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Original Article

Association of hsa-miR-5571-5p Expression with Clinicopathological Factors besides Identification of its Hub Target Genes and Key Pathway in Breast Cancer

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Abstract

Background: miRNAs are small non-coding RNAs; regulate gene expression using RNA degradation or translation repression. Dysregulation of miRNAs is involved in the initiation and progression of many cancers. We aimed to determine the relationship between miR-5571-5p expression and clinical factors and regulatory mechanisms in breast cancer.

Methods: Histopathologic sections approximately with 25 microns thick from FFPE tissues were achievement of Al-Zahra Hospital (Isfahan, Iran) in 2020-2021 years by Pathologist. miR-5571-5p expression, determined using real-time PCR. For miRNA target genes prediction, integrated miRNA target prediction tools, were used. Gene ontology and KEGG pathway analysis were accomplished to identify the biological function. A PPI network was constructed to display key target genes. For hub genes validation, GEPIA databases were used.

Results: miR-5571-5p was upregulated in breast tumor tissues, and its increase was significantly related to a poor prognosis in breast cancer (P<0.0001). At first, 324 target genes were predicted, and then 110 genes with a decrease in expression were selected. GO analysis showed that genes were mainly enriched in the regulation of the ERBB2 and EGFR signaling pathway. KEGG pathway analysis suggested that downregulated genes were enriched in glioma, the ErbB signaling pathway, and breast cancer. Finally, the ten hub genes (*EGF*, *PIK3R1*, *SOS1*, *PTEN*, *SHC1*, *CBLB*, *LIFR*, *LEP*, *PDE1C*, and *NT5C2*) were detected from the PPI network.

Conclusion: miR-5571-5p up-regulation is associated with breast cancer progression and worse survival. The current study identified ten genes associated with breast cancer, which might help to provide candidate targets for the treatment.

Keywords: Breast cancer; Bioinformatics; miRNA targets

Introduction

Breast cancer is the most common malignancy in women and the leading cause of cancer death in over 100 countries (1). It is a heterogeneous disease involving multiple oncogenic biological pathways and genetic alterations (2). Early detection and monitoring of patients in response to various therapies are important aspects of breast cancer therapy (3). Moreover, it is essential to

identify the molecular mechanisms involved in central processes of breast cancer, such as tumorigenesis and metastasis (4). MicroRNAs (miRNAs) play vital roles in the initiation and progression of different cancers, including breast cancer (5). These molecules are involved in proliferation, differentiation, apoptosis, metabolism, and immune system regulation (6). It is hade predicted that Human miRNAs regulate many genes (about 30% of all human genes). Dysregulation of miRNAs is common in different types of cancers such as breast cancer (7) and occurs through various mechanisms, including amplification/deletion, epigenetic and transcriptional dysregulation and defective miRNA biogenesis (8).

miR-5571 has been reported in lung adenocarcinoma as potentially biomarkers for the prognosis. In non-small cell lung cancer (NSCLC), tissue-specific and plasma miRNAs miR-5571-5p was detected in different staging lung adenocarcinoma (9). In gastric cancer stem cells, using miRNAs profiling, miR-5571-5p identifies with down-regulation and indicates that the role of this miRNA may be tumor suppressors (10). Using microarray-based miRNA expression profiling from plasma samples of dilated cardiomyopathy (DCM) patients compared with healthy control was shown that miR-5571-5p significantly upregulated and could be a diagnostic biomarker for DCM (11). However, how miR-5571 regulates different pathways in cells and how miR-5571 targets regulate biological processes are not discussed. The identification of miR-5571 target genes would provide a better understanding of the molecular mechanism causal miR-5571 induced downstream pathway and network. In breast cancer, only microarray data about miR-5571-5p shows its dysregulation in tumoral conditions. Therefore, in current study, we aimed to determine the expression of miR-5571-5p in breast cancer tissue and key molecular mechanism and functions of miR-5571-5p.

Material and Methods

Quantitative Real-Time PCR

Tissue sample from formalin-fixed, paraffin-embedded tissues (FFPE) were provided by the Pathological Laboratory. Histopathologic sections approximately with 25 microns thick from FFPE tissues were achievement of Al-Zahra Hospital (Isfahan, Iran) in 2020-2021 by Pathologist. The samples comprised of 50 breast tumors and matched non-tumor tissues.

The study guidelines adapted to the standards set by the Declaration of Helsinki, and the sampling was approved by the research ethics committee at Shahrekord University, Iran (IR.SKU.REC.1398.005). Written informed consent was obtained from the patients to operate their specimen.

Furthermore, demographic and clinical information such as patients' age, tumor size, metastasis, and tumor grade was also provided for each sample. Afterward, total RNA was purified from FFPE specimens using MN Kit (Nucleo Spintotal RNA FFPE, Germany) in accordance with standard protocol of kit. The first strand cDNA was synthesis by stem-loop method for mature miRNA extension. Real-time PCR was performed using SYBR Green method and Mic real-time PCR cycler machine (Bio Molecular Systems, Australia). The reaction for specific miRNA (miR-5571-5p) and internal control (U6) in a volume of 10 µl contained 1µl of a specific miRNA First strand cDNA, 5 µl of SYBR Green master mix and 0.5 µl of each the forward and reverse primers.

Prediction of miR-5571-5p Target Genes

The miR-5571-5p target genes were predicted by Integrated Target Prediction of miRNA MiRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/), which identifies miRNA binding sites inside the complete gene sequence, but also combines resulting from 12 existing miRNA-target prediction programs. As well as, the potential miR-5571-5p target genes analyzed with three

other highly recognizable miRNA-target prediction tools include Targetscan7.2: https://www.targetscan.org/vert_72/, miRDB: https://mirdb.org/, and miRmap: https://mirmap.ezlab.org/. To increase the reliability of future analysis of the selected miR-5571-5p target genes, the expression of overlapping target genes evaluated in the Gene Expression Profiling Interaction Analysis (GEPIA) database (12).

Gene ontology and KEGG Pathway Enrichment Analysis

To investigation the functions of down-regulated genes, the selected 324 overlapping genes were then conducted to the Enrichr database (13) and Metascape (metascape.org/gp/index.html) GO and KEGG pathway enrichment analysis. The GO terms were included 3 sections: biological processes (BP), molecular functions (MF), cellular components (CC). $P<0.05$ was regarded as the cutoff criterion.

Protein-Protein Interaction Analysis and Screening of Hub Genes

To understand the relationship between the selected genes, the down-regulated overlapping genes were submitted to Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/) database for construct protein-protein interaction (PPI) network. Genes with a confidence score of ≥ 0.4 were selected to construct a network model. Consequently, the result was visualized using Cytoscape software (3.7.1) (14). Molecular Complex Detection (MCODE) plugin in Cytoscape was used to screen potential cluster of the PPI network, which may assistance detect probability key target genes for miR-5571-5p. The criteria were set as, the degree cutoff value to 2 and the node score cutoff to 0.2. Maximal Clique Centrality (MCC) algorithm is most effective method of finding hub nodes. The MCC of each node was estimated and designed by CytoHubba plugin. In current study, the genes with the top 10 highest MCC score were considered as hub genes.

Hub Genes Expression Level and Prognostic Value

GEPIA and the Human Protein Atlas (https://www.proteinatlas.org/) databases were utilized to explore the expression level of the top 10 hub genes in breast cancer. The expression level of each hub gene between data from TCGA and the Genotype-Tissue Expression (GTEx) project (cancer and normal tissue) was plotted as a box plot graph. Log2FC > 2 and $P<0.05$ considered as significantly different in hub genes. Prognostic value of hub genes in breast cancer, were analyzed with overall survival rate using Kaplan-Meier Plotter (http://kmplot.com/analysis/) TCGA dataset. Prognostic value of hub genes in breast cancer, were analyzed with overall survival rate using Kaplan-Meier Plotter TCGA and microarray dataset (1,402 samples) dataset (15). The upper and lower 50% of gene expression were established as the standard for analysis.

Statistical Analysis

Analyze the real-time PCR data was perfumed using $2^{-\Delta\Delta Ct}$ method. Data analysis was conducted by SPSS (ver. 22, IBM Corp., Armonk, NY, USA). The *t*-test was used to define the significance of the gene expression in tumor and non-tumor samples, as well as to compare tumor size and metastasis. The ANOVA was used to investigate the relationship between gene expression and the grade of tumor sample of breast carcinoma. GraphPad Prism ver. 7.01 software was used to confirmed statistical analysis and drawn graphs.

Results

Correlation Between miR-5571 Expression and clinicpathological factors

Relative expression of miR-5571-5p was determined in tumor and non-tumor adjustment tissue. Expression of miRNA was normalized by the internal control gene (U6). Data revealed that expression of miR-5571-5p was significantly higher in tumor tissues compared with normal

tissues (P<0.001) (Fig. 1A). A Kaplan-Meier plot was analyzed to identify the effects of miR-5571 expression on survival rate (16). Fig. 1B showed significant differences (P<0.0001) in survival between the down-regulated and up-regulated miR-5571-5p groups. The relationship between gene expression and lymph node metastasis of the breast tumors, was also evaluated (Fig. 2A). Gene expression increased in metastasis than in non-metastasis but no significant change was observed (P>0.05). In the study of the association between gene expression and tumor size, since the average size of the tumors was estimated 4 cm, the samples were divided into two groups, the tumors smaller than 4cm and those larger and equal to 4 cm. The comparison of gene expression was performed in both groups (Fig. 2B), and no statistical difference was observed in expression of miR-5571-5p between these two groups of tumors (P>0.05). As well as, no statistically significant reduction of miR-5571-5p expression was observed in various tumor grade (Fig. 2C).

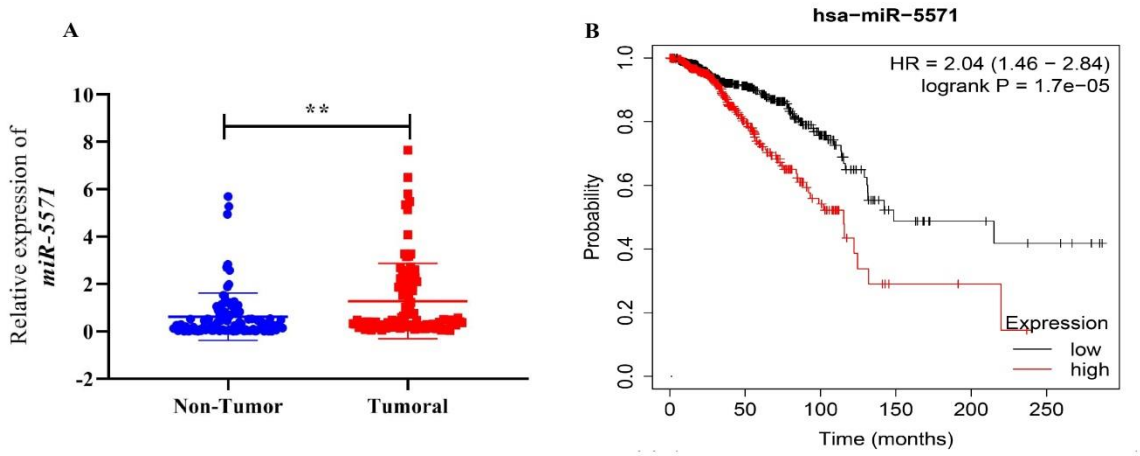

Fig. 1: A) The expression of miR-5571 in breast cancer tumor and adjust non-tumor tissue. B) Kaplan–Meier curves for miRNA-5571 in breast cancer based on TCGA data in Kaplan–Meier plotter database. The red line displays individual with high expression and blue line show cases with low expression. The x-axis indicates overall survival time (months), and the y-axis indicates the survival probability. Individual with high expression have a decrease in overall survival

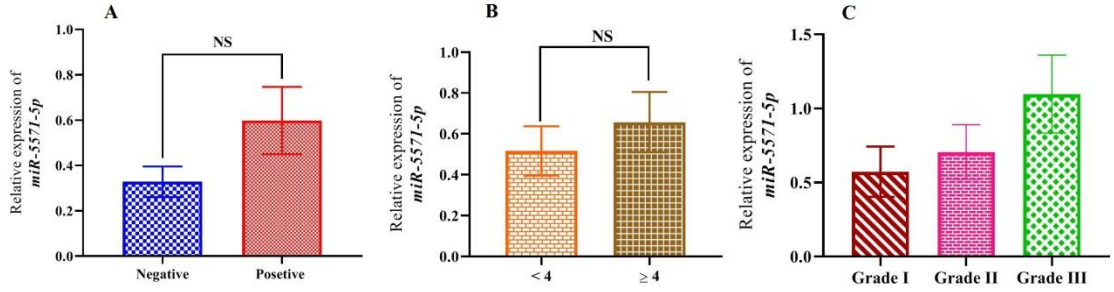

Fig. 2: Comparative expression levels of miR-5571-5p are presented for metastasis (A), tumor size (B) and various grades (C) of breast carcinoma

Predicting Target Genes

The potential targets of miR-5571-5p were identified with 3 favorable miRNA target prediction tools (miRWalk 3.0, TargetScane and miRmap). 324 overlapping genes were both predicted by the 3 tools (Fig. 3) (Supplementary Table 1: Not showed. Readers may contact authors if needed). There were 108 up- and 110 down-regulated tar-

get genes and 106 targets without alteration found on the basis of GEPIA. After intersection of the down-regulated genes in breast carcinoma were selected for future analysis and these genes might be involved in miR-5571-5p-regulated biological processes.

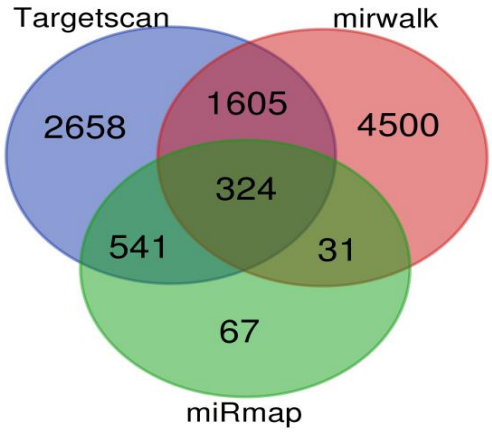

Fig. 3: Venn diagram of target genes of miR-5571-5p. Overlap targets in three databases is 324 and was used for following study

GO function and KEGG Enrichment Analyses

To further investigate the biological roles of down-regulated target genes, Enrichr and Metascape were performed to analyze functional and pathway enrichment. In Enrichr database, regarding GO BPs, 110 down-regulated targets were significantly enriched (P<0.05) in ERBB2 signaling pathway, epidermal growth factor receptor signaling pathway, and negative regulation of organ growth (Fig. 4A); in MF revealed enrichment of neurotrophin TRK receptor binding, transmembrane receptor protein tyrosine kinase adaptor activity, and I-SMAD binding (Fig. 4B); and in CC including cytoskeleton, phosphatidylinositol 3-kinase complex, and intracellular membrane-bounded organelle (Fig. 4C). KEGG pathway analysis revealed that downregulated genes were enriched in glioma, ErbB signaling pathway, breast cancer, and phospholipase D signaling pathway (Fig. 4D).

Furthermore, pathway and process enrichment analysis through Metascape database, showed that the down-regulated genes mainly participated in biological processes correlated with ERBB1pathway, embryonic stem cell pluripotency, receptor protein tyrosine kinase signaling pathway and so on (supplementary Fig. 1: Not published). Terms with a P<0.01, a count of three, and an enrichment factor >1.5 were collected and classified into clusters based on their relationship similarities.

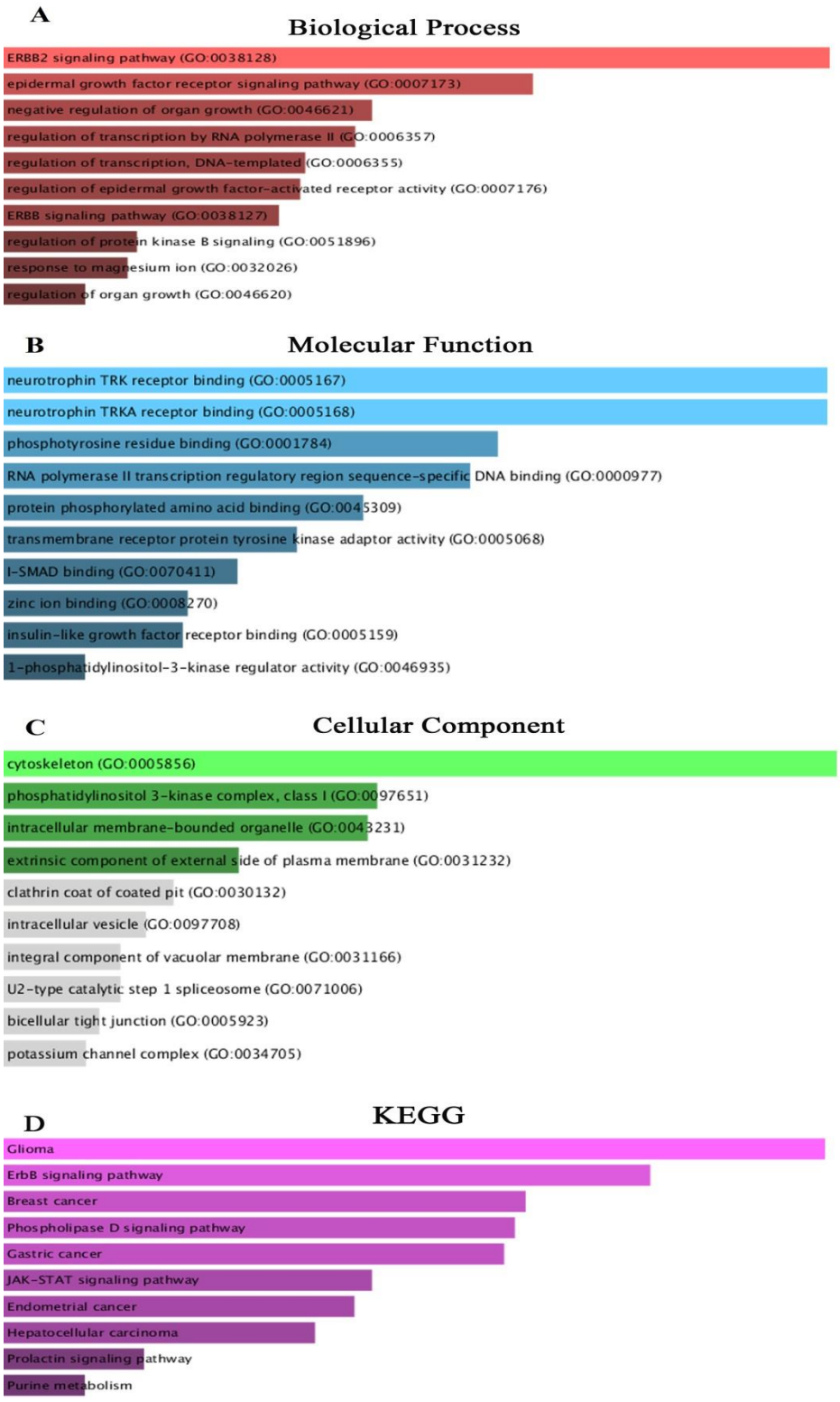

Fig. 4: GO and KEGG pathway enrichment analysis of predicted downregulated targets of miR-5571-5p. The top 10 enriched GO (A) biological processes, (B) molecular function and (C) cellular component terms as well (D) KEGG pathways

Construction of the PPI network and screening of hub genes

The down-regulated target genes were imported into the STRING database to construct the PPI network (Fig. 5A). The PPI network consisted of 110 nodes and 58 edges (average node degree of 1.05 and average local clustering coefficient=0.301). To find the potential interconnected regions in this network, MCODE plugin was utilized. A two smaller network were clustered including 5 nodes and 9 edges in cluster 1 and 3 nodes and 2 edges in cluster 2 (Fig. 5B, C). Nodes in first cluster were the son of sevenless homolog 1 (*SOS1*), phosphatidylinositol 3-kinase regulatory subunit alpha (*PIK3R1*), phosphatase and tensin homolog (*PTEN*), epidermal growth factor (*EGF*) and SHC adaptor protein1 (*SHC1*); nodes in another cluster were the cytosolic purine 5'-nucleotidase (*NT5C2*), ectonucleoside triphosphate diphosphohydrolase4 (*ENTPD4*), and phosphodiesterase1C (*PDE1C*). These genes might be central genes involved in miR-5571-5p regulated biological processes. Furthermore, the top 10 hub genes were identified based on their MCC algorithm (Fig. 6). *EGF* was the highest key gene with the maximum MCC score (score=36), followed by PIK3R1 at score= 32, SOS1 at score=30, *PTEN* at score= 26, SHC1 at score= 24, *CBLB* at score = 6, *LIFR*, *LEP* at score =4 and *PDE1C*, *NT5C2* at score=1. KEGG enrichment analysis revealed that the hub genes markedly associated with the ErbB signaling pathway, endometrial cancer, breast cancer, JAK-STAT, and FoxO signaling pathway (Table 1).

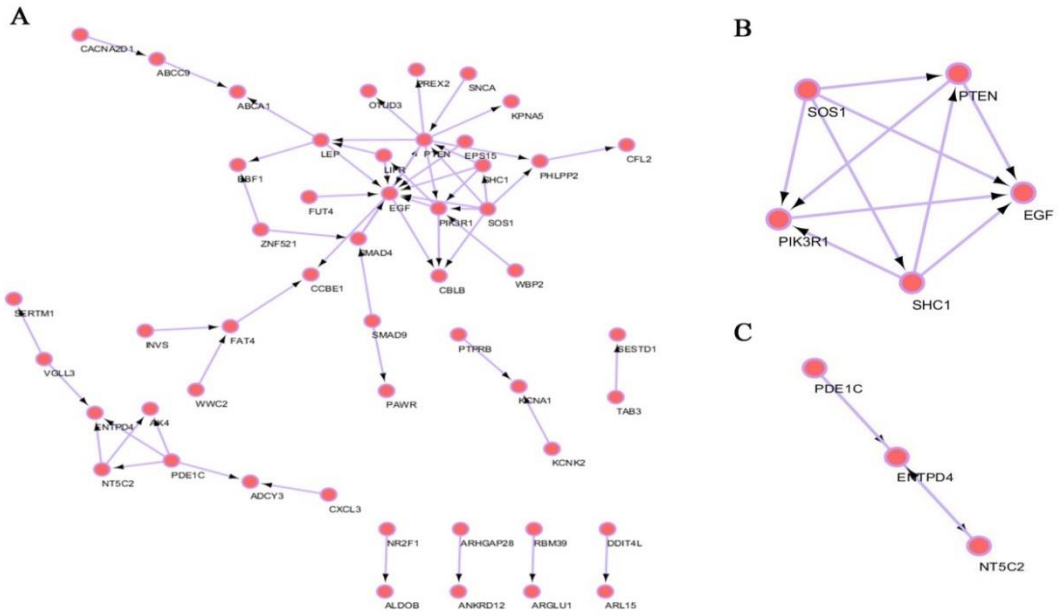

Fig. 5: The PPI network of down-regulated predicted genes. A) Network was constructed by STRING database with a medium confidence (interaction score >0.400) and visualized by Cytoscape 3.7.1 software. B and C) MCODE plugin was used to evaluate clusters in whole network

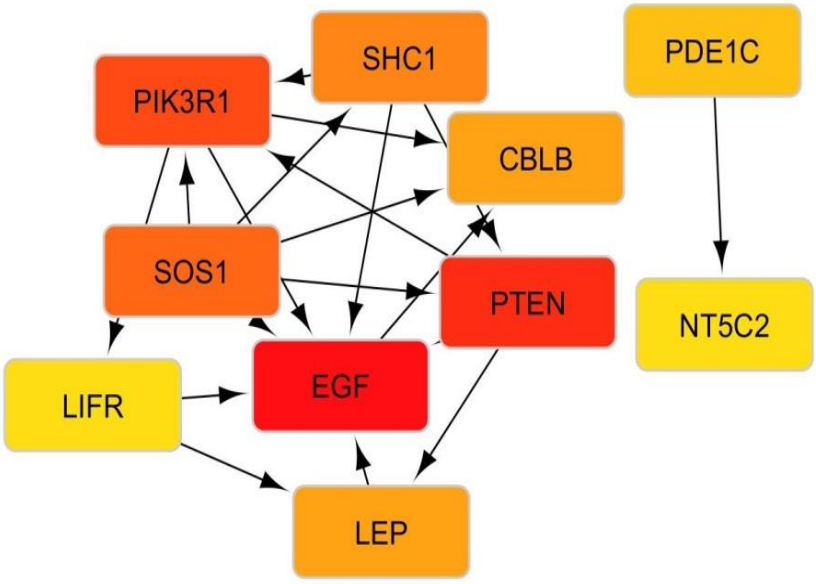

Fig. 6: The top ten hub genes in the PPI were selected by Cytoscape (v3.6.1) plugin cytoHubba based on MCC score. The identified hub genes are displayed from red (high score) to yellow (low score)

Table 1: KEGG pathway enrichment analysis of the 10 hub genes

Index	*Name*	*P-value*	*Adjusted P-value*	*Odds Ratio*	*Combined score*
1	Glioma	1.608e-10	1.814e-8	284.57	6417.37
2	ErbB signaling pathway	3.049e-10	1.814e-8	248.88	5453.09
3	Endometrial cancer	1.319e-8	3.140e-7	246.12	4465.54
4	Breast cancer	4.901e-9	1.944e-7	139.77	2674.44
5	JAK-STAT signaling pathway	7.992e-9	2.378e-7	126.32	2355.30
6	Prostate cancer	1.067e-7	0.000001815	142.63	2289.62
7	Focal adhesion	2.359e-8	4.680e-7	100.99	1773.61
8	Prolactin signaling pathway	0.000004841	0.00003921	127.44	1559.65
9	FoxO signaling pathway	3.580e-7	0.000005326	104.27	1547.61
10	Melanoma	0.000005272	0.00003921	123.73	1503.75

Expression and Confirmation of Potential Hub Genes

The GEPIA database was used to identify mRNA expression patterns of ten hub genes in breast cancer and normal tissues in the TCGA data. Ten hub genes were significantly lower in the breast tumor tissues than in normal tissues (Fig. 7). Furthermore, the protein expression levels of hub genes in breast cancer were explored using the Human Protein Atlas database (Fig. 8). Immunohistochemical analysis of breast cancer sections shown that most of the hub genes (*EGF*, *LIFR* and *PDE1C* were not available in the database) have low to moderate levels of expression. Particularly, the protein levels of *SHC1*, *CBLB* and *LEP* were highly expressed in normal breast tissues, whereas low and medium expression levels of these genes were observed in breast cancer tissues.

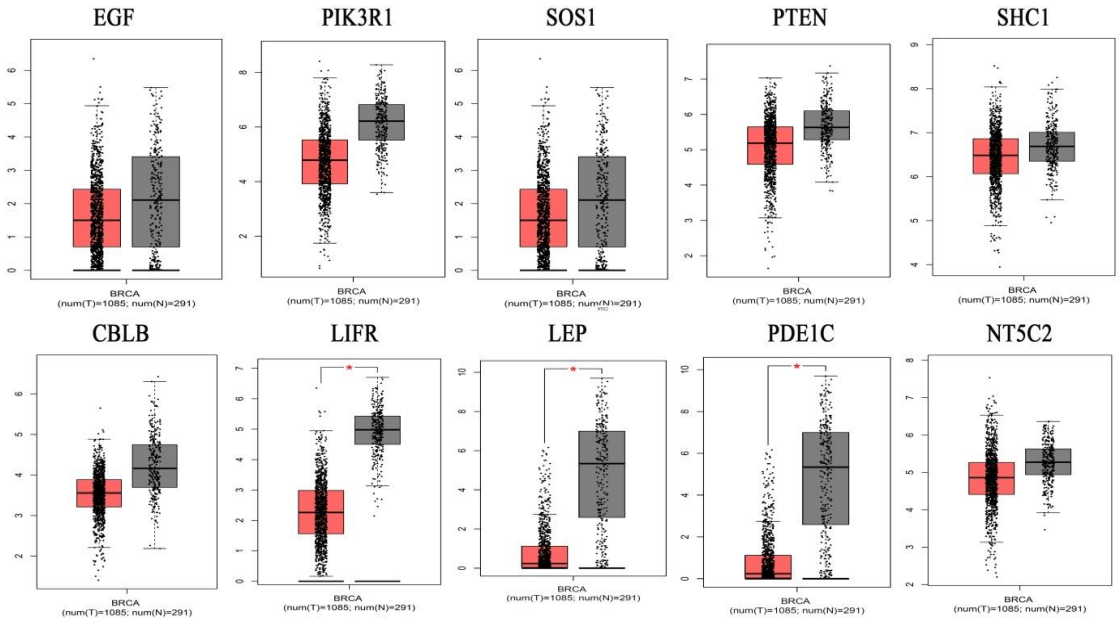

Fig. 7: Expression of hub genes in breast cancer and normal tissues, based on Gene Expression Profiling Interactive Analysis (GEPIA). Red indicates tumor tissue and gray indicates normal tissue. The box display the interquartile range of data, the middle line is the median, and the upper and lower whiskers indicate the maximum and minimum values, respectively. Each dot represents a patient sample. Red asterisks indicate $P<0.05$. BRCA, breast cancer adenocarcinoma

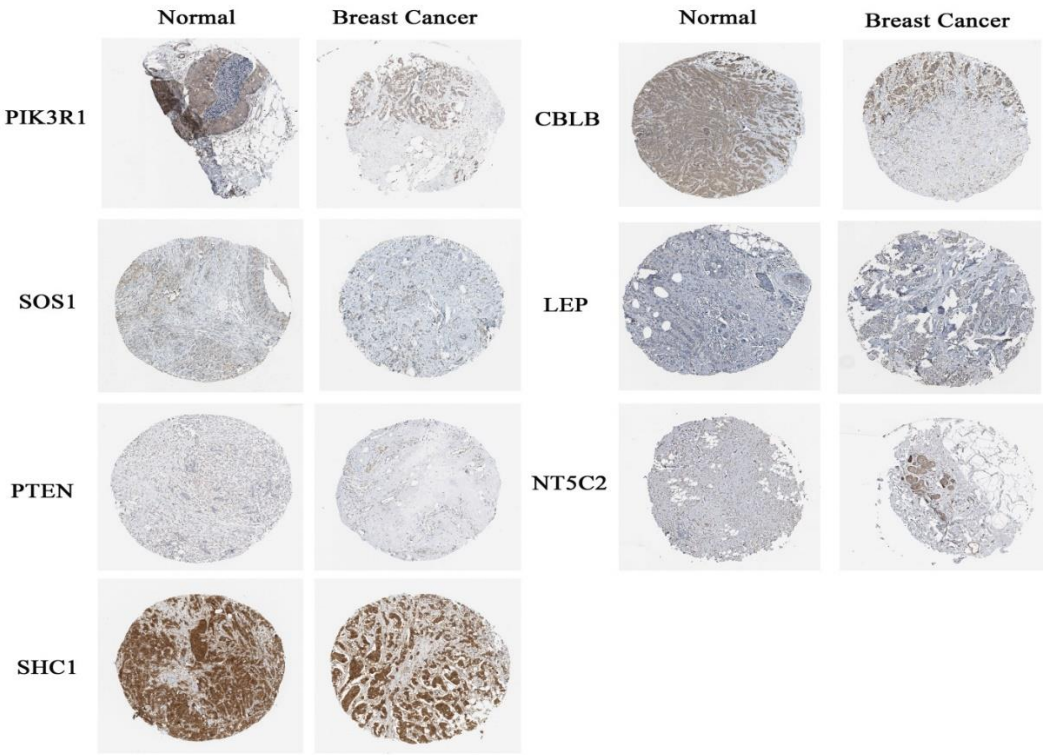

Fig. 8: Descriptive immunohistochemistry images of hub genes in breast cancer and non-cancerous breast tissues derived from the Human Protein Atlas database

Prognostic Possible of the Hub Genes

The prognostic values of the ten hub genes identified by the PPI network in breast cancer were determined by Kaplan-Meier plotter (Fig. 9). Low expressions of *EGF* (HR=0.83, *P*=0.00022), *PIK3R1* (HR=0.7, *P*=6.1e-12), *CBLB* (HR=0.68, *P*=8.4e-07), LIFR (HR=0.65, *P*=1.6e-08), and *PDE1C* (HR=0.8, *P*=1.3e-05) was negatively associated with patient survival in breast cancer; PNT5 (HR=1.27, *P*=3.4e-06) lower expression was associated with better overall survival and there was no statistical significance between the other 4 hub genes.

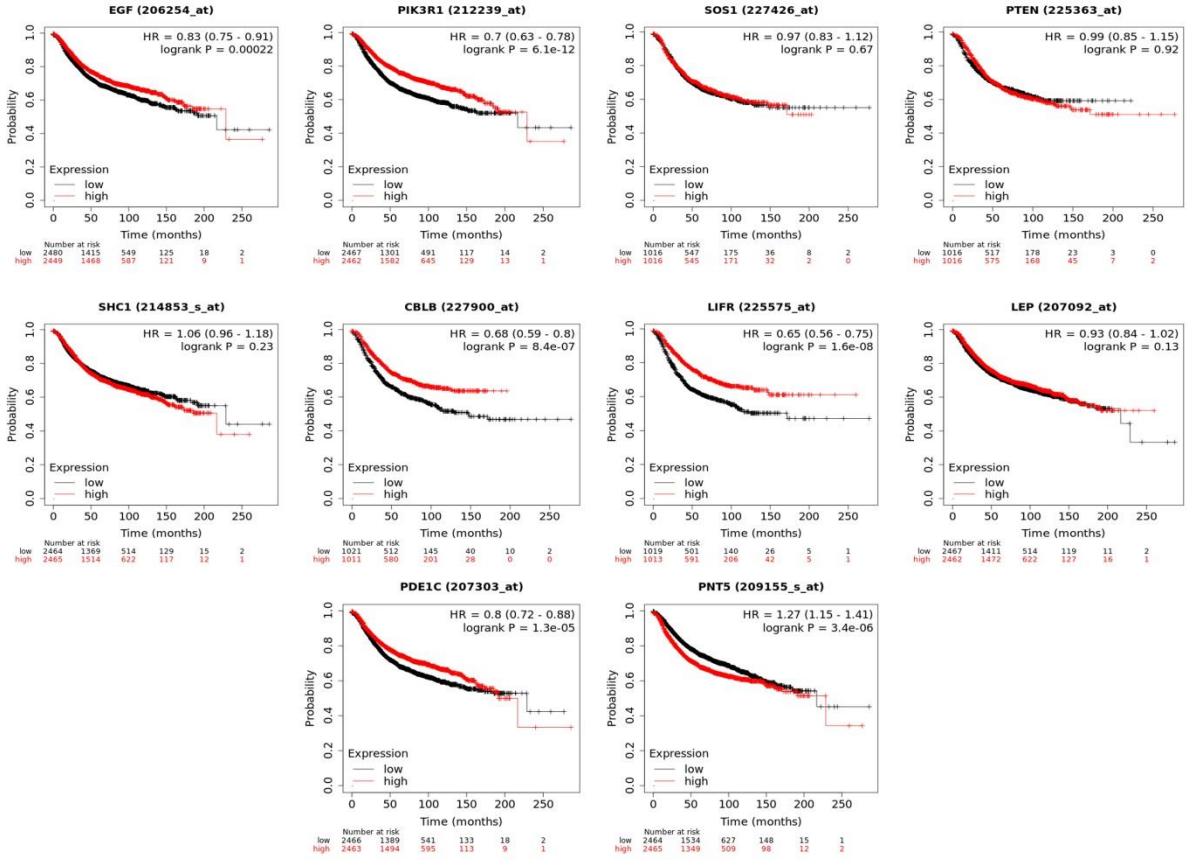

Fig. 9: The Kaplan–Meier curves of ten hub targets in breast cancer. The overall survival rate of 10 hub targets by Kaplan-Meier survival analysis based on 4929 samples downloaded from Kaplan-Meier plotter tool. The red line display individual with high expression and blue line show cases with low expression. Individual with low expression for *EGF*, *PIK3R1*, *CBLB*, *LIFR*, and *PDE1C* have a decrease in overall survival

Discussion

In this study, miR-5571-5p was upregulated in breast cancer tissues. miR-5571 is located on the q11.22 arm of human chromosome 22, that this region is involved in some malignancy including breast cancer, leukemia (17, 18). As well as, progression or inhibition of cancer in miRNA-correlated studies is depending on its main target genes in a related cell with a specific miRNA expression profile. The role of 5571 in many cancers has not been elucidated completely. In a study on lung adenocarcinoma (LUAD), based on the recognition in both tissues and plasma of patients, revealed that miR-5571-5p was a promising biomarker of LUAD with different stages (19). In a recent study, miR-5571 was predicted as the upregulator of murine double minute 2

(MDM2) in cervical cancer cells and miR-5571 up-regulation induced cell apoptosis and reactive oxygen species accumulation (20).

In the current study, we used 3 favorable miRNA target prediction tools, miRWalk 3.0, miRmap, and TargetScan 7.2 , to explore the possible target genes of miR-5571-5p. At first, we found 324 genes at the connection of the prediction of the three tools, the next step, 110 genes were selected with down-regulation in breast cancer TCGA data. To further explore the roles of target genes and the central regulatory pattern of miR-5571-5p, we used these down-regulation genes to carry out GO and KEGG pathway enrichment analysis. GO annotation items such as ERBB2 signaling, EGF signaling, transmembrane receptor protein tyrosine kinase adaptor activity, and sites of PIK3 complex and cytoskeleton were enriched. In addition, KEGG pathway analysis revealed that the glioma, ErbB signaling pathway, breast cancer, and phospholipase D signaling pathway were the most significant pathways. These results of GO and KEGG analysis indicate the potential roles of the candidate targets of miR-5571-5p in breast cancer. Following, a PPI network was constructed and the top ten hub genes were identified that had the high score of confidence, indicating that all of them might as a key regulator in miR-5571-5p pathways and phenotypes. Therefore, GEPIA and Human Protein Atlas database were used to examine the differential expression of these genes in breast cancer. Most of these hub genes have been identified as main regulators in different types of cancers such as endometrial cancer, breast cancer, prostate cancer, and melanoma.

PIK3R1 gene is a tumor suppressor, inhibited proliferation, invasion, and metastatic properties of breast cancer cells (21). *PIK3R1* was significantly down-regulated in MDA-MB-231 compared with MCF-7 cells line, thus possibly contributing to breast cancer metastasis (22). As well as, down-regulation of *PIK3R1* is associated with decreased breast cancer patient survival and poor prognosis (23). The *PTEN*, as a significant tumor suppressor gene, is involved in cell proliferation, cell cycle progression, and invasion in cancers (24). The loss of *PTEN* function was reported in up to almost half of breast cancer tumors (25). Several miRNAs are acted as an oncogenic miRNA that promoted tumorigenesis at the initiation and metastatic stage in breast cancer by *PTEN* targeting, consequently activating Wnt/β-catenin pathway (10, 26). *CBLB* is an E3 ubiquitin-protein ligase that prevented cell migration, invasion, and metastasis of breast cancer cells both in vitro and in vivo. Additionally, *CBLB* may also be a potential prognostic biomarker for the invasion and metastasis of breast cancers (27). Leukemia inhibitory factor receptor (*LIFR*) is a member of the gp130 receptor family. It becomes heterodimer with gp130 and functions as an interleukin-6 signal transducer (28). *LIFR* is downregulated in breast cancer, and it functions as a breast cancer metastasis suppressor through the Hippo-YAP pathway. Furthermore, a decrease in the expression of *LIFR* also associates with a poor prognosis of breast cancer (29). Leptin (*LEP*) is produced by adipocytes, mammary epithelium, and placenta and is known as protein hormone. *LEP* plays vital roles in metabolism regulation, immune responses, angiogenesis, and oxidation of lipids (30). *LEP* was downregulated in ductal breast cancer tissues compared to normal tissues (31). In addition, low expression in *LEP* was correlated with higher stage, lymph node status, HER2 positive, ER+, PR+, and its down-regulation was associated with poor prognosis (32). Then, the predictive biomarkers value of these genes was evaluated with ROC plotter. The results showed that the high expression of *PIK3R1*, *SOS1*, *SHC1*, and *NT5C2* might be potential biomarkers for a good chemotherapy response.

Conclusion

The study validates miR-5571-5p up-regulated in breast cancer and associated with low overall survival. Bioinformatics analysis shown that miR-5571-5p might be involved in the ERbB signaling, breast cancer and JAK-STAT signaling pathway.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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