RESEARCH ARTICLE

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Tumor Suppressor miRNA-based Signatures in Triple Negative Breast Cancer: A Study Based on Big Data Analysis of Gene Expression Omnibus (GEO) Datasets and Its Validation

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Abstract

Background: Emerging evidence highlights the therapeutic potential of microRNAs (miRNAs) in cancer, positioning them as key molecular tools in personalized medicine. In this study, we aim to identify miRNAs as novel indicators of poor prognosis in Triple Negative Breast Cancer (TNBC) patients and to explore their potential therapeutic options for TNBC. **Materials and Methods:** Potent tumor suppressor miRNAs were obtained from four available datasets (*GSE38167, GSE40049, GSE86278, GSE154255*) of the Gene Expression Omnibus database comprising a total of 94 TNBC-positive and 40 normal tissue samples were analyzed using DESeq2 software. Further, TargetScan was used to predict the targets of differentially downregulated miRNAs and the functional and pathway enrichment analyses were performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics tool. The data obtained were validated by quantitative real-time PCR (qRT-PCR). Finally, survival analysis was performed in the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) cohort to check the impact of these miRNAs in TNBC patients. Results: Differential expression analysis revealed that 110 miRNAs were upregulated and 243 miRNAs were downregulated in TNBC samples compared to the normal breast tissue samples. The top five downregulated miRNAs were miR-204, miR-6068, miR-139, miR-26a and miR-215. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology analysis showed that these miRNAs are involved in various hallmarks of cancer. Further validation using qRT-PCR analysis showed that miR-204, miR-139, and miR-26a were significantly downregulated in TNBC cell lines, MDA-MB-231, MDA-MB-468 and HCC1937 compared to non-tumorigenic cell line, MCF 10A. Kaplan-Meier analysis showed that the survival rate of patients with low miR-204 expression was significantly lower compared to the miR-204 upregulated group. **Conclusion:** miR-204 can be a potential therapeutic molecule in TNBC. Strategies aimed at restoring the expression of miR-204 through miRNA replacement therapies could offer novel therapeutic approaches for TNBC patients.

Keywords: TNBC- miRNA- tumor suppressor

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs of 22-25 nucleotide length that modulate gene expression by binding to 3' untranslated regions (UTR) of messenger RNA (mRNA), leading to mRNA degradation or translational repression [1]. In recent years, miRNAs have emerged as crucial regulators of gene expression [2]. They serve as master regulators of several biological processes, including cell growth, cell cycle, differentiation, development, immune response and apoptosis [3]. Over the last several years, increasing evidence has shown the expression of several miRNAs that are dysregulated in human cancers through various mechanisms, including miRNA gene amplification or deletion, dysregulated transcriptional machinery and epigenetic changes [4]. The dysregulated miRNAs act as oncogenes or tumor suppressors and are implicated in various aspects of cancer biology, including tumorigenesis, progression, apoptosis, metastasis and angiogenesis [5].

Triple Negative Breast Cancer (TNBC) is an aggressive subtype of breast cancer lacking the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2).

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Despite advancements in cancer treatment, TNBC patients often confront poor prognosis and have limited therapeutic options compared to other breast cancer subtypes [6]. Identifying miRNAs could pave the way for developing miRNA-based targeted therapies for TNBC patients. Studies over the last several years have highlighted the potential of miRNAs as therapeutic molecules for various cancers, including TNBC [7]. Several studies have reported downregulation of tumor suppressor miRNAs, such as miR-206, miR-203, miR-770, miR-148a, and miR-200 in TNBC, suggesting their involvement in the development and progression of this aggressive breast cancer subtype [8-12].

The Gene Expression Omnibus (GEO) is a publicly accessible database curated by the National Center for Biotechnology Information (NCBI), serving as a valuable tool for scientists investigating the underlying mechanisms of several disease conditions. In the current study, we utilized bioinformatics tools to analyze gene expression profiling data from the GEO database to identify differentially expressed miRNAs in TNBC compared to normal tissues. Subsequently, we used Gene Ontology (GO) functional annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, quantitative real-time PCR (qRT-PCR), and Kaplan-Meier analysis of the top five downregulated miRNAs to identify tumor suppressor miRNAs with therapeutic potential. By integrating these analyses, our study aims to identify key tumor suppressor miRNAs downregulated in TNBC and analyze their therapeutic potential.

Materials and Methods

miRNA-Seq Datasets

The miRNA datasets GSE38167, GSE40049, GSE86278, and GSE154255 were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The consolidated dataset with 94 TNBC and 40 normal samples was selected for the study.

miRNA Expression Analysis

Differential miRNA expression analysis of miRNA-seq data was conducted using DESeq2 software. The differentially expressed miRNAs in TNBC compared to the normal tissues were identified. A volcano plot with $\log_2$ fold change ($\log_2$FC) and $-\log_{10}$ (p-value) of each miRNA was created to represent the findings visually. miRNAs with p-value < 0.05, $\log_2 FC \geq 1$ (upregulated) and $\log_2 FC \leq -1$ (downregulated) were considered statistically significant.

Functional Enrichment Analysis

The targets of the identified miRNAs were predicted using TargetScan. Further, the GO and KEGG pathway enrichment analyses of the miRNA target genes were performed using the bioinformatics tool Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/tools.jsp). The GO analysis included biological processes, cellular components, and molecular function categories. GO terms and pathways with $p < 0.05$ were considered statistically significant.

Cell Culture

The non-tumorigenic mammary epithelial cell line, MCF 10A and TNBC cell lines, MDA-MB-231, MDA-MB-468, and HCC1937 used for the study were collected from the RGCB Central Cell Repository facility. The cells were validated by STR analysis and were cultured at 37°C in a humidified 5% CO_2 incubator. MCF 10A cells were grown in Mammary Epithelial Cell Growth Basal Medium (MEBM®, Lonza) supplemented with SingleQuots® (MEGM® BulletKit®, Lonza). MDA-MB-231 were cultured in Dulbecco's Modified Eagle Medium (DMEM), high glucose supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin (5000 Units/mL)-streptomycin (5000 µg/mL). MDA-MB-468 and HCC1937 were cultured in Roswell Park Memorial Institute (RPMI), supplemented with 10% FBS and 1% Penicillin (5000 Units/mL) - streptomycin (5,000 µg/mL).

RNA Extraction and qRT- PCR Validation

Each cell line was cultured in triplicates for RNA extraction using the Trizol method as per the manufacturer's protocol (Invitrogen, USA). The quantification assessment of the extracted RNA was performed using the NanoDrop 2000 spectrophotometer. The extracted RNA underwent reverse transcription following the Mir-X™ miRNA First-Strand Synthesis Kit (Takara Bio). qRT-PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) in technical triplicates. The primers are shown in Table 1. The miRNA expression levels were normalized to U6, the normalized expression of the technical triplicates was then averaged, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

Survival Analysis

Survival curves were constructed using the Kaplan-Meier plotter online tool (http://kmplot.com/analysis/). Systemically untreated TNBC patient samples were selected from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset for the study. The log-rank test was used to analyze the relationship between miRNA expression and survival time; $p < 0.05$ was considered statistically significant.

Statistical Analysis

All quantified data represents an average of triplicate samples or as indicated. Statistical analysis was performed using a two-way ANOVA followed by Dunnett's multiple comparison test using GraphPad Prism 8.0 Software

Table 1. List of Primers

Sl.No	Primer	Sequence (5' to 3')
1	miR-204	CCTTTGTCATCCTATGCC
2	miR-6068	CTGCGAGTCTCCGGCGGT
3	miR-139	ACAGTGCACGTGTCTCC
4	miR-26a	TCAAGTAATCCAGGATAGGC
5	miR-215	TGACCTATGAATTGACAGA

(GraphPad Software, Inc., CA, USA). Data are presented as the mean ± standard deviation, and $p<0.05$ are considered statistically significant.

Results

Identification of Differentially Expressed miRNA

The analysis of differentially expressed miRNAs in TNBC using miRNA seq data from TNBC patients and normal tissue samples yielded 353 differentially expressed miRNAs, including 110 upregulated and 243 downregulated miRNAs, as shown in the volcano plot (Figure 1). miR-204, miR-6068, miR-139, miR-26a and miR-215 were the top five downregulated miRNAs identified.

KEGG Pathway and GO Enrichment Analysis of the Downregulated miRNAs

KEGG pathway and GO enrichment analysis were conducted to elucidate the functional roles of the top five downregulated miRNAs. The analysis revealed several enriched KEGG pathways and GO terms associated with the downregulated miRNAs, miR-204, miR-6068, miR-139, miR-26a and miR-215. The top 10 enriched KEGG pathways and GO terms with $p<0.05$ are correlated with various pathways and hallmarks of cancer (Figures 2 and 3). The top 10 GO enrichment terms and KEGG pathways of the downregulated miRNAs demonstrated a diverse spectrum of pathways, biological processes, cellular components and molecular functions influenced by miRNAs. The enriched GO terms highlighted their involvement in critical cellular processes such as regulation of transcription, signal transduction, phosphorylation, cell differentiation, apoptotic process, cell cycle, cell division, cell proliferation, and angiogenesis. It was found that miR-204, miR-139, and miR-26a were associated with molecular functions like protein binding, and miR-204 and miR-139 were enriched with metal ion binding, and RNA polymerase II core promoter proximal region activity. These findings highlight the multifaceted roles of the miRNAs in modulating critical biological processes and suggest their potential significance in various cellular and molecular pathways in TNBC.

qRT-PCR Validation of Downregulated miRNAs in TNBC Cell Lines

To further validate the analysis, qRT-PCR was performed for the top five downregulated miRNAs (miR-204, miR-6068, miR-139, miR-26a, and miR-215). Among them, miR-204 showed the most significant downregulation. Additionally, miR-139 and miR-26a were significantly downregulated in all the TNBC cell lines, MDA-MB-231, MDA-MB-468 and HCC1937, compared to the non-tumorigenic cell line MCF 10A (Figure 4).

Survival Prediction of miRNAs

To further validate the findings, Kaplan-Meier analysis of systemically untreated TNBC patients from the METABRIC cohort was performed to assess the overall survival (OS) probability associated with the miRNAs and explore the effect of their differential expression. The analysis showed that patients with higher expression of miR-204 had a significantly higher survival rate (Figure 5). Therefore, it can be speculated that the expression of miR-204 may have a profound impact on the survival rate of TNBC patients.

Discussion

TNBC is a highly heterogeneous disease with limited therapeutic options, underscoring the urgent need to identify therapeutic targets and predictive biomarkers [13]. Various novel strategies have been clinically evaluated in TNBC patients, such as targeting poly ADP-ribose

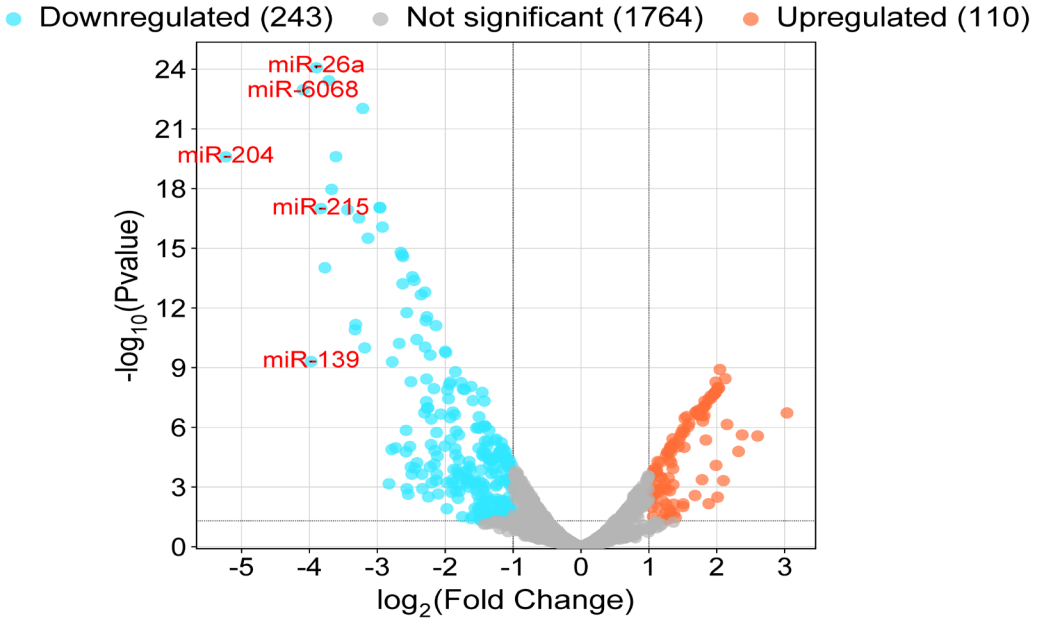

Figure 1. Volcano Plot of Differentially Expressed miRNAs in TNBC. miRNAs with p-value < 0.05, $\log_2FC \geq 1$ (upregulated, red) and $\log_2FC \leq -1$ (downregulated, blue).

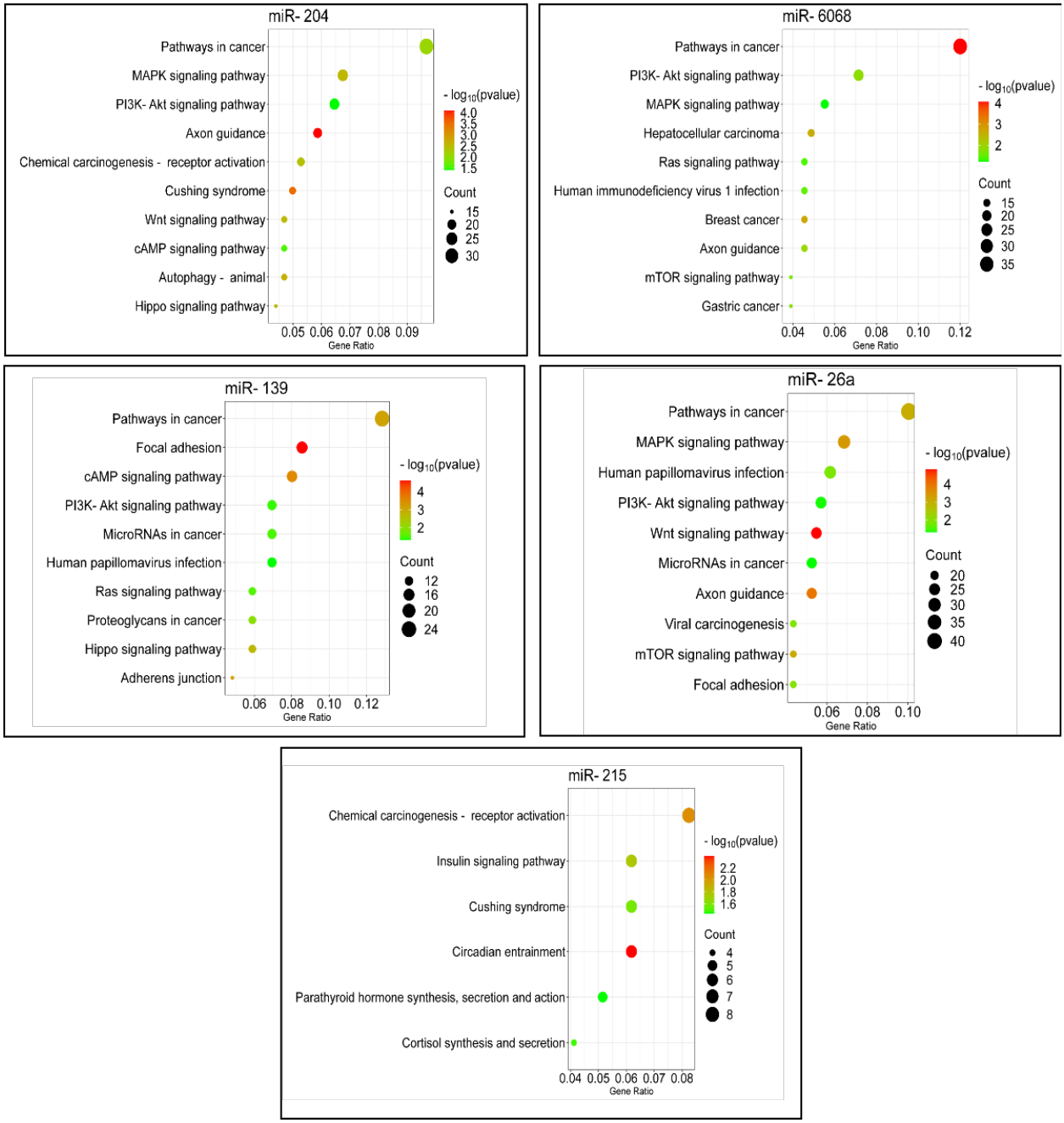

Figure 2. Bubble Plot of the Top 10 KEGG Pathway Enrichment of the Target Genes of miRNAs (miR-204, miR-6068, miR-139, miR-26a and miR-215) with p value<0.05. Bubble color and size correspond to p-value and gene count, respectively. Gene ratio indicates the number of differentially expressed genes to the number of total genes in a pathway.

polymerase (PARP), epidermal growth factor receptor (EGFR), receptor tyrosine kinases, etc [14]. Unfortunately, these treatments have resulted in significant drug resistance in patients. Tumor suppressor miRNAs play a vital role in regulating various cellular processes involved in tumorigenesis and metastasis [15, 16]. By leveraging the extensive gene expression data available in GEO datasets, we systematically identified the downregulated miRNAs that may act as tumor suppressors in TNBC, offering potential therapeutic options for this challenging disease. Three GEO microarray datasets and one deep sequencing dataset containing the miRNA expression profiles from the TNBC patients and healthy controls were chosen for differential expression analysis to identify the tumor suppressor miRNA candidates. Our study identified miR-204, miR-6068, miR-139, miR-26a and miR-215 as the top five downregulated miRNAs. Of these miRNAs, miR-215 was found to be downregulated in breast cancer [17], non-small cell lung carcinoma (NSCLC) [18] and colorectal cancer [19], whereas it is upregulated and promotes the progression of nasopharyngeal cancer [20] and gastric cancer [21]. Studies in breast cancer [22-24], nasopharyngeal cancer [25-27], NSCLC [28-30], gastric cancer [31-33] and colorectal cancer [34-36] have shown that miR-204, miR-139, miR-26a are downregulated in tumor samples compared to the normal samples and thus

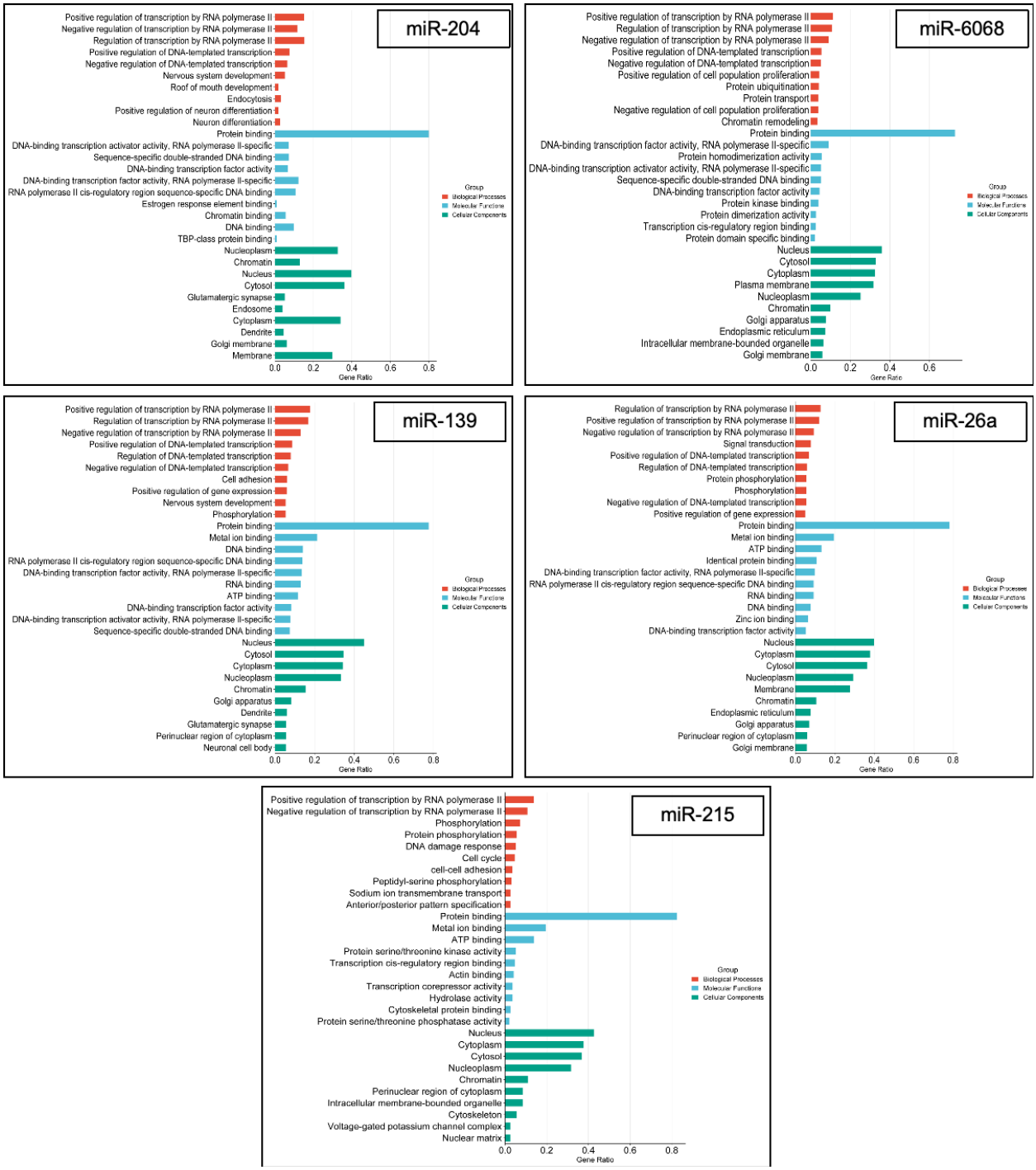

Figure 3: Top 10 GO Terms of Three Categories (Biological Processes, Cellular Components, Molecular Functions) of the target genes of miRNAs (miR-204, miR-6068, miR-139, miR-26a and miR-215) with p value<0.05. Gene ratio is the ratio of the number of genes present in this GO term over the total number of genes in this category.

indicates the therapeutic potential of these miRNAs. However, not many studies have explored the levels of miR-6068. Our subsequent qRT-PCR validation confirmed that miR-204, miR-139 and miR-26a were significantly downregulated in TNBC cell lines compared to non-tumorigenic breast cell lines. This validation underscores the potential therapeutic significance of these miRNAs in TNBC.

To understand the molecular functions of these miRNAs, miRNA targets were predicted using the TargetScan database and GO, and KEGG pathway analysis was performed using the DAVID online bioinformatics tool. The enriched GO terms revealed that miR-204, miR-139, and miR-26a are linked to key cellular processes like transcription regulation, cell cycle progression, and apoptosis. These miRNAs show associations with protein binding, with miR-204 and miR-139 also involved in metal ion binding. miR-204, miR-139, and miR-26a participate in RNA polymerase II promoter activity, which is important for regulating cell cycle dynamics in cancer cells [37-39]. Static cancer cells hinder cancer therapy by halting transcription and RNA polymerase II,

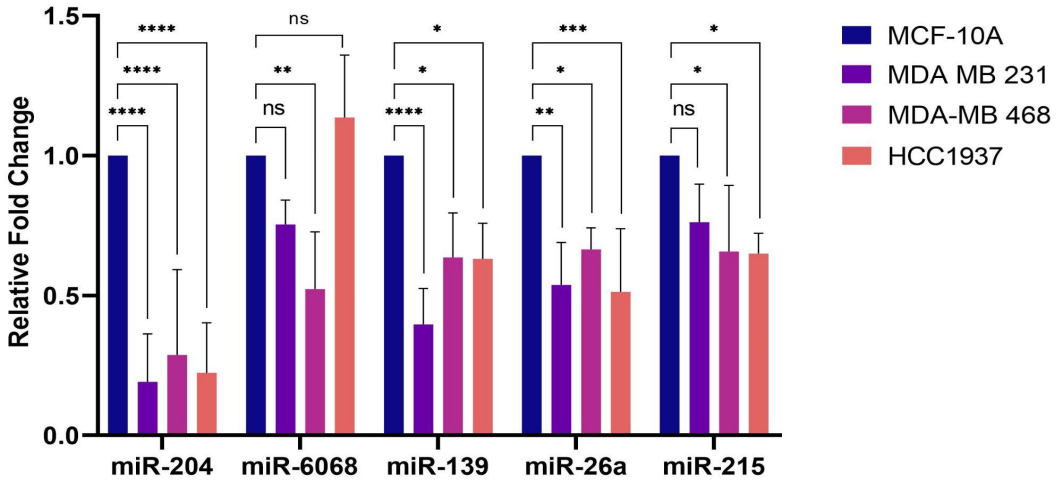

Figure 4. Validation of Differential miRNA Expression by qRT-PCR. Relative miRNA expression of miR-204, miR-6068, miR-139, miR-26a, and miR-215 in TNBC and control cell lines. Results are presented as the mean±standard deviation of three independent experiments and were analyzed by two-way ANOVA followed byDunnett's multiple comparison test using GraphPad PRISM 8.0 software (GraphPad Software, Inc). Means were considered significant if $p < 0.05$ indicated as; * $p<0.05$, ** $p<0.01$, ****$p<0.001$, ****$p<0.0001$.

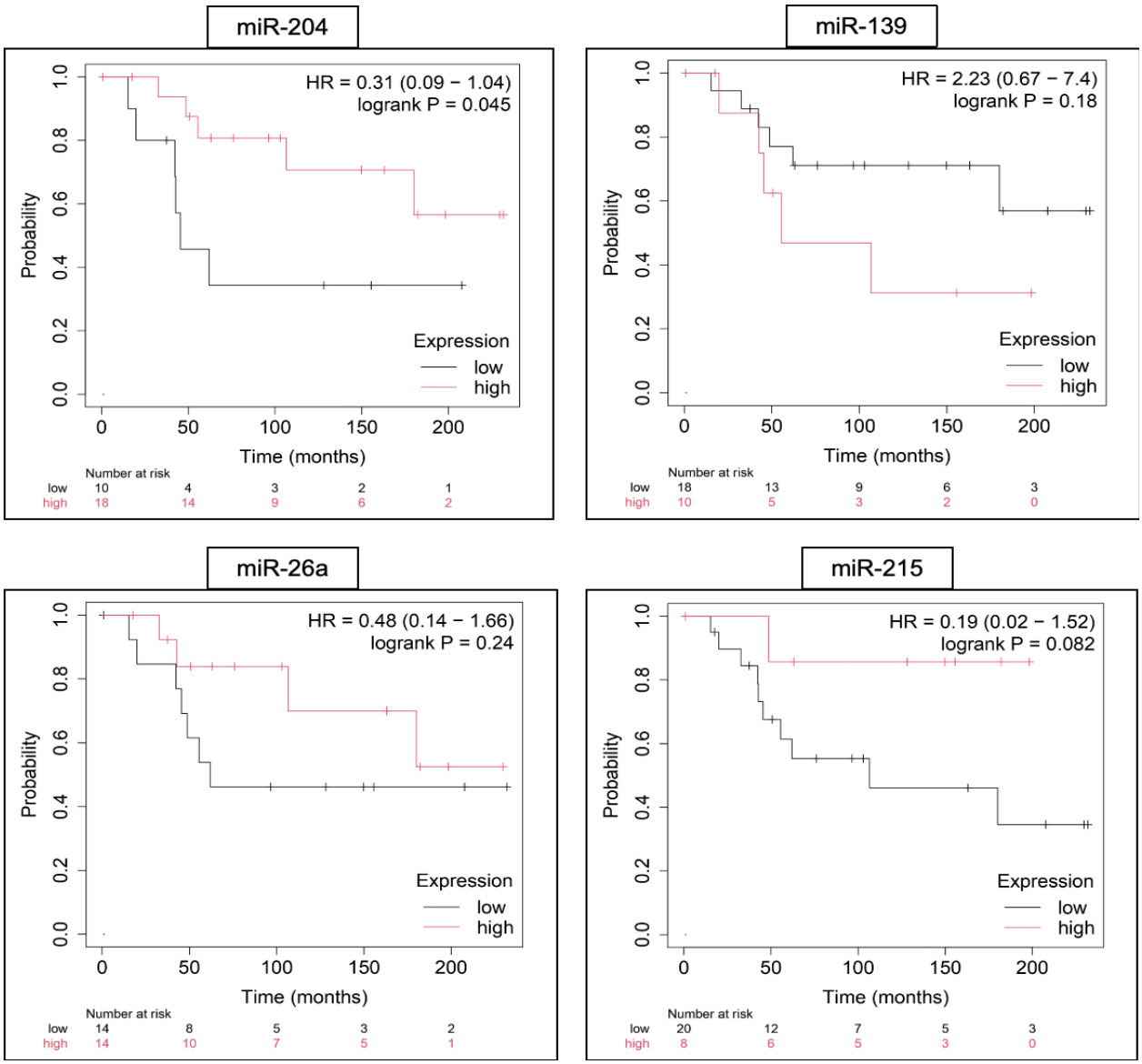

Figure 5. Kaplan-Meier Survival Analysis of the Differentially Expressed miRNAs, miR-204, miR-139, miR-26a and miR-215. Survival analysis curves with log rank p test < 0.05 were considered statistically significant.

leading to DNA double-strand breaks and chromosomal aberrations, which drive cancer progression [40, 41]. We hypothesize that regulating those identified miRNAs can disrupt this dormancy and inhibit TNBC development. Interestingly, the immune system plays a major role in miR-26a, miR-139 and miR-6068 in the pathway analysis.

It is significant to identify a miRNA signature capable of predicting survival in TNBC patients. This is the first study reporting that miR-204 could be a therapeutic option for TNBC based on patient data analysis, with patients with high miR-204 expression showing better survival outcomes. miR-26a and miR-215 showed no significant association with survival, while miR-139 was linked to poorer survival outcomes. However, miR-26a, miR-215, and miR-139 have been reported as tumor suppressors in TNBC, suggesting their roles in inhibiting tumor growth and progression in this subtype [42-44]. No data was available to ascertain the role of miR-6068 in patient survival, potentially due to its low expression levels in tumor samples or limited data points in the patient cohort for this specific miRNA. Recently, one study reported the lower expression of miR-6068 in renal cancer cell carcinoma [45], and further studies are required to understand its role in TNBC. Fewer studies have shown miR-204 as a tumor suppressor and highlighted the therapeutic potential of miR-204 in TNBC [46, 47]. Studies have shown that miR-204 inhibits tumor growth and metastasis by targeting key regulatory pathways [48-50]. This underscores the importance of miR-204 in tumorigenesis and suggests that restoring its expression could have therapeutic implications in TNBC. While these findings are promising, the limited patient cohort in our study constrains the statistical strength of the results, underscoring the need for further validation in larger, more diverse cohorts to confirm the therapeutic value of miR-204. More studies must include miR-204 in larger, TNBC-specific cohorts that could clarify its potential role as a therapeutic molecule. To our knowledge, no clinical trials have been performed to evaluate the therapeutic potential of miR-204 in TNBC.

Several limitations need to be considered in this study. Firstly, the heterogeneity of tumor samples within GEO datasets poses a significant challenge. Tumor samples collected from different patients at varying stages of disease progression and subjected to other treatments may display considerable variability in miRNA expression profiles. This inherent heterogeneity increases the risk of false-positive or false-negative results and limits the reproducibility and generalizability of findings. Variations in experimental protocols and platforms, such as microarray or RNA sequencing technologies, may lead to discrepancies in miRNA expression data. Additionally, the need for standardized protocols for data preprocessing and normalization further complicates comparing and integrating data from different studies. The heterogeneity of tumor samples within GEO datasets, differences in sample processing and miRNA profiling platforms, and the lack of matched normal tissue samples may introduce variability and bias into the results.

In conclusion, TNBC is a highly heterogeneous disease with limited therapeutic options, necessitating the identification of therapeutic targets and predictive biomarkers. Various novel strategies have been explored, including targeting PARP, EGFR, and Src tyrosine kinase, but significant improvements in patient outcomes have not been achieved. Leveraging big data analysis of GEO datasets and further validation, this study identified miR-204 as a promising therapeutic option for inhibiting tumor progression and managing TNBC.

Author Contribution Statement

Methodology and Writing – original draft: KU; Data curation: KU; Supervision, Idea, Writing – review & editing: PS and RMRK; Data analysis: KCS; All authors approve the preparation and submission of the final manuscript.

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Availability of Data

The datasets in this study are available from GEO (https://www.ncbi.nlm.nih.gov/geo/), TargetScan (https://www.targetscan.org/), and Kaplan-Meier plotter (http://kmplot.com/analysis/)

Conflict of Interest

All authors declare no competing interests

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