNBC cell lines vs the normal cells. These findings suggest that microRNAs could be used as potential biomarkers to diagnose TNBC and could potentially be used as drug-targeted therapies in the future.***

Keywords: breast cancer, TNBC, microRNA, miRNA, qPCR.

1. Introduction

Breast cancer is a heterogeneous disease that will affect 12% of all women, and therefore is one of the most common type of cancer diagnosed in women in the USA [1]. Triple-negative breast cancer (TNBC) accounts for about 15% of all breast cancers, and has high mitotic indexes and is highly invasive. TNBC is characterized by the absence of the estrogen, progesterone, and HER2/Neu receptors and therefore do not respond to hormone therapies [2]. TNBC also has no known biomarkers that could be used in early diagnosis and in targeted therapy.

MicroRNAs (miRNAs) are single-stranded short RNA molecules ranging from 20-23 nucleotide bases in length. MicroRNAs regulate the expression of genes by destabilizing a gene's mRNA and preventing the translation of the mRNA into protein and thus reducing or inhibiting protein expression. This reduction in protein expression has been implicated in various cellular processes including inflammation, cell cycle regulation, stress responses, apoptosis, differentiation, and cancer [3].

MicroRNAs have been reported to act as oncogenes [4] or as tumor suppressors [5] thereby playing a role in tumorigenesis. It has been reported that the expression of miRNAs are altered in cancerous versus normal tissues, thereby suggesting that the altered miRNAs could be used as diagnostic or prognostic markers [6, 7]. Recently, miRNAs have been reported to play a role in TNBC [8, 9], or could serve as biomarkers for TNBC that could potentially be targeted for drug therapy.

No molecular mechanisms for miRNAs in triple-negative breast cancer have been identified. Most reports that suggest the role that miRNAs play in TNBC have been clinical or from tissue samples. Here, we show that TNBC cell lines differentially express miRNAs that have been previously reported to play a role in triple-negative breast cancer when compared to normal breast cell lines. Through the identification of miRNAs that are over or under-expressed in TNBC cells, the pathways associated with the molecular mechanisms of miRNAs in gene expression can be determined and potentially used as diagnostic or prognostic tools. More importantly, these cell lines could be used as a model to establish the roles that these miRNAs play in the propagation of triple-negative breast cancer.

2. Materials and Methods

2.1. Cell Lines and Culture

Triple-negative breast cancer cell lines HCC1806, HCC70, and MDA-MB-157 were purchased from American Type Culture Collection (Manassas, VA). Normal breast cell line A11132 was purchased from Coriell Institute for Medical Research (Camden, NJ). Cell lines HCC1806 and HCC70 were both grown in RPMI-1640 (ThermoScientific; Rockford, IL) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Hyclone Laboratory; Logan Utah). AG11132 was grown in MEGM (Lonza; Walkersville, MD). MDA-MB-157 was grown in L-15 (ThermoScientific; Rockford, IL). All cell lines were maintained in 5% CO2 at 37°C. All of the cells were grown to 95% confluence, counted, pelleted, and then stored at -80°C.

2.2. Microarray Hybridization and Partek Genomic Suite Analysis

A total of eight cell pellets, two of each cell line (AG11132, HCC1806, HCC70, and MB157) ran as duplicates, designated as 1&2and were sent frozen on dry ice to Beckman Coulter Genomics (Morrisville, NC) in accordance with Beckman Coulter Genomics' specifications for microRNA extraction and processing. One hundred nanograms of total RNA were labeled with fluorescent dye Cy3 and then hybridized to an Agilent Human miRNAs Microarray 8X15K Release 14.0 array and the generated data was analyzed using Partek Genomics Suite at North Carolina A&T State University. The controls used in this experiment were the normal breast tissue cell line AG11132, and the TNBC cancer cell lines were the MDA-MB-157, HCC1806, and the HCC70 cell lines. The mean microRNA signals and gene name were filtered out of the original cell line data, and then the data from the duplicate cell lines were averaged and merged. Hierarchical clustering and the production of heat maps of the microRNA signal expression profiles were performed using Partek Genomics Suite. Text files of the microRNA expression data of the g-mean signal were imported into Partek Genomics Suite software. Controls used in this experiment were designated as the normal breast tissue cell line AG11132 (1&2) and TNBC cancer cell lines were identified as MDA-MB-157 (1&2), HCC1806 (1&2), and HCC70 (1&2). Determination of under- and overexpression of the g-mean hybridization signal values were in part determined through literature reviews.

2.3. Real-Time Quantitative PCR Analysis

Cell lines (TNBC cell lines HCC1806 and HCC70; AG11132 normal breast cells) were grown to 95% confluency, pelleted and stored at -80°C. The microRNA was extracted from the frozen cell pellets using the miRNEasy extraction kit (Qiagen) and its standard mini-prep protocols. The purity and concentrations of the extracted microRNAs were measured using the NanoDrop 1000 (ThermoScientific). Approximately 10ug of microRNA from each cell line was converted to cDNA using the cDNA Synthesis Kit (Exiqon) and its standard protocols. The concentration and quality of cDNA was measured using the NanoDrop 1000 (ThermoScientific). Equal amounts of cDNA from each cell line was subjected to Real-Time Quantitative PCR Analysis. Approximately 4ul of cDNA was added to 5ul PCR master mix and 1ul Primer mix (Exiqon). The following primers were purchased from Exiqon and used in the subsequent real-time qPCR analysis (hsa-miRNA-103, hsa-miR-21, hsa-miR-141, hsa-miR-34a, and hsa-let-7a). Real-time qPCR analysis was performed using the CFX Connect Real-Time System (BioRad) under the following parameters and conditions: Polymerase Activation/Denaturation (95°C for 10mins); Forty-five amplification cycles (95°C for 10secs; 60°C for 1min; Ramp-rate 1.6°C/s; Optical Read). A melting curve was performed. Real-Time PCR reaction expression levels are indicated by Cq values. Cq is a quantification cycle that corresponds to a threshold cycle; the point where the fluorescence signal is high enough to be differentiated from the background noise. When there is more DNA or high expression levels then there are in fact fewer cycles, which result in lower Cq values. However, when there is less DNA or lower expression levels then more cycles occur, demonstrating higher Cq values thereby providing an inverse relationship to microRNA expression levels in the cell. *Statistical analysis*: An unpaired student's t-test was performed on the Real-Time qPCR analysis between the AG11132 normal cells that served as the control and the TNBC cell lines HCC1806 and HCC70, and if the standard deviation of the Cq values were determined to be $p<0.05$ it was accepted as significantly different and indicated with an asterisk (*) above the bar graph.

3. Results

3.1 Microarray Analysis

Table 1 displays the g-mean hybridization signal averages of microRNAs hybridized to Microarray 8X15K Release 14.0 arrays that have been reported to play a role in breast cancer.

Table 1: MicroRNA average g-mean hybridization signal

MicroRNA	*AG11132*	*MDA-MB-157*	*HCC1806*	*HCC70*
Hsa-mir-34a	256	48	98	68
Hsa-mir-141	100	65	95	127
Hsa-mir-21	716	528	908	774
Hsa-mir-103	103	121	121	148
Hsa-let-7i	158	148	225	107
Hsa-let-7f	471	380	233	198
Hsa-let-7a	1299	573	743	602

Partek Genomic Suite analysis was used to perform a hierarchical cluster of seven selected microRNAs that have been reported to play a role in breast cancer and is graphically displayed in a heatmap in Figure 1.

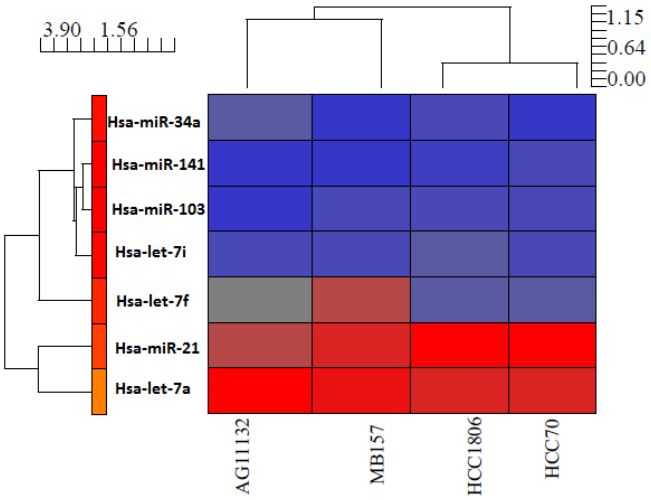


Figure 1: Heatmap of Breast Cancer Associated MicroRNAs g-Mean Hybridization Signals in Breast Cell Lines.

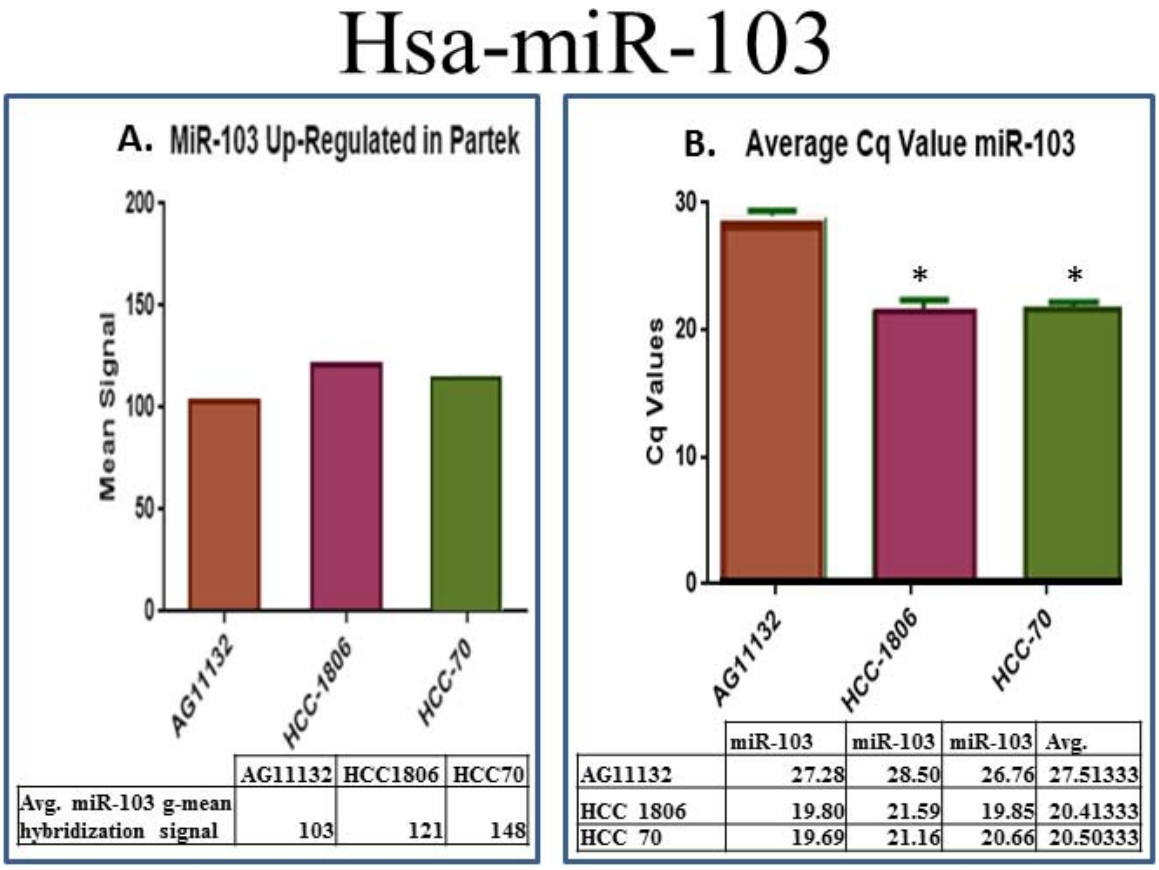
Table 2 lists the five microRNAs that were chosen for real-time quantitative PCR (RT-qPCR) analysis. Table 2 also describes the role that the microRNA play in control of the cell cycle.

Table 2: Cell Control Properties of MicroRNAs

miRNA	*Function*	*Oncogene/Tumor Suppressor*	*Reference*
Hsa-miR-103	Increased metastatic potential	Oncogene	Jansson et al, 2012 [7]
Hsa-miR-141	Promotes Metastasis	Oncogene	Chang et al, 2012 [10]
Hsa-miR-21	Increase cell growth & proliferation; Decreased apoptosis	Oncogene	Hanna-Dellago et al, 2013 [11]
Hsa-let-7a	Inhibits Cell Proliferation	Tumor Suppressor	Hu et al, 2013 [12]
Hsa-miR-34a	Promotes Apoptosis	Tumor Suppressor	Misso et al, 2014 [13]

3.2 qPCR Analysis

According to Martello et al, the microRNA family 103/107 displays high expression levels which are associated with metastasis and poor outcome [1, 14]. Figure 2 represents the expression analysis of miR-103 in AG11132 "normal" breast cells and in triple-negative breast cancer cell lines HCC1806 and HCC70. Figure 2A reveals through microarray analysis performed using Partek Genomics Suite the differential expression of miR-103. MicroRNA-103 is upregulated in TNBC cell lines HCC1806 and HCC70 compared to AG11132 normal breast cells. Figure 2B shows that the Cq values of miR-103 are lower in the TNBC cell lines than in the normal breast tissue cell lines. This indicates an up-regulation of microRNA-103 in the TNBC cell lines (HCC 1806 &HCC70) vs. the normal breast tissue cell line (AG11132). These results are consistent with our findings which indicate microRNA-103 is overexpressed in the TNBC cells versus the normal breast cells.

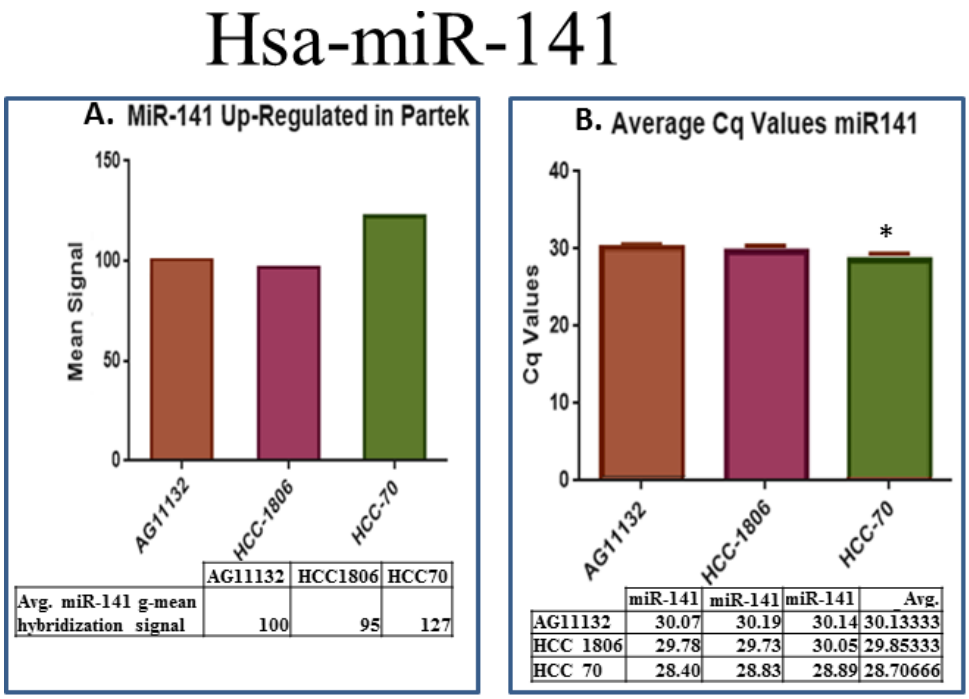


	AG11132	HCC1806	HCC70
Avg. miR-103 g-mean hybridization signal	103	121	148

	miR-103	miR-103	miR-103	Avg.
AG11132	27.28	28.50	26.76	27.51333
HCC 1806	19.80	21.59	19.85	20.41333
HCC 70	19.69	21.16	20.66	20.50333

Figure 2: Hsa-miR-103 Expression Analysis. (A) Microarray analysis (B) Real-time qPCR expression analysis. TNBC RT-qPCR Cq values displaying significant difference ($p<0.05$; unpaired student t-test) from AG11132 control cells indicated with * symbol.

MicroRNA-141 is part of the microRNA-200 family and studies by Chang et al indicate an over-expression of this microRNA in breast cancer which promotes metastasis and drug resistance[10]. Figure 3 represents the expression analysis of miR-141 in AG11132 "normal" breast cells and in triple-negative breast cancer cell lines HCC1806 and HCC70. Figure 3A reveals through microarray analysis performed using Partek Genomics Suite the differential expression of miR-141. MicroRNA-141 is upregulated in TNBC cell line HCC70 while downregulated in TNBC cell line HCC1806 compared to AG11132 normal breast cells. Figure 3B shows and confirms that the Cq values of miR-103 are lower in the TNBC cell line HCC70 while no differences in the Cq value was detected in TNBC cell line HCC1806 when compared to the normal breast tissue cell line AG11132. This indicates a differential expression pattern of microRNA-141 in the TNBC cell lines (HCC 1806 &HCC70) vs. the normal breast tissue cell line (AG11132).

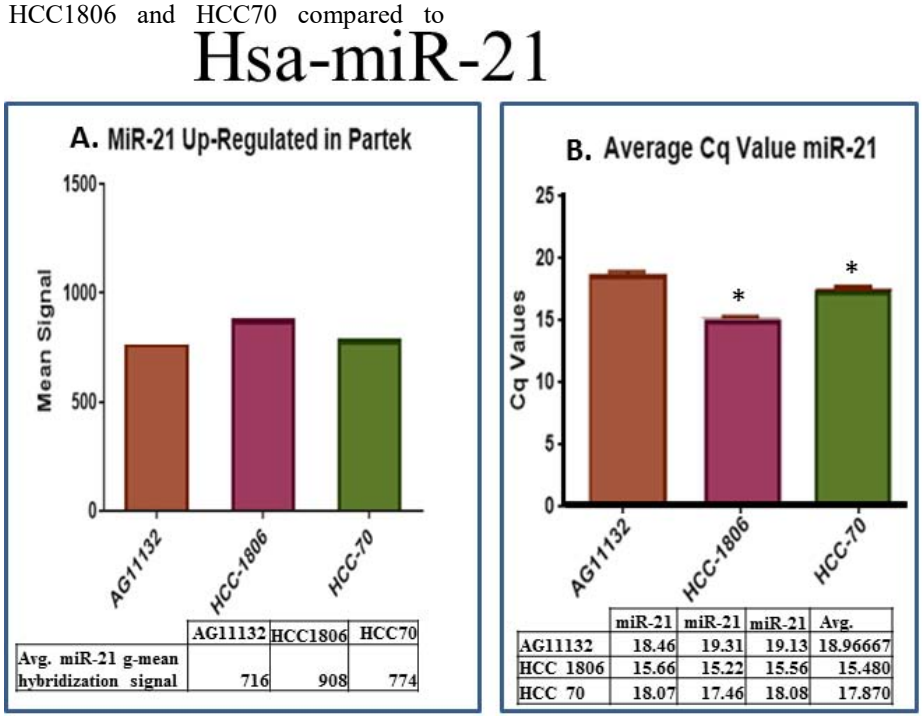


	AG11132	HCC1806	HCC70
Avg. miR-141 g-mean hybridization signal	100	95	127

	miR-141	miR-141	miR-141	Avg.
AG11132	30.07	30.19	30.14	30.13333
HCC 1806	29.78	29.73	30.05	29.85333
HCC 70	28.40	28.83	28.89	28.70666

Figure 3: Hsa-miR-141 Expression Analysis. (A) Microarray analysis (B) Real-time qPCR expression analysis. TNBC RT-qPCR Cq values displaying significant difference ($p<0.05$; unpaired student t-test) from AG11132 control cells indicated with * symbol.

According to Hanna Dellago et al, microRNA-21 is described as being an oncogenic [11]. Figure 4 represents the expression analysis of miR-21 in AG11132 "normal" breast cells and in triple-negative breast cancer cell lines HCC1806 and HCC70. Figure 4A reveals through microarray analysis performed using Partek Genomics Suite the differential expression of miR-21. MicroRNA-21 is upregulated in TNBC cell lines HCC1806 and HCC70 compared to AG11132 normal breast cells. Figure 4B shows that the Cq values of miR-21 that are lower in the TNBC cell lines than in the normal breast tissue cell lines. This indicates an up-regulation of microRNA-21 in the TNBC cell lines (HCC 1806 &HCC70) vs. the normal breast tissue cell line (AG11132).



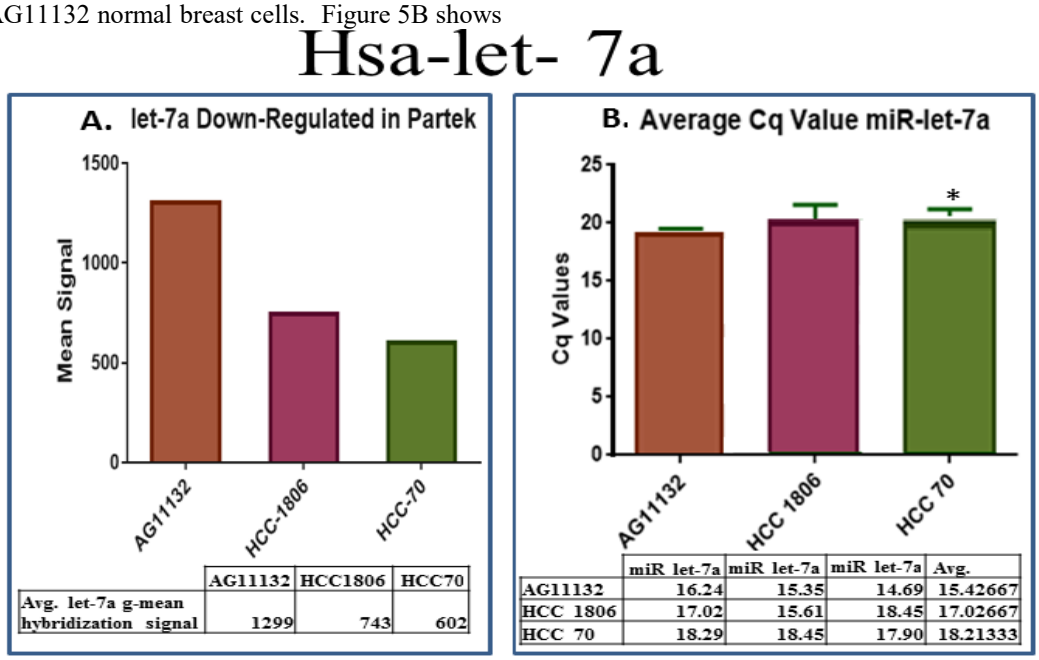
	AG11132	HCC1806	HCC70
Avg. miR-21 g-mean hybridization signal	716	908	774

	miR-21	miR-21	miR-21	Avg.
AG11132	18.46	19.31	19.13	18.96667
HCC 1806	15.66	15.22	15.56	15.480
HCC 70	18.07	17.46	18.08	17.870

Figure 4: Hsa-miR-21 Expression Analysis. (A) Microarray analysis (B) Real-time qPCR expression analysis. TNBC RT-qPCR Cq values displaying significant difference ($p<0.05$; unpaired student t-test) from AG11132 control cells indicated with * symbol.

Let-7a is microRNA described as a heterochronic gene which serves as a tumor suppressor by targeting various oncogenic pathways in cancer cells as stated by [12]. Figure 5 represents the expression analysis of let-7a in AG11132

"normal" breast cells and in triple-negative breast cancer cell lines HCC1806 and HCC70. Figure 5A reveals through microarray analysis performed using Partek Genomics Suite the differential expression of let-7a. Let-7a is significantly downregulated in TNBC cell lines HCC1806 and HCC70 compared to AG11132 normal breast cells. Figure 5B shows that the Cq values of let-7a are higher in the TNBC cell lines than in the normal breast tissue cell lines. This indicates a down-regulation of let-7a in the TNBC cell lines (HCC 1806 &HCC70) vs. the normal breast tissue cell line (AG11132).

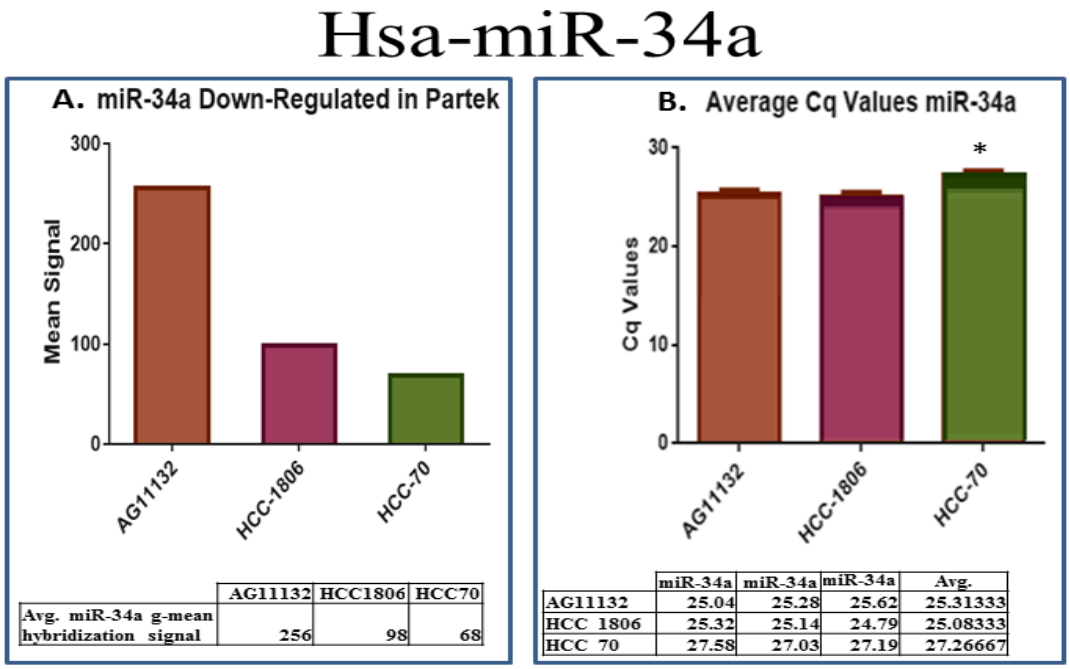


	AG11132	HCC1806	HCC70
Avg. let-7a g-mean hybridization signal	1299	743	602

	miR let-7a	miR let-7a	miR let-7a	Avg.
AG11132	16.24	15.35	14.69	15.42667
HCC 1806	17.02	15.61	18.45	17.02667
HCC 70	18.29	18.45	17.90	18.21333

Fig. 5. Hsa-let-7a Expression Analysis. (A) Microarray analysis (B) Real-time qPCR expression analysis. TNBC RT-qPCR Cq values displaying significant difference ($p<0.05$; unpaired student t-test) from AG11132 control cells indicated with * symbol.

Figure 6 represents the expression analysis of miR-34a in AG11132 "normal" breast cells and in triple-negative breast cancer cell lines HCC1806 and HCC70. Figure 6A reveals through microarray analysis performed using Partek Genomics Suite the differential expression of miR-34a. MicroRNA-34a is significantly downregulated in TNBC cell lines HCC1806 and HCC70 compared to AG11132 normal breast cells. Figure 6B shows that the Cq values of miR-34a are higher in the TNBC cell line HCC70 while there was no significant difference in the Cq values of TNBC cell line HCC1806 than in the normal breast tissue cell lines. This suggests a down-regulation of miR-34a in the TNBC cell lines (HCC 1806 &HCC70) vs. the normal breast tissue cell line (AG11132).



	AG11132	HCC1806	HCC70
Avg. miR-34a g-mean hybridization signal	256	98	68

	miR-34a	miR-34a	miR-34a	Avg.
AG11132	25.04	25.28	25.62	25.31333
HCC 1806	25.32	25.14	24.79	25.08333
HCC 70	27.58	27.03	27.19	27.26667

Figure 6: Hsa-miR-34a Expression Analysis. (A) Microarray analysis (B) Real-time qPCR expression analysis. TNBC RT-qPCR Cq values displaying significant difference ($p<0.05$; unpaired student t-test) from AG11132 control cells indicated with * symbol.

4. Discussion

This study compared reported breast cancer associated microRNA expression profiles using microarray and real-time qPCR analysis on triple-negative breast cancer and normal breast tissue cell lines. Hierarchical clustering and heatmaps of microarray analysis of microRNA expression profiles revealed a differential expression pattern between triple-negative breast cancer cells and normal breast tissue cells. MicroRNA-21 has been reported to be an oncogene, and here we show that miR-21 is over-expressed in the TNBC breast cancer cell lines. Previous studies have suggested that microRNA-21 has a poor prognostic value in cancer patients [11, 15]. The study revealed a decrease in the expression of the tumor suppressor microRNA let-7a which correlated with other studies implicating this microRNA's role in targeting oncogenes RAS and CMYC [15].

MicroRNA-34a has been reported to be down-regulated in TNBC breast cancer cell lines and tissues, compared with normal cell lines and the adjacent non-tumor tissues, respectively [16]. Our study also demonstrated a down-regulation of microRNA-34a in the TNBC cell lines. Martello et al, suggests in human breast cancer, high levels of microRNA-103/107 are associated with metastasis and poor outcome [14]. Data from our study shows an up-regulation of microRNA-103 in the TNBC cells compared to normal breast tissue cells. Previous studies indicated that miR-141 is upregulated in cancer [10, 17], while other studies suggest that miR-141 is down-regulated in cancer [18]. We show that miR-141 is upregulated in the HCC70 cell line, while miR-141 is slightly down-regulated in HCC1806 cells.

These findings support the hypothesis that microRNAs may play a role not only in breast cancer, but other cancer types as well and suggests that miRNAs may be used as biomarkers for diagnosis, therapeutic targets, and prognostic indicators or predictors. The usage of cell lines may serve as models for future research studies and may provide less error and variation that may result from using patient tissue samples.

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