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MiRnome Profiling of Breast Cancer Cells: Seeking a Reliable Biomarkers

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Abstract

Background: Breast cancer (BC) is elevating global wide and causing risky neoplasia in women at young ages. Besides, it is the most known disease that causes fear in women as it affects their self-esteem and confidence. **Aim:** The current study aimed at establishing a prediction model for BC based on epigenetic and genetics readings. **Material and Methods:** In this study, we used MCF-7 breast cancer cells to recognize the role of 95 different types of microRNAs (miRNAs). **Results:** The results showed that among 95 different miRNAs profiled, 6 miRNAs were found to be up-regulated, and 26 miRNAs were found to be down-regulated. After analysis, there was a link between the development of BC and the 6 up-regulated miRNAs. **Conclusion**: To reach early BC diagnosis, the 6 up-regulated miRNAs can be used as a biomarker. Specifically, has-miR-23b-3p was overexpressed in MCF-7 breast cancer cells and can be considered as a potential biomarker. Notwithstanding, more investigations and further studies are required to support these findings.

Keywords: BC, miRNA, MCF-7, Profiling, Epigenetic, Biomarker Potential, Immunology.

Introduction

Cancer is a general term that encompasses a wide range of illnesses that can affect various organs in the body. Cells have a natural tendency to divide and die in a predictable pattern [1,2]. On the other hand, cancer cells have the ability to divide uncontrollably indefinitely [3,4]. There are over 200 distinct cancer kinds and with 8.8 million fatalities each year, making cancer the second leading cause of death on the planet [5,6]. Breast cancer (BC) is the most common invasive malignant tumor in women and the second leading cause of cancer related deaths in women, with 1.5 million new diagnosed cases each year worldwide [7-11]. BC is a heterogeneous disease that involves changes in both mRNAs and microRNAs (miRNAs), causing BC cell division abnormalities [12-16].

Early detection using and regular monitoring of the BC patients' therapeutic reactions are urgently needed to properly monitor BC [17,18]. However, due to the many undiscovered BC biomarkers; it is challenging to early detect or diagnose the disease. The use of miRNAs at the early detection of breast cancer and other malignancies has recently received a lot of attention [19-22]. Given the simplicity with which miRNAs may be isolated, characterized, and quantified, it could be used as a trustworthy biomarker.

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miRNAs could be employed as prognostic or predictive biomarkers in addition to early diagnosis of BC [23-24].

In BC miRNAome, extensive study has revealed remarkable discoveries, some of these have already been accepted for use in therapeutic settings [23]. miRNA research is currently ongoing, and it shows great potential in terms of finding new therapeutic targets and biomarkers [25-27]. MiRNAs are a type of short non-coding RNA molecules with a single strand that have evolved to be evolutionary preserved, and have a big role in the post-transcriptional regulation of the genome [28-30]. Some miRNAs are well-known for acting as major negative regulators in a variety of biological processes that contribute to BC development [31]. Expression of different miRNA in associated to BC development reveal a good starting point for investigation them as biomarkers [32,33]. Further, unregulated miRNAs discovered in BC may help us better understand the tumor microenvironment, which promotes a better understanding into their function in cancer cells growth and metastasis [34-36].

In the last decade, several types of studies have focused on establishing the relationship between distinct miRNA and BC by miRNAome profiling in cancer cells and patients [37-39]. This aids in elucidating the functional role and molecular mechanisms of down/up-regulated microRNAs in the formation and progression of BC, which is required for miRNA-based therapy to progress [13,15].

Materials and Methods

Cell Culture

MCF-7, the human breast cancer cell line, was provided by VACSERA (The Holding Company for

Biological Products and Vaccine, Cairo, Egypt). In a 12-well plate, cells were seeded at a density of

2×10^4 cells/well and cultured in RPMI 1640 media supplemented with 10% FBS (Hyclone, Logan, UT) and 1% antibiotic mix at laboratory conditions (37 °C and 5% CO2). To control genomic drift due to instability, the MCF-7 cell line was involved in the study throughout the first 10 passages from the flask which was originally bought. Every three days, the RPMI-1640 was altered, and the cells were transfected when they reached 65% - 80% confluency.

MTT assay

The cell viability was assessed using 3-(4&5-dimethylthiazolyl-2)- 2&5 diphenyl tetrazine bromide assay, known as MTT assay. First, each well was seeded in a 96-well plate, as $5x10^4$ cells/well and incubated until cell confluency reached 65%. Then, MTT assay was carried out directly after applying siRNA. Many wells are left and considered as control wells. The control and treated wells were loaded with media, supplied with 20 µL (5 mg/mL), followed by 3 hours incubation at 37 °C and 5% CO2 incubator. Then, 50 µL of media was aspirated and 180 µL of DMSO was added to each well. After that, the plate was transferred to a water bath shaker at 37 °C, then centrifuged at 250 rpm for 30 min. to dilute the formazan crystals.

Total RNA Extraction

The RNeasy kit was used to extract total RNA from MCF-7 cells (Qiagen, Germany). DNase I (Boehringer-Mannheim, Mannheim, Germany) was used to treat RNA for 50 min. before

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purification according to the kit's instructions. Spectrophotometry and ethidium bromide agarose gel electrophoresis was used to assess the quality and integrity of the RNA.

First-strand cDNA synthesis

About 200 ng poly RNA was mixed with provided enzymes and primers, and the volume was brought to 200 µL using ribonuclease-free water. The reaction was performed according to the kit's instructions.

Double-stranded cDNA synthesis

About 11 µL of first-strand poly(A) cDNA, 74 µL of Milli-Q water, 10 µL of 10x PCR buffer, 2 µL of 10 mM dNTP mix, 1 µL of 25 mM 5′ SMART PCR primer, and 2 µL of 50x Polymerase Mix (Clontech) were mixed together. A 100-L reaction mixture was incubated at 95°C for 1 min., 68 °C for 20 min, then 70 °C for 10 min to extend the primers.

PCR array

The generated cDNA has been subjected to RT-PCR against specific primers (1 µL for each primer), gene expression was calculated according to the cyclic threshold (CT), and the total volume of all reactions was 25 µL containing 12.5 µL of SYBR Green master mix. Program of the thermal cycler was 95 °C for 15 min., and 40 cycles of 94 °C for 15 sec, 54 °C for 30 sec, and 72 °C for 30 sec. Internal control was achieved by using GAPDH housekeeping primer.

Data Analysis

The CT values were collected and uploaded to Qiagen's online analysis tool [40], where data validation and 2-CT calculations for each miRNA were carried out.

Statistical analysis

IBM-SPSS-24 (IBM-SPSS- Inc. Chicago- IL) was used for conducting the data analyses, in addition to the ± SD in the expression of all values. Determining the significance of the different values in a multiple comparison was achieved using analysis of variance with t-test. P values differences were less 0.05.

Results

Up-regulated miRNAs in BC

In this work, 84 miRNAs were identified in BC cells (MCF-7). Four miRNAs, hsa-miR-21-5p, hsa- miR-10a-5p, has-miR-23b-3p, hsa-miR-106a were shown to be elevated in BC cells (Figure 1).

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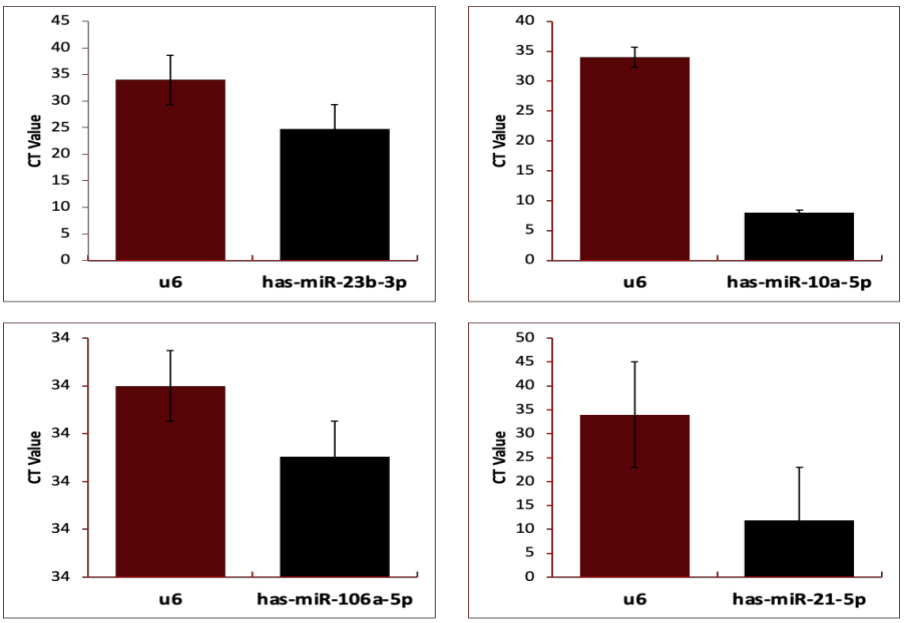
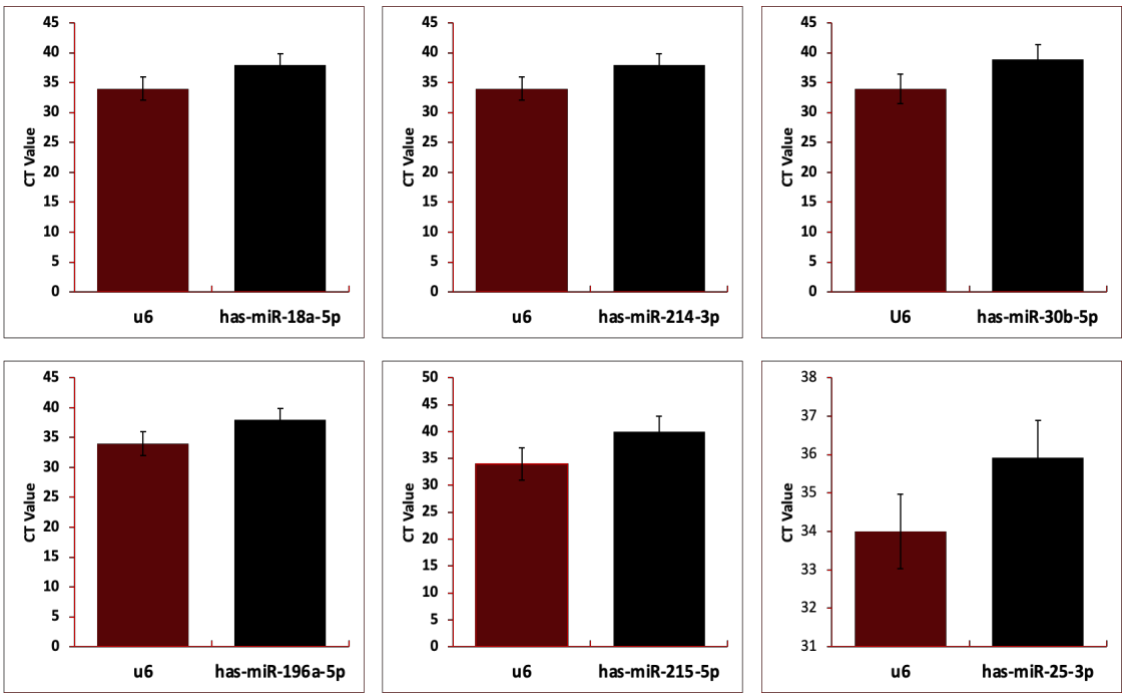


Fig. 1: The upregulated miRNA detected in BC cells.

The significant differences were estimated between all the studied miRNA ($p<0.001$) compared to U6 snRNA, with one exception for hsa-miR-106a-5p as there is no significant difference in the p- value compared to U6.

Down-regulated miRNAs in BC

In this study, 84 miRNAs were profiled, with 12 miRNAs being down-regulated in BC cells; miR-218a, miR-214, miR-30b, miR-196a, miR-215, and miR-25 (Figure 2a), and miR-206, miR-10b, hsa-let-7b-5p, hsa-let-7g-5p, hsa-let-7c-5p, and hsa-203-3p (Figure2b).



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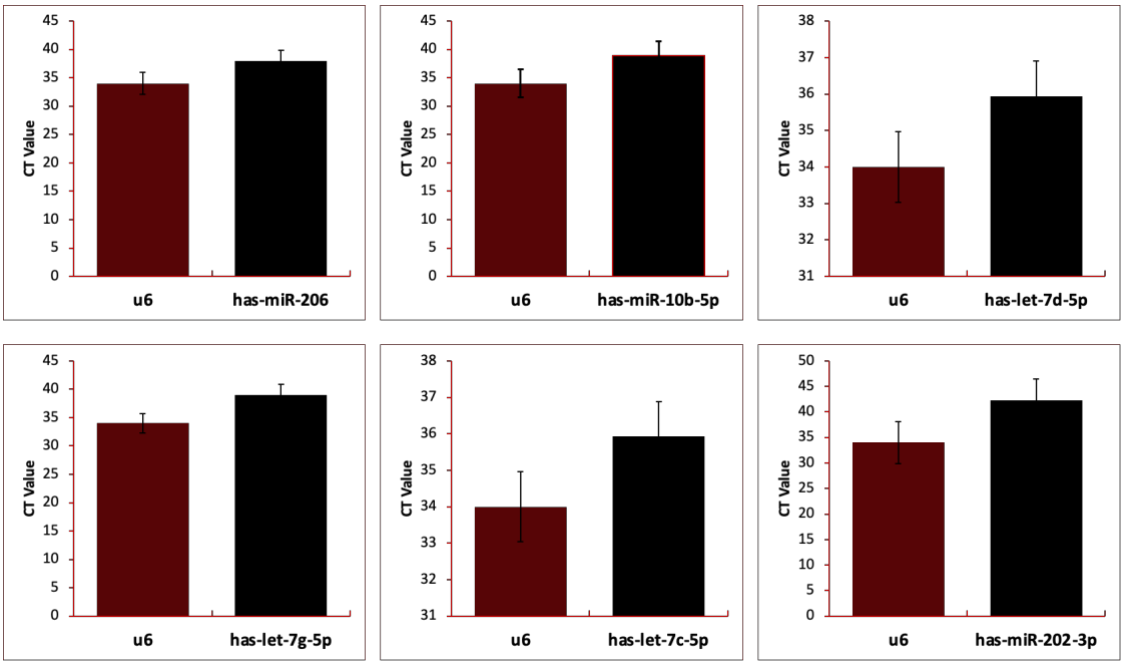

Fig. 2: The downregulated miRNAs in BC cells.

The statistical differences in the *p*-values between U6 snRNA and the expression level of the target miRNAs were significant ($p<0.01$ and 0.001). However, the expression level of hsa-let-7c-5p, hsa- let-7d-5p, has-miR-206, has-miR-25-3p, has-miR-196a-5p, has-miR-30b-5p, hsa-let-7g-5p, has- miR-18a-5p, and hsa-214-3p do not appear to be statistically significant compared to U6.

Discussion

In the current study, the profiling of 95 miRNAs was achieved in the MCF-7 BC cells for the aim of finding BC potential biomarkers. Some miRNAs found to be down-regulated (26 out of 95 miRNAs), while some found to be up-regulated (6 out of 95 miRNAs) in a comparison to U6 sn- RNA as an internal control in RT-PCR.

Up-regulated miRNAs

The clear differences between U6 sn-RNA and these miRNAs were revealed by statistical analysis. It has been reported that in "hsa-miR-196a-5p," the levels of apoptosis is elevated resembling GC-2 treated cells, which means that it is involved in cancer cells apoptotic machinery [38]. Both hsa- miR-21 and hsa-miR-17-5p were significantly high in cell lines and tissues of breast cancer, where AIB1 gene was down-regulated primarily via translational inhibition. Meanwhile, overexpression of hsa-miRNA-17-5p promotes and controls the proliferation of cells which induces growth of tumor [39,40].

The "has-miR-195 5p" suppresses the proliferation, invasion, and migration of carcinoma squamous cells. Many miRNAs that are known to be up-regulated, such as; "has-miR-103a-3p, has-miR-21-5p, and has miR-195-5p," should be employed as BC diagnostic biomarkers [40-42]. The up-regulation of "miRNA-19a-3p" reduced cancer growth and metastasis in both the bladder and the breast by inducing macrophage polarization down-regulating the expression of the "Fra-1" proto- oncogene in vivo [43], and in the oral cavity via targeting TRIM14 in vivo [44].

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Down-regulated miRNAs

About 95 of the miRNAs investigated in this study were found to be down-regulated in breast cancer cells, with about 26 of them being down-regulated. In a study, it was known that the "hsa- miR-30c" enhances BC metastasis through the invasive phenotype by NOV/CCN3 targeting. Thus, it was expected that “hsa-miR-30c” is up-regulated to accomplish its role, however, it was down- regulated in this study. Antitumor activity is also a feature of miR-99a, which is achieved in human BC cells by targeting the mTOR/p-4E-BP1/p-S6K1 pathways, and in human urothelial and carcinoma cells found in the urinary bladder by activating the RAD001- triggered apoptotic pathway [45]. These outcomes are connected to our study according to the findings.

Moreover, it has been documented that that “hsa-miR-206” crushes the invasion and proliferation of BC cells via Cx43 targeting [46]. In addition, hsa-miR-206 also suppresses the metastasis and of BC by MKL1/IL11 targeting pathway [47]. However, it has been indicated that when hsa-miR-206 is down-regulated in BC cells, that can suppress the proliferation of cells through "cyclin-D2" up- regulation, and these results match our results [48].

miRNA profiling studies in transcriptional expression across cancer cell lines and tumor tissues have showed that "miR-29-a" down-regulates the cancer related genes. miR-29-a controls invasion and growth of ER-positive breast cancer cell [49]. However, another study showed that enforced expression of miR-29-a controls apoptosis by controlling the expression of "MCL-1" and reduce tumor growth in ALCL cell lines. Subsequently, miR-29-a synthesis could work as a new early diagnostic tool for cancer [50,51].

Furthermore, miR-145 is a dual-stranded tumor suppressor that operates via "MTDH" targeting in lung squamous cell carcinoma and via direct or indirect "TGF-1" regulatory expression to restrict the kidney cells invasion via regulating PAK5 gene. Also, miR-106a-5p could be used as a suppressor for the potential tumors’ growth [52,53]. The expression of miR-106a-5p was low in this study, which is expected, considering that it suppresses cancer promoting genes.

Conclusion

About 95 miRNAs were analyzed in MCF-7 BC cells in this study. Based on RT-PCR data analysis, 6 of the 95 total miRNAs were found to be up-regulated in BC cells, while approximately 26 were found to be down-regulated. Further investigations need to be done to reach identify the

main role of these miRNA in BC. The miRNAs that were found to be up-regulated, which are "hsa- miR-21-5p, hsa-miR-10a-5p, has-miR-23b-3p, hsa-miR-223-3p, unisp-31pc, and unisp-3pc" can be employed as biomarkers for BC for the early diagnosis of the disease. Finally, we conclude that the significantly up-regulated miRNA "has-miR-23b-3p" could be cancer progression.

Acknowledgements

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Conflict of Interest

No conflicts of interest the authors have to declare.

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