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miRNA Profiling in MCF-7 Breast Cancer Cells: Seeking a New Biomarker

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Abstract

Cancer is an out-of-control cell proliferation that can attack any cell in the human body. One of the most prevailed cancers in women is breast cancer, with elevated incidence and mortality rates, that makes it a major health threat globally. In the present investigation, we used MCF-7 breast cancer cells to identify the role of 25 different miRNAs. The study revealed that out of the 25 miRNAs profiled, 11 miRNAs were upregulated, and 14 miRNAs were down-regulated. Target gene prediction was conducted using MirNet online web building tool, and results indicated that up-regulated miRNAs interact with several cancer-related genes such as FRS2, TNFRSF10B, BTBD7, MYC, and TPRG1L. The down-regulated miRNAs were also interacting with a list of tumor-related genes (e.g., GABPB2, GNB1, CCND1, ERCC1, ZNF417, GLO1, EIF4G2, DRAXIN, HOXD11, and KPNA6). Disease prediction has also been conducted to identify the relatedness of, especially, the up-regulated miRNA with common disease including cancer. This study concludes that the 11 up-regulated miRNAs could be used as biomarkers to early diagnose BC, but we can highlight hsa-miR-30d-5p as a potential biomarker for its high expression profile. However, these data need more investigations to confirm this finding.

Keywords: Breast cancer; MCF-7; miRNA; Profiling; Biomarker

Introduction

Cancer describes a wide range of diseases that can affect different organs in the body. The normal behavior of cells is to divide and die in an orderly manner [1,2], however, cancer cells are capable to continually divide in an out-of-control way [3,4]. There are about 200 different types of cancers [5-7]. Cancer is the second leading cause of death globally with 8.8 million deaths every year [8].

Breast cancer, however, is the most common invasive malignant tumors and the second leading cause of death by cancer in females after lung cancer, with 1.5 million new cases per year and more than 500,000 deaths globally per year [9-11]. It is a heterogeneous disease comprising various types of neoplasms, which involves different profile changes in both mRNA and micro-RNA (miRNA) expression [12-16].

To effectively monitor BC, early diagnosis with reliable tools and close observing of the patient's response to treatment are needed [17,18]. However, these tasks are surprisingly difficult to tackle because of the lack of approved sensitive biomarkers to early detect or diagnose the disease [17,18]. A lot of evidence recently highlighted the potential use of miRNA in the early diagnosis of BC and other cancers [19-22]. miRNA also could be employed as a reliable biomarker, given the ease of isolation, characterization, and quantification. miRNA could not only be used in early diagnosis of BC but also as prognostic or predictive biomarkers [22-24].

Extensive research in BC miRnome has yielded fascinating discoveries, some of which have been approved to be utilized in

clinical settings [23]. On-going miRNA research indicated incredible potential for the advancement of novel biomarkers and therapeutic targets [25-27].

miRNAs are a class of small single-stranded non-coding RNA molecules that are evolutionarily conserved and encoded by nearly 1% of the genome in most species. These are involved in the regulation of gene expression at the post-transcriptional level [28-30]. It is well known that some miRNAs function as key negative regulators in several biological processes underlie breast cancer [31]. Abnormal microRNA expression involved in the cancer process provides a suitable entry point to explore its functional role in cancer [13,32,33]. Deregulated miRNAs identified in BC might put us a step forward towards understanding the tumor microenvironment, which prompt the investigate of their role in cancer progression and spreading [34-36].

Several types of research have focused in the last decade on identifying the association between different miRNA and BC *via* profiling the miRnome in either BC cells or patients [20,37-39]. This profiling helps in understanding the functional role and molecular mechanism of down/up-regulated microRNAs in BC development and progression, which, in turn, is needed to advance miRNA-based therapy [13,15,24].

Materials and Methods

Cell line maintenance

Breast cancer cell line (MCF-7) was obtained from the Holding Company for Biological Products and Vaccines (VACSERA, Giza, Egypt). Cells were seeded at a density of 10^4 cells/cm^2 in 12-well plate and were cultured under standard laboratory conditions; at 37°C and 5% CO_2 and maintained on RPMI 1640 media (GIBCO/Invitrogen Life Technologies, Carlsbad, USA) supplemented with 10% FBS (Hyclone, Logan, UT) and 1% antibiotic mix. MCF-7 cell line was enrolled in the study within the first 10 passages from the originally purchased flask to control genomic drift due to instability. RPMI-1640 was changed every 3 days and cells were passaged whenever it reached 65% to 80% confluently.

RNA extraction

Total RNA was extracted from the MCF-7 cells with the RNeasy kit (Qiagen, Germany). RNA was treated with DNase I (Boehringer-Mannheim, Mannheim, Germany) for 50 min. and purified according to the kit's protocol. The quality and integrity of RNA were checked by spectrophotometry and ethidium bromide agarose gel electrophoresis.

First-strand cDNA synthesis

In a 0.2 mL PCR tube, the following items were combined: 200 ng of poly RNA, 0.6 µL of 25 M semi-random primer, and ribonuclease-free water in a 4.75 µL volume. The mix was incubated at 72°C in a hot-lid thermal cycler for 3 min., cooled down on the ice for 2 min., and incubated again at 25°C for 10 min. The following mixture was prepared and added to the tube: 2 µL of 5x SMARTScribe buffer, 0.5 µL of 25 µM 5'SMART tag, 1 µL of 10 mM deoxynucleotide triphosphate (dNTP) mix, 0.25 µL of 100 mM DTT, 0.5 µL of RNaseOUT (Invitrogen), and 1 µL of SMARTScribe Reverse Transcriptase (100 U) (Clontech). The mixture was incubated at 42°C for 90 min and then at 68°C for 10 min in a thermal block. To digest RNA, 1 µL of RNase H (Invitrogen) was added to the mixture, and then the mixture was incubated at 37°C for an extra 20 min.

Double-stranded cDNA synthesis

About 11 µL of the prepared first-strand poly(A) cDNA was mixed with 74 µL of Milli-Q water, 10 µL of 10x PCR Buffer, 2 µL of 10 mM dNTP mix, 1 µL of 25 µM 5' SMART PCR primer, and 2 µL of 50x Polymerase Mix (Clontech). A 100 µL volume of the reaction mixture for primer extension was incubated at 95°C for 1 min, 68°C for 20 min, and then 70°C for 10 min. Double-stranded cDNA was stored at -20°C until being used.

Real-time PCR

Using miScript PCR array for tumor suppressor genes (Qiagen, Germany), real-time-based expression analysis of 25 miRNA was performed in StepOne-Plus thermal cycler (Applied Biosystems). The thermal profile was as follows: 95°C for 1 min., 57°C for 45s, and 72°C for 1 min. The fold change was calculated using the 2-ΔC_T method. U6 snRNA (as a housekeeping gene) was involved in the reaction to control the fidelity of the real-time amplification.

Data analysis

The retrieved data of C_T values were uploaded to the online analysis tool provided by Qiagen [40], where the validation of data and calculation of 2-$\Delta\Delta C_T$ for each miRNA were performed.

Statistical analysis

Statistical analyses were performed using the SPSS software package (SPSS, Inc., Chicago, IL). All values were expressed as mean ± SD. Analysis of variance with t-test was used to determine the significance of the difference in a multiple comparisons. Differences with a P value of less than 0.05 were considered statistically significant.

Target prediction

The up-regulated and down-regulated miRNAs in BC cells were submitted to miRNet.ca [41] online target prediction tool to identify the target gene(s) for both the up-and down-regulated miRNAs.

Results

Up-regulated miRNA in BC

In the present study, 25 miRNAs were profiled in breast cancer cells (MCF-7). Eleven miRNA were found to be up-regulated in BC cells; hsa-miR-141-3p, hsa-miR-16-5p, hsa-miR-196a-5p, hsa-miR-17-5p, hsa-miR-19a-3p, hsa-miR-181b-5p,

hsa-miR-195-5p, hsa-miR-30b-5p, hsa-miR-103a-3p, hsa-miR-21-5p, and hsa-miR-30d-5p (**Figure 1 and Table 1**). Significant differences were obtained between U6 snRNA and all the miRNA studied (p=0.001), with one exception for hsa-141-3p where the differences was not significant at the same p-value.

Down regulated miRNAs in BC

Of the 25 miRNAs profiled In the present study, 14 miRNAs were found to be down-regulated in BC; hsa-miR-194-5p, hsa-miR-31-5p, hsa-miR-30c-5p, hsa-miR-99a-5p, hsa-miR-133a, hsa-miR-206, hsa-miR-23b-3p, hsa-miR-24-3p, hsa-miR-20b-5, hsa-miR-143-3p, hsa-miR-145-5p, hsa-miR-106a-5p, hsa-let-7a-5p, and hsa-let-7b-5p (**Figure 2 and Table 1**).

Table 1: Statistical analysis of all miRNAs enrolled in this study (p=0.001).

miRNA name	Mean	p-value	Sig.
hsa-let-7a-5p	19.048	0.0848	ns
hsa-let-7b-5p	37.972	0	***
hsa-miR-103a-3p	8.583	0	***
hsa-miR-106a-5p	30.777	0	***
hsa-miR-141-3p	13.183	0.0723	ns
hsa-miR-143-3p	35.751	0	***
hsa-miR-145-5p	33.218	0	***
hsa-miR-17-5p	10.083	0.0005	***
hsa-miR-181b-5p	9.484	0.0001	***
hsa-miR-195-5p	8.816	0	***
hsa-miR-196a-5p	10.931	0.0023	**
hsa-miR-19a-3p	9.742	0.0002	***
hsa-miR-20b-5p	37.413	0	***
hsa-miR-21-5p	8.4139	0	***
hsa-miR-23b-3p	30.629	0	***
hsa-miR-24-3p	28.952	0	***
hsa-miR-30d-5p	6.489	0	***
hsa-miR-31-5p	28.75	0	***
hsa-miR-206	35.516	0	***
hsa-miR-30b-5p	8.8	0	***
hsa-miR-99a-5p	24.688	0	***
hsa-miR-133a	18.307	0.1983	ns
hsa-miR-16-5p	11.212	0.0037	**
hsa-miR-194-5p	32.553	0	***
hsa-miR-30c-5p	21.886	0.001	***
U6 snRNA	16.179		

The differences between the level of expression of the target miRNA and U6 snRNA were significant (p=0.001). Two exceptions were recorded; hsa-let-7a-5p and hsa-133a where the differences in expression level were not significant at the same p-value (**Figure 3**).

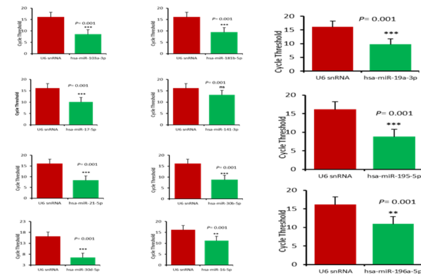

Figure 1: Up-regulated miRNAs in BC cells (11 miRNAs) compared to U6 snRNA and the significance of differences.

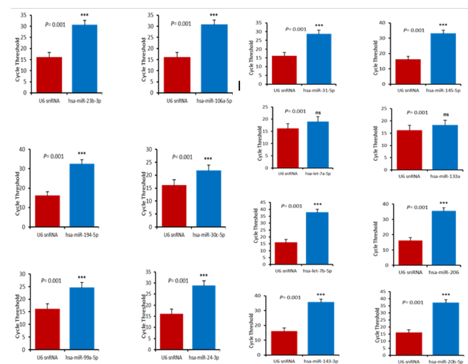

Figure 2: Down-regulated miRNAs in BC cells (14 miRNAs) compared to U6 snRNA and the significance of differences.

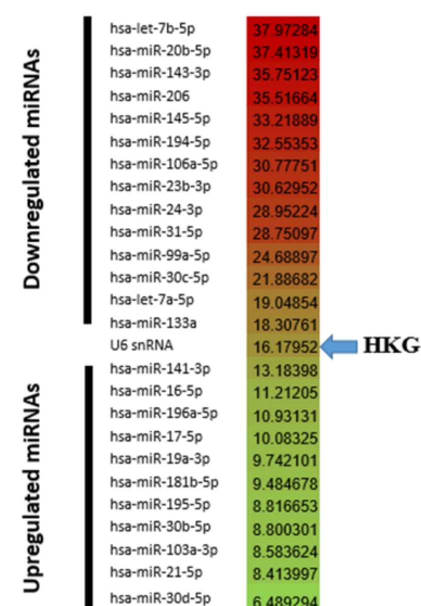

Figure 3: Down-regulated and up-regulated miRNAs heat map based on C_T values.

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Target Prediction

The up- and down-regulated miRNAs detected in BC cells were uploaded to OmicsNet miRNA target prediction online tool (URL provided in the Materials and Methods section). Up-regulated miRNAs were found to interact with several genes (**Figure 4**). FRS2, TNFRSF10B, BTBD7, MYC, and TPRG1L genes were the highlighted genes with which up-regulated miRNAs interact. The online database retrieved (for the 11 up-regulated miRNAs) 3202 nodes (miRNAs: 4, Targets: 3198) and 3718 edges.

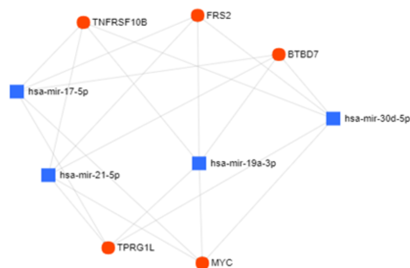

Figure 4: Target gene prediction of up-regulated miRNAs in BC cells as revealed by miRNet miRNA target prediction online tool [1].

For down-regulated miRNAs, a wide range of target genes was obtained (**Figure 5**). These target genes include GABPB2, GNB1, CCND1, ERCC1, ZNF417, GLO1, EIF4G2, DRAXIN, HOXD11, KPNA6, HSPA3, ZNF578, ACER2, CLS35F6, BDELK4, HIST1H2B, EIF4A2, COX6B1, HMGB1, HIF1A, ANKRD52, MFSD8, FOXK1, AGO3, CDKN1A, GATA6, and SLC30A7. The online database retrieved (for the 14 down-regulated miRNAs) 3079 nodes (miRNAs: 7, Targets: 3072) and 3608 edges. Two miRNAs with their target genes are shown in this study to avoid the complexity of the network retrieved using the seven miRNAs indented by the tool.

Figure 5: Target gene prediction of down-regulated miRNAs in BC cells as revealed by the use of miRNet miRNA target prediction online tool [1].

Disease correlation prediction

Besides predicting the target genes with which up- and down-regulated miRNAs in BC cells interact, disease prediction also was performed using the same tool. hsa-miR-17-5p was presented as a model for the up-regulated miRNAs (**Figure 6**).

A wide range of diseases was found to be correlated with the up-regulated miRNAs, these diseases include lymphoma, pancreatic cancer, lung cancer, prostate cancer, colorectal cancer, breast cancer, bladder cancer, and others.

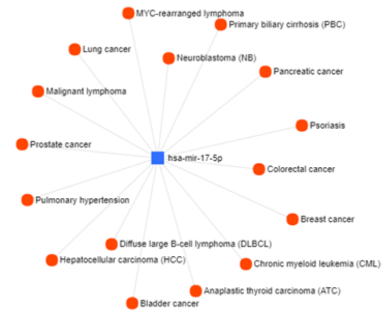

Figure 6: Disease prediction of up-regulated miRNAs in BC cells as retrieved from miRNet miRNA target prediction online tool [1].

Several diseases also were found to be correlated with the down-regulated miRNAs in BC cells (**Figure 7**); these diseases include leukemia, prostate cancer, hepatic cancer, ulcerative colitis, and others. hsa-miR-24-3p and hsa-miR-106a-5p were presented as representative of the down-regulated group of miRNAs.

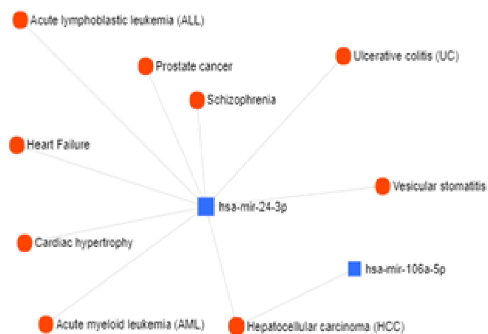

Figure 7: Disease prediction of down-regulated miRNAs in BC cells as retrieved from miRNet miRNA target prediction online tool [1].

Discussion

In the present study, 25 miRNAs were profiled in breast cancer cells (MCF-7) with the aim to identify a potential biomarker. Of these miRNAs, 14 were found to be down-regulated and 11 were found to be up-regulated in BC cells as compared to U6 snRNA as an internal control in real time PCR-based expression analysis.

Up-regulated miRNAs

Statistical analysis revealed the significant differences between these miRNAs and U6 snRNA expect hsa-miR-141-3p where a non-significant difference was obtained (**Table 1**). This

profile has been obtained earlier as Ranjha et al. [42] indicated that hsa-miR-141-3p was up-regulated in CRC, while Minn, Lee [43] indicated its elevated expression in breast cancer. hsa-miR-16-5p overexpression significantly inhibited the proliferation and colony formation in MCF-7 cells [44]. It has been indicated that apoptosis levels were significantly increased in hsa-miR-196a-5p mimic treated GC-2 cells, meaning that it is involved in the apoptotic machinery in cancer cells [45]. It could, likewise, be used as a potential cancer biomarker for digestive tract cancers [46]. The hsa-miR-17-5p and hsa-miR-21 were found to be elevated in breast cancer tissues and cell lines, where it down-regulates AIB1 gene primarily through translational inhibition. Meanwhile, hsa-miRNA-17-5p overexpression promotes cell proliferation and induces tumor growth [47-49]. The hsa-miR-30 was also found to play a role in neoplastic transformation, metastasis, and clinical outcomes in breast cancer development by controlling critical signaling pathways and relevant oncogenes. It also promotes the invasive phenotype of metastatic breast cancer cells by targeting NOV/CCN3 [50].

Several up-regulated miRNAs *i.e*., hsa-miR-103a-3p hsa-miR-21-5p, and hsa-miR-195-5p could be employed as diagnostic biomarker for breast cancer [48,51-54].

The up-regulation of hsa-miRNA-19a-3p inhibited breast and bladder cancer progression and metastasis by inducing macrophage polarization through the down-regulated expression of Fra-1 proto-oncogene *in vivo* [55], and this miRNA might exhibit the same action in MCF-7 cells *in vitro*. It also plays a suppressive role in bone metastasis of prostate cancer, which makes it a potential candidate as an effective cancer therapy miRNA [56]. The oncogenic miR-181b was shown to be highly expressed in BC cells and many malignancies such as pancreatic, head and neck, and bladder cancer [57], as it mediates TGF-β1-induced epithelial-to-mesenchymal transition in non-small cell lung cancer stem-like cells derived from lung adenocarcinoma A549 cells, and this profile makes it a potential biomarker [58].

The hsa-miR-195-5p suppresses the proliferation, migration, and invasion of oral squamous cell carcinoma by targeting TRIM14 *in vivo* [59]. It works through the inhibition of tumor progression by targeting RPS6KB1 in cancer cells [60].

Down-regulated miRNAs

In the present investigation, of the 25 miRNAs studied, 14 were found to be down-regulated in BC cells. Although these 14 miRNAs were down-regulated in BC cells, some of them were up-regulated with tumor regulatory action such as hsa-miR-194 in gastric cancer [61].

hsa-mir-30c promotes the invasive phenotype of metastatic breast cancer cells by targeting NOV/CCN3 [48], meaning it must be up-regulated to perform its function, although it was down-regulated in our study. Shukla et al. [62] also indicated that hsa-miR-30c negatively regulates NF-κB signaling and cell cycle progression through down-regulation of TRADD and CCNE1 in breast cancer. It has been indicated that hsa-miR-30 family is involved in the breast cancer development, and thus, it might serve as promising biomarkers and may bring a novel insight in molecular targeted therapy of breast cancer [63].

miR-99a has antitumor activity, and this activity is achieved by targeting the mTOR/p-4E-BP1/p-S6K1 pathway in human breast cancer cells [64], and by enhancing RAD001-induced apoptosis in human urinary bladder urothelial carcinoma cells [65]. These results were in accordance with the results obtained in the present study.

miR-133a was found to be up-regulated and negatively controls the expression of LASP1 in breast cancer cells [66], and it has potential use as a biomarker for breast cancer detection [67]. However, our investigation indicated an opposite profile for the expression of this microRNA. Nonetheless, our results were observed earlier when Wu et al. [68] indicated that miR-133a expression was associated with poor survival of breast cancer and restoration of miR-133a expression inhibited breast cancer cell growth and invasion [68].

Gao et al. [69] indicated that hsa-miR-206 represses the proliferation and invasion of breast cancer cells by targeting Cx43. This microRNA also inhibits the stemness and metastasis of breast cancer by targeting MKL1/IL11 pathway [70]. However, Zhou et al. [71] showed that it is down-regulated in breast cancer and found to inhibit cell proliferation through the up-regulation of cyclinD2, and these results are in agreement with our results.

MiR-23b is a tumor suppressor that functions by regulating Zeb1 in bladder cancer [72], cervical cancer [73], and ovarian cancer [74]. Behaving like a tumor suppressor miRNA indicates its down-regulation in cancer cells, and this profile was observed in the present study.

In cancer, loss-of-function of let-7 miRNAs has been linked to tumorigenesis *via* increased expression of target oncogenes [75]. MicroRNA let-7b regulates genomic balance by targeting Aurora B kinase [76]. Let-7, however, functions as a regulator of the ERα signaling pathway in human breast tumors and breast cancer stem cells [77], and acts as a novel suppressor by targeting the AKT2 gene [78]. Furthermore, let-7a suppresses breast cancer cell migration and invasion through down-regulation of C-C chemokine receptor type 7 [79]. These findings are in concordance with our obtained results in terms of the down-regulation of let-7 miRNAs in BC cells.

Transcriptional profiling studies of miRNA expression across tumor tissues or cancer cell lines have revealed that miR-29 is down-regulated in the majority of cancers and up-regulated in the minority [80]. It regulates ER-positive breast cancer cell growth and invasion [81]. However, other study found that enforced miR-29a expression modulates apoptosis through inhibition of MCL-1 expression in ALCL cell lines, with a concomitant tumor growth reduction. Therefore, synthetic miR-29a might serve as a potential new tool for early diagnosis of cancer [82].

miR-24-3p functions as a tumor suppressor that is involved in cancer progression by regulating cell migration and invasion [83]. It might play a key role in breast cancer invasion and metastasis, and thus, could potentially be a target for cancer

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intervention [84]. miR-24 was down-regulated in BC cells where it may function as a tumor suppressor, in our study.

miR-24-3p acted as a tumor suppressor in hepatocellular carcinoma, as it inhibited cells proliferation through regulating CCDN1 and caspase3 expression [85]. It might function as a tumor suppressor in BC cells also as it found to be down-regulated.

MiR-20b was found to promote cell growth of breast cancer cells [86] and esophageal cancer cells [87] via targeting phosphatase and tensin homolog (PTEN). However, in our study, hsa-miR-20b was found to be down-regulated in BC cells.

hsa-miR-143 was markedly down-regulated in triple-negative breast cancer [88]. However, it inhibits cell proliferation and invasion by targeting DNMT3A in gastric cancer [89] with its tumor suppressor action [90], especially in BC cells [91].

miR-145 is dual-strand tumor suppressor that functions via targeting MTDH in lung squamous cell carcinoma [92], and via inhibiting proliferation and migration of breast cancer cells by directly or indirectly regulating TGF-β1 expression [93].

Being capable to inhibit the proliferation and migration of astrocytoma cells [94], and invasion of renal cell carcinoma PAK5 [95], miR-106a-5p could be a potential tumor suppressor. This function is concordant with its low expression in BC as revealed in the present study.

Conclusion

In the present investigation, 25 miRNAs were profiled in MCF-7 breast cancer cells. Real-time PCR-based expression analysis data indicated that 11 miRNAs of the 25 studied miRNAs were up-regulated in BC cells, while 14 were down-regulated. Prediction analysis using miRNet online tool revealed that for up-regulated miRNAs were found to interact with several genes such as FRS2, TNFRSF10B, BTBD7, MYC, and TPRG1L. The down-regulated miRNAs were found to interact with a wide array of genes that includes GABPB2, GNB1, CCND1, ERCC1, ZNF417, GLO1, EIF4G2, DRAXIN, HOXD11, KPNA6, HSPA3, ZNF578, ACER2, CLS35F6, BDELK4, HIST1H2B, EIF4A2, COX6B1, HMGB1, HIF1A, ANKRD52, MFSD8, FOXK1, AGO3, CDKN1A, GATA6, and SLC30A7. Based on disease prediction, the up-regulated miRNAs (hsa-miR-141-3p, hsa-miR-16-5p, hsa-miR-196a-5p, hsa-miR-17-5p, hsa-miR-19a-3p, hsa-miR-181b-5p, hsa-miR-195-5p, hsa-miR-30b-5p, hsa-miR-103a-3p, hsa-miR-21-5p, and hsa-miR-30d-5p) could be used as biomarkers for the early detection of BC. We could conclude that hsa-miR-30d-5p, the highly up-regulated miRNA could essentially be employed as a potential diagnostic biomarker for BC. However, these data need further investigations to deeply identify the main role of this miRNA in developing/progression of breast cancer.

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Conflict of Interest

The authors declare no conflict of interests.

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