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Research article

The endogenous association among MMP2/miR-1248/ Circ_0087558/miR-643/ MAP2K6 axis can contribute to brain metastasis in basal-like subtype of breast cancer

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ABSTRACT

Brain metastasis in basal-like breast cancer poses a significant challenge in cancer management due to its aggressive nature and limited treatment options. This study conducted a comprehensive analysis to explore the potential role of circular RNAs (circRNAs) as members of endogenous networks in developing breast cancer brain metastasis.

Here, we utilized RNA sequencing data from primary breast cancer and brain metastasis tissue with basal-like subtype (n = 11). After quality controlling and preprocessing of fastq files, gene expression of mRNA and circRNAs were extracted from matched samples and normalized. Then, we employed the weighted gene co-expression network analysis approach to identify brain metastasis-associated circRNA modules (*Spearman* Correlation > 0.5, P − value < 0.05). Moreover, we found five protein-coding genes of *PHLDA1*, *SLC12A2*, *MMP2*, *RGP1*, and *MAP2K6*, significantly upregulated in brain metastatic tissues compared to primary breast cancer (FDR < 0.05). These genes were enriched in the "GnRH signaling pathway" and "Fluid shear stress and atherosclerosis" pathways (FDR < 0.05).

Next, to explore the potential interactions between circRNAs and protein-coding genes, we reconstructed a competing endogenous RNA (ceRNA) network using mutual miRNAs between the circRNA module and upregulated mRNAs. Notably, we could detect two axes of *circ*_0087558/ miR-*604/MMP2* and *MMP2/miR-1248/Circ*_0087558/miR-*643/MAP2K6* in ceRNA network.

In conclusion, the identified circRNA-miRNA-mRNA axes might be therapeutic targets or diagnostic biomarkers for this challenging subtype of breast cancer. However, due to the small number of samples, further experimental validations are essential.

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1. Introduction

Breast cancer is one of the three most common cancers worldwide and remains the second leading cause of cancer-related death in women in the United States [1]. Approximately 90 % of cancer-related deaths are attributed to metastasis [2]. Although breast cancer metastasis has decreased and survival time for patients has improved, in patients with breast cancer, metastatic recurrence is a serious problem. According to recent observations, different tumor types display distinct organ tropisms [3]. Breast cancer metastasis organ tropism is mainly reported to the bone, lungs, liver, and brain as the primary target sites and shows diverse molecular mechanisms of metastatic heterogeneity. Nevertheless, these regulatory mechanisms remain unclear and have not been fully understood, specifically for aggressive breast cancer subtypes, such as basal-like [2].

Breast cancer is classified into five intrinsic molecular subtypes, namely Luminal A, Luminal B, human epidermal growth factor receptor 2 (HER2-enriched), Basal-like, and Claudin-low [1]. The Basal-like subtype has been previously identified as triple-negative breast cancer, which is characterized by the presence of basal and myoepithelial markers and the absence of amplification in the genes for ER, PR, and HER2. Basal-like tumors exhibit a higher incidence of brain, lung, and distant nodal metastases but a significantly lower occurrence of liver and bone metastases [4]. Notably, brain metastasis is more prevalent in breast cancer patients with Basal-like and HER2+ breast cancer [5].

The diagnosis, prognosis, prediction, and effective management of cancers, including breast cancer, heavily rely on biomarkers [6]. These biomarkers play a vital role in distinguishing between benign and malignant diseases, monitoring disease progression, detecting recurrences, and assessing therapy response [7]. Consequently, the discovery of novel biomarkers holds the potential to identify early-stage cancer progression and guide appropriate treatment strategies.

Circular RNAs (circRNAs), which are single-stranded RNA molecules, are produced through messenger RNA (mRNA) back-splicing from exonic or intronic sequences and are conserved [8,9]. CircRNAs were previously considered the result of splicing errors. Still, they are recently identified as endogenous small non-coding RNAs showing different mechanisms including, acting as competing endogenous RNAs (ceRNA), regulating gene transcription in the nucleus, and binding to coding proteins [10]. CircRNAs competitively can bind to miRNAs, subtly regulating the production of downstream target gene mRNA, thus contributing to breast cancer progression. These miRNA sponges have complementary sequences with their target mRNAs, ultimately reducing miRNA activity while increasing the expression of target genes. A diverse array of circRNAs with distinct miRNA-binding sites has been identified, offering numerous prospects for identifying breast cancer metastasis biomarkers and developing therapeutic strategies [11]. For instance, Circular RNA *circBCBM1* promotes breast cancer brain metastasis by modulating as an endogenous *miR-125a* sponge to inhibit *miR-125a* activity, and *circ_0001944* may be involved in breast cancer brain metastasis through sponging up *miR-509* and interfering with its binding to the downstream targets [12,13].

Currently, only a few studies have reported the functions of circRNAs in breast cancer brain metastasis. For example, in one study, Meng An. identified long noncoding RNA (lncRNA), miRNA, and mRNA expression profiles associated with breast cancer brain metastasis in 231-BR cells compared with MDA-MB-231 cells [14]. In this study, our research group analyzed the gene expression profiles of circRNA and mRNA in primary breast cancer tissues and brain metastasis tissues. By reconstructing the circRNA-miRNA-mRNA network using human tissue gene expression data, we successfully identified the brain metastasis module.

2. Materials and methods

2.1. Data collection

Fastq data were obtained from the Genotypes and Phenotypes database (dbGaP). The data set is a controlled access data set containing patients with basal-like primary tumors (n = 5) and brain metastasis tissue with basal-like (n = 6) subtypes from dbGaP with accession number phs000676 (Table 1) [15,16].

Table 1
Individual information.

Sample	Trait	Age range	Subtype	Sample status
A5-PRIMT030065B-RNA	primary	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A11-PT-FFPE-RNA	primary	45.5 yr(30–66yr)	basal	no treatment exposure
A15-PRIMT070427B-RNA	primary	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A20-Primary-FFPE-RNA	primary	45.5 yr(30–66yr)	basal	no treatment exposure
A23-PTcore-2009-FFPE-RNA	primary	45.5 yr(30–66yr)	basal	no treatment exposure
A11-CELEB-MET-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A11-BRAIN-MET-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A20-BrainMet-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment sample
A23-BrainMet-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A23-BrainMet-3-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment and post-radiation
A23-BrainMet-2-RNA	Brain metastasis	45.5 yr(30–66yr)	basal	post-treatment and post-radiation

2.2. Data quality control and preprocessing

The FastQC tool was applied to check the quality of the raw data [17]. In the next step, the Trimmomatic tool was utilized to trim each sample and remove low-quality bases, adapter sequences, and other artifacts that might negatively impact downstream analysis [18]. Default parameters were applied in each step.

2.3. mRNA extraction

Extraction of mRNA was gained through HISAT2 protocol. To reduce the effect of technical noise and non-biological changes in each expression data, CPM (Counts per Million) normalization was conducted. To detect differentially expressed (DE) mRNAs, we utilized the limma package [19]. The Benjamini-Hochberg method was used to determine the adjusted P-value (FDR < 0.05). We used SRPLOT for the visualization of DE mRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was conducted to ascertain the signaling cascade and GO biological processes that the DE mRNAs participate in ($FDR < 0.05$) [20,21].

2.4. Circular RNA detection

The highly precise CIRIquant tool was utilized to identify circRNAs. The YAML-formatted configuration file was prepared for the setting up of CIRIquant. In this tool, two aligners including BWA and HISAT2 were used for alignment to the reference genome. In fact, CIRIquant uses CIRI2 (which relies on BWA) for the first round of circRNA detection. Then, CIRIquant uses HISAT2 for the second round of back splice junction (BSJ) detection and false positive removal. This is the main step in CIRIquant so HISAT2 is mandatory. Due to finding gene expression matrix, StringTie as a transcript assembler, also, was applied during the CIRIquant setup [22]. In our study, hg19 was used as the reference genome. Following the extraction of circRNA expression, normalization was carried out to obtain count per million (CPM), and a filter was applied to exclude some circRNAs (rowSums < 2).

2.5. CircRNA co-expression network construction

We established the co-expression network for circRNAs using gene expression data of primary and brain metastasis breast cancer samples. Normalized data were used to reconstruct the co-expression network employing the weighted gene correlation network analysis (WGCNA) approach [23,24]. The WGCNA has several steps, including co-expression network reconstruction using the Pearson correlation and Topological Overlap Measure (TOM), extracting modules using hierarchical clustering and cutting the dendrogram, merging similar modules, and finally finding the disease-related module.

In the first step, to check outliers, the goodSampleGenes function (default parameters) was used. Then, the adjacency matrix was transformed into a more generalized form of the Topological Overlap Measure (TOM). TOM is a technique for identifying groups of genes with high connectivity [23]. After that, hierarchical clustering was implemented, and groups of circRNAs with a similar expression were identified as circRNAs modules. In this step, the "deepSplit" argument value was 4, and each module contained at least 5 genes (minModuleSize $= 5$). In the next step, we needed to calculate the module eigengene (ME), the first principle component in each module which shows the highest percentage of variance for expression values of all circRNAs in a module [25]. The MEs were calculated and association to brain metastasis trait was assessed by calculation of the Spearman coefficient correlation (SCC) ($|SCC| > .5, Pvalue < 0.05$). SCC is a non-parametric analysis which after checking the distribution of the variables was selected.

2.6. Construction of circRNA–miRNA–mRNA network

The miRNAs were predicted for the brain-metastasis-associated circRNAs and DE mRNAs using the Circular RNA Interactome database and MicroRNA Target Prediction Database, respectively [26,27]. Then, we overlapped these predicted miRNAs to find mutual ones between selected circRNAs and DE mRNAs. For the reconstruction of the circRNA–miRNA–mRNA network, the pairs of miRNA-mRNAs and miRNA-circRNA with mutual miRNAs were included. The Cytoscape 3.6.1 software was utilized to create the regulatory network involving circRNA-miRNA-mRNA interactions [24,28].

3. Results

3.1. Significant brain metastasis-associated circRNA modules

The WGCNA approach was primarily developed for the analysis of biological networks [23]. In the first step, we did not detect any outlier samples in our analyses (Supplementary Figure S1). Then, by analyzing transcriptome data obtained from primary breast cancer and brain cancer metastases, we identified groups of co-expressed circRNAs, which are referred to as modules in this study. Based on our scale-free checking, we found that the R-square value does not exceed 0.1. Therefore, we utilized a power value of $\beta = 1$, which led to the conclusion that our network is not scale-free. (Supplementary Figure S2). After merging similar modules, we identified four co-expressed modules (Supplementary Figure S3). From large to small in containing gene numbers, these modules are blue, turquoise, brown, and green, respectively (Supplementary Table S1). After examining the results, we observed that the brown module had the strongest correlation with brain metastasis, as evidenced by a significant P-value and a high SCC. Consequently, we decided to select the brown module for further analysis. The module trait heatmap for Spearman Correlation coefficients for each module was

provided in Figure 1(SCC for brown module $= -0.505, P - value 0.002$) (Supplementary Table S1, Figure 1).

3.2. mRNA analysis

Primary breast cancer and brain metastasis gene expression were employed to investigate the function of mRNAs in the progression of breast cancer brain metastasis. Our findings indicated many differentially expressed genes among which we selected five genes with a significant false discovery rate (FDR < 0.05) (Supplementary Table S2, Figure 2). The five upregulated genes are *PHLDA1*, *SLC12A2*, *MMP2*, *RGP1*, and *MAP2K6*. The results of DEG analyses were reported in Supplementary File 1.

3.3. Competing Endogenous RNA network in brain metastasis

Twelve circRNAs from the brown module (breast-brain metastasis module) of co-expression networks and five significant DE mRNAs with FDR < 0.05 were carried out to explore the association between circRNA-miRNA-mRNA (Supplementary Table S2, S3, Figure 3). MiRNAs for these circRNAs and DE mRNAs were predicted and then, we chose 12 mutual miRNAs to reconstruct the circRNA-miRNA-mRNA interaction network (Supplementary Table S4). As depicted in Figure 3, we identified two upregulated circular RNAs (circ_0004503 and circ_0087558) that share common microRNAs with differentially expressed mRNAs, including PHLDA1, SLC12A2, MMP2, RGP1, and MAP2K6.

3.4. Breast-brain metastasis biological pathways

We utilized the Enrichr webserver to perform functional enrichment analysis on the differentially expressed mRNAs, and the significant KEGG pathways and biological pathways were displayed in Fig. 4a and b. Our results indicated that the most significant pathways were the "*Gonadotropin-releasing hormone (GnRH) signaling pathway*" and "*Fluid shear stress and atherosclerosis*".

4. Discussion

Several studies have identified specific circRNAs that are dysregulated in breast cancer and are associated with breast cancer metastasis and invasion [9,29,30]. In our study, our main objective was to explore changes in circRNAs' expression and their potential effect on the development of breast cancer brain metastasis. Therefore, we used the WGCNA approach to detect brain metastasis-associated modules. We discovered twelve new circRNAs in the context of brain metastasis in breast cancer, which had not been previously documented. Additionally, we observed the upregulation of five protein-coding genes in brain metastatic tumor tissues compared to primary breast cancer. Through our analysis, we predicted the involvement of twelve mutual miRNAs in constructing the circRNA-miRNA-mRNA network. All the results were based on a small number of samples. Therefore, the experimental validations are needed.

In our study, *MMP2* and *MAP2K6* were two mRNAs that showed increased expression in brain metastasis; interestingly, these genes appeared to be regulated by *miR-1248*, *miR-520h*, and *miR-604*, and *miR-643*, respectively. According to our findings, these miRNAs could be sponged by circ_0087558. Moreover, our analysis of cellular pathways revealed that both *MMP2* and *MAP2K6* were strongly

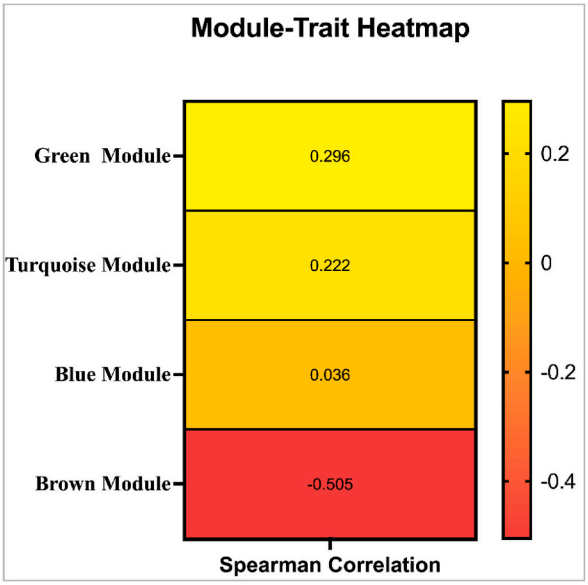

Fig. 1. Module-trait Heatmap. the values in the heatmap indicate the Spearman correlations for each module.

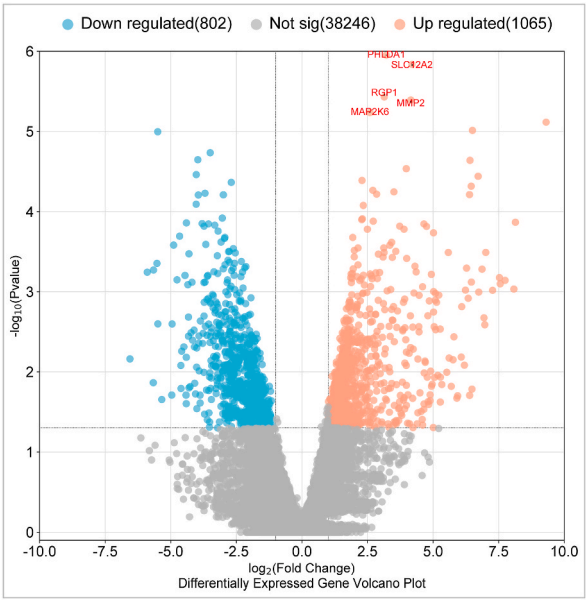

Fig. 2. Volcano plot for DEGs. The graphic compared the DEGs between the dataset's brain metastasis and primary breast cancer. These are the representations: Y-axis: log10 of a p-value; x-axis: logFC. Single genes are depicted as dots. The genes were upregulated (in red) and downregulated (in blue) (p-value <0.05). Genes with a significant False Discovery Rate (FDR<0.05) are labeled with their gene name.

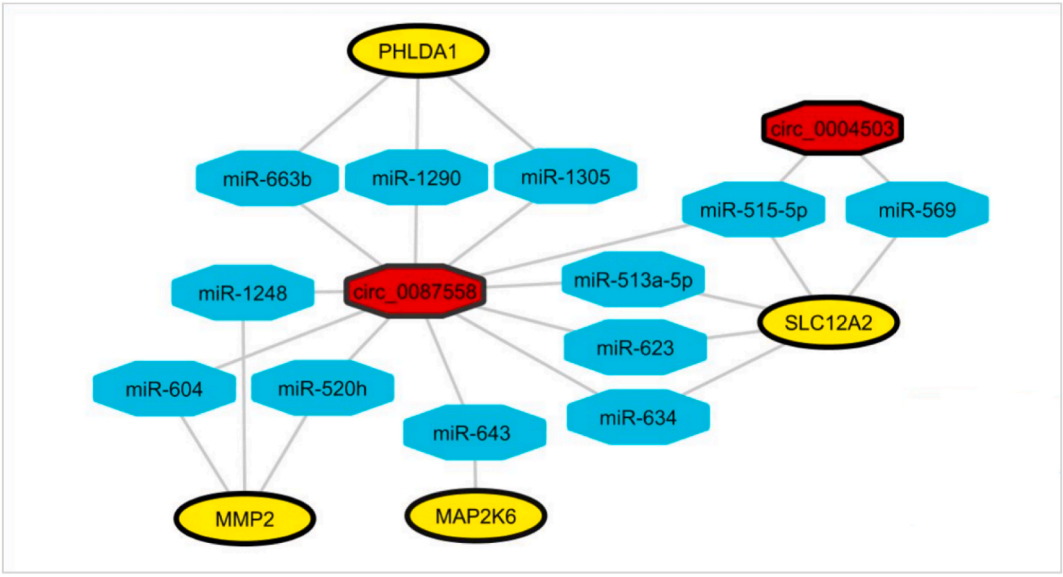

Fig. 3. The circRNA-miRNA-mRNA network. The network consists of two circRNAs (i.e., circ_0004503, circ_0087558) (red nodes), miRNAs (cyan nodes), and their target mRNAs (yellow nodes). The black border shows upregulation.

associated with the "GnRH signaling pathway" and "Fluid shear stress and atherosclerosis". Peluffo et al. also noted the connection of GnRH signaling pathway to breast cancer brain metastasis and tumor progression [31]. Past studies have found that low expression of GnRH can increase the invasion and migration of ovarian cancer cells by activating and up-regulating the *MMP2* [32]. Another study indicates that GnRH-II agonists promoted cell motility of endometrial cancer cells via the phosphorylation of ERK1/2 and JNK and the subsequent MAPK-dependent activation of matrix metalloproteinase-2 **(***MMP2*) [33].

Another pathway is fluid shear stress and atherosclerosis. The endothelial cells (ECs) that line the inner surface of blood vessels are continually subjected to shear stress due to the frictional forces generated by the flow of blood. Endothelial cells elicit a response to fluctuations in local shear stress by modulating intracellular signaling pathways. This modification, in turn, results in various alterations, such as changes in gene expression, morphological transformations of the cell, and structural remodeling. The role of *MMP2* in

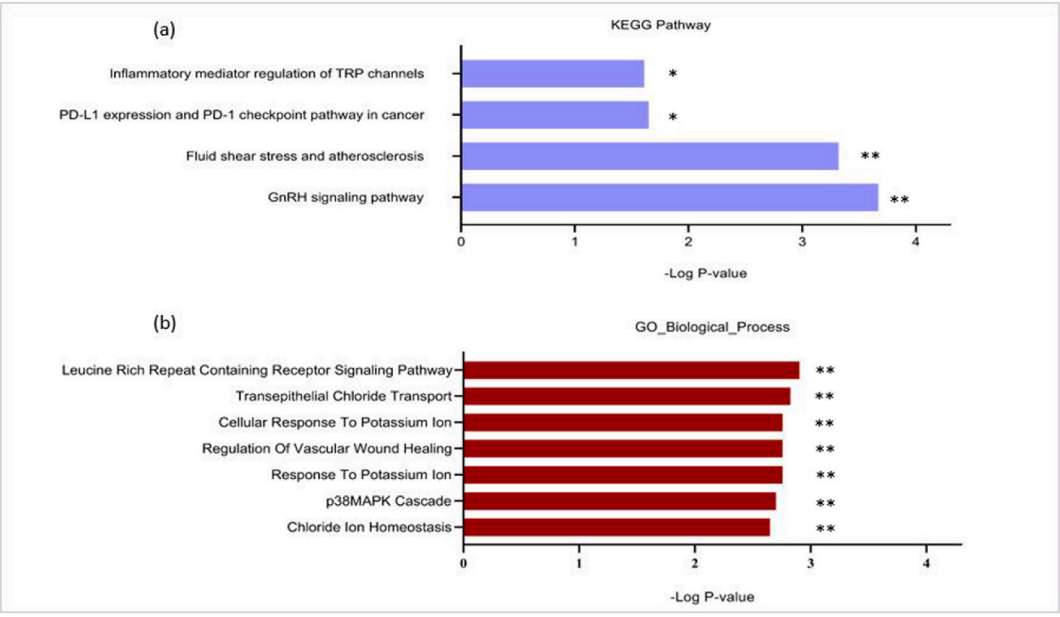

Fig. 4. KEGG pathways and biological processes analysis of DE mRNAs. (a),(b)*: p-value<0.05, **: FDR<0.05.

facilitating the invasion and migration of endothelial cells during angiogenesis has been established in various scientific literature. Overexpression of this protein has been seen in many malignancies and our study, which may help invasion and angiogenesis through the Fluid shear stress signaling pathway [34]. It also has been seen that initiation of the GnRH signaling pathway and Fluid shear stress and atherosclerosis pathway activate various MAPKs [35,36]. As a result, *MMP2* and *MAP2K6* were strongly associated with the GnRH signaling and Fluid shear stress and atherosclerosis pathway.

According to our findings, we detected the association between *MMP2*, *miR-1248*, *miR-520h, miR-604,* and circ_0087558 in the case of breast-brain metastasis in the Basal-like subtype. Various studies on lung and colorectal cancers have indicated that *miR-1248* is sponged by various circular RNAs [37–39]. We found the upregulation of *circ_0087558* in breast-brain metastasis as well as the upregulation of *MMP2.* Moreover, the downregulation of *miR-1248* in colorectal cancer metastasis was previously investigated [39]. Similarly, *miR-604*, influenced by various circular RNAs, has been tied to the progression of colorectal, gastric, and liver cancers [40]. According to all evidence from previous studies as well as our findings, we concluded that the module of *circ*_0087558/-miR-*1248/MMP2* and *circ*_0087558/miR-*604/MMP2* axis might play a significant role in breast-brain metastasis. However, experimental investigations are essential.

Combining earlier research with our discoveries, *MMP2* has a substantial impact on the metastasis of breast cancer to the brain that plays a crucial role in various processes including, morphogenesis, cancer invasion, and metastasis [41]. The upregulation or enhanced functionality of *MMP2*, in particular, is associated with the breakdown of the extracellular matrix and basement membrane, facilitating the infiltration of malignant cells into adjacent tissues and promoting metastatic spread [42]. Although the significant role of *MMP2* in breast-brain metastasis has been investigated in previous studies, the interplay of miRNAs/circular RNAs and *MMP2* as endogenous networks in Basal-like primary and brain metastasis tumors was not studied. On the contrary, our study specifically targets this subtype and investigates its regulatory connections. Nevertheless, experimental validations are essential in this context.

We noted increased levels of *MAP2K6* in brain metastasis compared to primary breast cancer in Basal-like. This might suggest a potential role for *MAP2K6* in cancer growth in the brain as a member of *MAPK*. Additionally, we found evidence hinting that *circ_0087558* could interact with *miR-643*, potentially affecting *MAP2K6* expression. *MAP2K6* influences processes like cell growth and inflammation within cells. Previous research has shown heightened levels of *MAP2K6* in various cancer types, including breast cancer, indicating its potential role in cancer progression [43]. On the other hand, *miR-643* has been identified as a potential tumor suppressor in different cancers. Its effects on cell viability, colony formation, and invasion indicate its potential role in inhibiting cancer progression [44,45]. Altogether, the upregulation of *MAP2K6* as a member of the *MAPK* pathway as well as the up-regulation of *MMP2* as a maker of breast-brain metastasis, and the up-regulation of *Circ_0087558* in our study may indicate the activation of *MMP2/miR-1248/Circ*_0087558/miR-*643/MAP2K6* in breast-brain metastasis. However, more investigations are needed to determine the role of predicted microRNAs and their association with breast-brain metastasis markers.

We also identified two genes, *PHLDA1* and *SLC12A2* that showed upregulation and have complex roles in various cancers. In estrogen receptor-positive breast tumors, elevated *PHLDA1* mRNA levels are associated with an increased likelihood of distant metastasis [46]. In this study, the expression of *PHLDA1* was found to be increased as well. This higher expression may be attributed to the action of *circ_0087558*, which acts as a sponge for miRNAs, such as *miR-1305* and *miR-1290*. Although the upregulation of *PHLDA1* is consistent with our results, experimental validations are needed for the effect of miRNAs on this gene.

Similarly, Based on recent research, *miR-663b* serves as an oncomir, and its suppression has the potential to diminish cell migration and proliferation; However, its precise role in various types of cancer remains unknown [47]. In previous studies has been reported that *miR-1305* is a tumor suppressor that can downregulate the downstream oncogenic genes [47–50]. Therefore, the upregulation of *circ_0087558* may contribute to the overexpression of *PHLDA* by the change in *miR-1305* expression. These results are in line with our findings.

Overall, our findings indicate the presence of possible regulatory subnetworks that consist of Circular RNAs and microRNAs.

5. Conclusions

In this research, we effectively identified endogenous modules linked to the metastasis of breast cancer to the brain. Among them, two modules involving the interactions of *circ*_0087558/miR-*1248/MMP2* and *Circ*_0087558/miR-*643/MAP2K6* stand out as potentially more significant. This is because both *MMP2* and *MAP2K6* were found to be in pathways associated with breast-brain metastasis. Moreover, upregulated genes were enriched in the GnRH signaling pathway" and "Fluid shear stress and atherosclerosis" pathways (FDR < 0.05). Finally, All the results in this study were based on bioinformatic analyses of Basal-like primary samples and brain metastasis. According to the small number of samples in our study as well as limited studies of circular RNAs on basal-like breast cancer subtype, there would be an essential need for further experimental validations.

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Samane Khoshbakht: Writing – original draft, Visualization, Methodology, Formal analysis. **Fatemeh Zomorodi Anbaji:** Writing – original draft, Visualization, Formal analysis. **Mohammad Darzi:** Formal analysis, Data curation. **Rezvan Esmaeili:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

ceRNA	competing endogenous RNA
circRNA	Circular RNAs
dbGaP	Genotypes and Phenotypes database
HER2-enriched	human epidermal growth factor receptor 2
CPM	Counts per Million
DE	Differentially expressed
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
FDR	false discovery rate
GnRH	Gonadotropin-releasing hormone
ME	module eigengene

WGCNA	weighted gene correlation network analysis
lncRNA	long noncoding RNA
SCC	Spearman coefficient correlation
MMP2	matrix metalloproteinase-2
TOM	topological overlap matrix

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33195.

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