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Original Research Article

Role of miR-301b-3p/5p in breast cancer: A study based on the cancer GenomeAtlas program (TCGA) and bioinformatics analysis

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ABSTRACT

Background: Breast cancer is one of the most common cancer type of women in the world. miR-301b-3p/5p were paired miRNAs derived from the same pre-miRNA, which may have different clinical roles in tumor and requires more exploration and research.

Methods: In order to investigate the differential expression, clinical significance, diagnostic and prognostic value of miR-301b-3p/5p and explore their function in breast cancer, we extracted information of miRNAs from TCGA data sets for clinical correlation analysis, and the potential function was explored by GO、KEGG enrichment and immunoinfiltration analysis.

Results: miR-301b-3p/5p were both highly expressed in breast cancer, there is a positive correlation between them. miR-301b-3p and miR-301b-5p have different clinical features. In breast cancer, miR-301b-3p can be used as a potential diagnostic marker while miR-301b-5p can be used as a prognostic molecule. GO, KEGG enrichment and immunoinfiltration analysis reveals that miR-301b-3p focuses on molecular functions, miR-301b-5p focuses on regulation of angiogenesis, and it is correlated with immune cells.

Conclusions: miR-301b-3p and miR-301b-5p are both tumor promoter in breast cancer, miR-301b-3p can be used as a potential diagnostic marker, while miR-301b-5p can be used as a prognostic molecule and an underlying therapy target. Although miR-301b-3p/5p is a pair of miRNAs from two arms of the same pre-miRNA, they may promote the progression of breast cancer together through different pathway.

1. Introduction

Breast cancer is one of the most common cancer type of women worldwide [1]. The incident of breast cancer in 2020 has topped that of new cancer cases in women around the world [2]. It is particularly important to study the pathogenesis of breast cancer because of its increasing incidence. MicroRNA is a series small RNAs widely present in animals and plants, they were found to exist in a large number of tissues of various animals, plants and humans, regulating various physiological and pathological processes. Widespread discovery of miRNAs in various organisms, as well as the discovery and confirmation of important biological functions, have led to the increasing knowledge of the important role of non-coding miRNAs in life. Dysfunction of microRNA is one of the manifestations of breast cancer and leads to dysregulation of tumor suppressors and oncogenes during breast cancer progression.

Even in the past, it was thought that only one strand could become mature during the formation from one pre-miRNA to miRNA, and then played a role in vitro or in vivo, while the other strand was degraded, which called miRNA*(miRNA star) at one time. With the progress of technology, we have found that mature miRNAs derived from either arms of the same pre-miRNA may both play important roles in the subsequent regulation of protein transcription, and we now distinguish them by marking -3p or -5p at the end of their names, depending on which arm they came from. Researchers have found that the regulation of miRNA on tumor cells is far more complex than expected, the same miRNA may play a dual role in the genesis and development of tumor cells [3–5]. even two mature miRNAs derived from the same precursor have different or opposite roles in the occurrence and development of tumors, either as oncogenes or as tumor suppressor genes [6].

Studies have shown that miRNA-5p and miRNA-3p in the diseased samples show different nucleotide composition, guanine(G) is dominant in miRNA-5p (over 32.82%), and cytosine (C) is the main nucleotide in miRNA-3p [7], the expression profile of miRNA can be modulated by arm selection and/or arm switching to dynamically adjust miRNA to

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adapt to functional and/or evolutionary stress. miRNA-5p and miRNA-3p have relatively independent expression patterns and target mRNAs data sets, which may also contribute to arm selection or arm switching phenomenon.

However, the involvement of miRNA arm selection preferences in breast cancer remains unclear. Mature miRNAs from two arms of the same precursor may play different roles in tumors, some of which are oncogenes and the others are tumor suppressors. Whether this phenomenon is an accidental behavior or a universal one, and what the possible mechanism of this disequilibrium phenomenon is still unclear. miR-301b-3p/5p are paired miRNAs derived from pre-miR-301b, whether they have the same roles in breast cancer or not is still unknown, which requires more exploration and research.

2. Materials and methods

2.1. Pan-cancer analysis of miR-301b-3p/5p and the relation between them in breast cancer (TCGA)

TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) has a lot of high throughput data on cancer including RNA and miRNA in pan-cancer, helping people know more about the comprehensive analysis of the expression of them in various cancer types. In our study, we obtained miR-301b-3p/5p expression of various types of human cancers and their adjacent normal tissues from TCGA pan-cancer project. Expression of miR-301b-3p and miR-301b-5p in breast cancer were from miRNAseq of level 3 BCGSC miRNA Profiling in TCGA invasive breast cancer project, spearman correlation test was used to analyze the molecular correlation between them, $p < 0.05$ was considered statistically.

2.2. Clinical significance of miR-301b-3p and miR-301b-5p in breast cancer

Expression of miR-301b-3p and miR-301b-5p including their clinical information in breast cancer were from miRNAseq of level 3 BCGSC miRNA Profiling in TCGA invasive breast cancer project. In clinical data table statistics, chi-square test was used when each group met the theoretical frequency >5 and total sample size >40; otherwise, fisher exact test was used. Ggplot2 (RRID:SCR_014601) was used to make visualizations. $p < 0.05$ was considered statistically.

2.3. Diagnosis significance and survival Kaplan-Meier of miR-301b-3p/5p in breast cancer

Expression of miR-301b-3p and miR-301b-5p in breast cancer, including their clinical information and their prognostic information all came from miRNAseq of level 3 BCGSC miRNA Profiling in TCGA invasive breast cancer project. First, log2 transformation was performed on miRNAseq data, then R (version 3.6.3) [8] and Prism7 were used for statistical analysis; survival package of (version 3.2–10) was used for statistical analysis of survival data; timeROC (version 0.4) was used for time-dependent ROC curves; Mann-whitney *U* Test was used for unpaired comparison; Kruskal-Wallis test was used for intra-group comparison; survminer (version 0.4.9) was used to make KM survival curve; pROC (version 1.17.0.1) was used for ROC curves; $p < 0.05$ was considered statistically.

2.4. Prediction of miRNA target genes

miRDB (miRDB.org) , Targetscan (TargetScanHuman 7.1) and miRwalk (http://mirwalk.umm.uni-heidelberg.de/) are all online databases or website. miRDB (RRID:SCR_016509) is an database for miRNA target prediction and functional annotations [9]; Targetscan is a website that predicts biological targets of miRNAs by searching for the presence of conserved 8mer, 7mer, and 6mer sites that match the seed region of each miRNA [10]; miRwalk stores predicted data including experimentally verified miRNA-target interactions. In our study, these three websites were all used to predict the target genes of miR-301b-3p and miR-301b-5p, and got common target genes by venn (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.5. GO 、KEGG enrichment and immunoinfiltration analysis

GO (Gene Ontology) is a very useful method for our daily large scale of functional enrichment research. GO analysis can be classified into different gene functions, including biological process (BP), molecular function (MF), and cellular component (CC). KEGG (Kyoto Encyclopedia of Genes and Genomes) [11] (RRID:SCR_012773) is also widely used nowadays, which often applies to genes functional annotation to understand related functions and pathways of genes, and it stores plenty data about biological pathways, diseases and chemical substances. In our study, we used DAVID (https://david.ncifcrf.gov/) [12] (RRID: SCR_001881) to analyze genes enrichment of BP, CC, MF and KEGG pathways. $p < 0.01$ was considered statistically.

Expression of 26 immune cell in breast cancer and the expression miR-301b-3p and miR-301b-5p were all from miRNAseq of level 3 BCGSC miRNA Profiling in TCGA invasive breast cancer project [13]; ssGSEA (immunoinfiltration algorithm) in GSVA (version 1.34.0) was used in immunoinfiltration analysis [14]. $p < 0.05$ was considered statistically.

3. Results

3.1. miR-301b-3p/5p were both highly expressed in invasive breast cancer

When we analyzed the expression of miR-301b-3p and miR-301b-5p in pan-cancer, we found that they were both highly expressed in invasive breast cancer. Furthermore, in bladder urothelial carcinoma, cervical squamous cell carcinoma and adenocarcinoma, esophageal carcinoma, head and neck squamous cell carcinoma, renal clear cell carcinoma, renal papillary cell carcinoma, hepatocellular carcinoma, lung adenocarcinoma, lung squamous cell carcinoma, prostate cancer, gastric cancer and endometrial carcinoma, they were also high expressed. However, they had low expression in thyroid cancer (Fig. 1A). miR-301b-3p and miR-301b-5p are both highly expressed in breast cancer. Spearman's correlation analysis showed that there was a positive correlation between them. (Fig. 1B, $r = 0.520$, $p < 0.001$).

3.2. Expression of miR-301b-3p/5p with different clinical factors in breast cancer

3.2.1. miR-301b-3p with different clinical factors in breast cancer

Based on statistical data in TCGA, we analyzed the differential expression of miR-301b-3p with different clinical factors, results showed that the differential expression of miR-301b-3p was related to the race, histological type, ER and PR status and genotype of breast cancer patients. miR-301b-3p was low expressed in Asian population and Luminal A type of breast cancer patients, while it was highly expressed in patients with invasive ductal carcinoma, ER or PR receptor negative breast cancer, and left-side breast cancer. Analysis of overall survival rate (OS), progression-free interval (PFI) and disease-related survival rate (DSS) showed that alive patients usually had a low miR-301b-3p expression.

3.2.2. miR-301b-5p with different clinical factors in breast cancer

When we analyzed expression of miR-301b-5p and their clinical feathers in breast cancer, results showed that its expression was related to patient's age, histopathological type, ER or PR status, the patients' menstrual status, and different breast cancer genotypes. miR-301b-5p was low expressed in Luminal A breast cancer patients, and high expressed in breast cancer patients younger than 60 years-old, breast

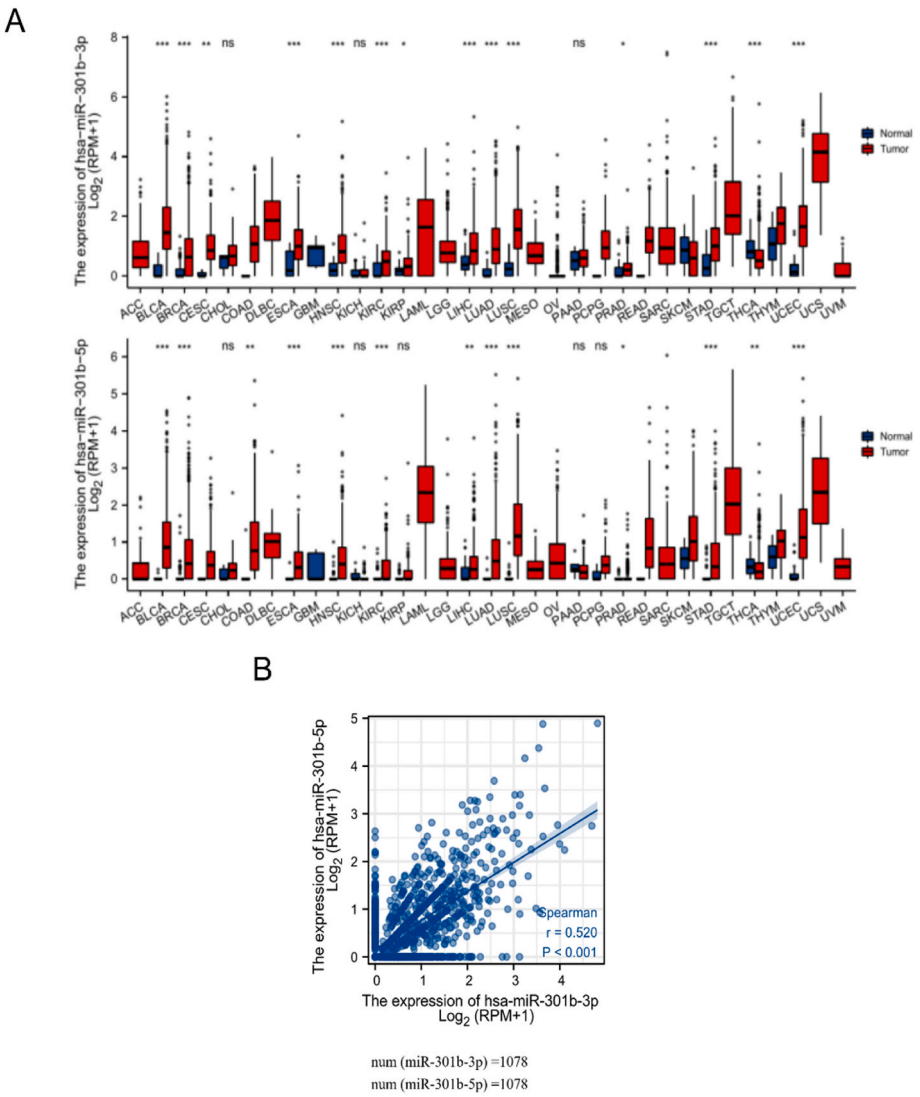

Fig. 1. A. Expression of miR-301b-3p/5p in pan-caner. B.miR-301b-3p and miR-301b-5p have a positively expression in breast cancer($r = 0.520$, $p < 0.001$)(TCGA) miR-301b-3p and miR-301b-5p have a positively expression in breast cancer ($r = 0.520$, $p < 0.001$) (TCGA) .

cancer patients with invasive ductal carcinoma, ER or PR negative breast cancer patients, premenopausal breast cancer patients, and left breast cancer patients. Analysis of OS, PFI and DSS showed that alive patients usually had a low miR-301b-5p expression either.

3.3. Visualization of clinical factors of miR-301b-3p/5p in breast cancer

miR-301b-3p/5p was highly expressed in breast cancer in our perious study, then, we performed visual analysis with different clinical factors and miR-301b-3p/5p expression. Results showed that miR-301b-3p/5p expression was higher in patients with invasive ductal carcinoma than in patients with invasive lobular carcinoma. They were highly expressed in breast cancer patients younger than or equal to 60 years-old Their expression was significantly increased in ER negative or PR negative breast cancer patients. The expression levels of miR-301b-3p/5p were significantly reduced in Luminal A breast cancer patients. OS, PFI and DSS analysis showed that miR-301b-5p was highly expressed in the dead patients. In this analysis, we could find that in the same kind of breast cancer samples, when the differential expression of miR-301b-3p/5p considered significant, the relative expression of miR-301b-3p was always higher than that of miR-301b-5p, in other words, the expression level of miR-301b-5p is much lower than that of miR-301b-3p. (Fig. 2).

3.4. Diagnostic significance of miR-301b-3p/5p in breast cancer

In order to compare the diagnostic value of miR −301b-3p/5p in breast cancer, we extracted relevant information from TCGA database for ROC analysis. Results showed that the ROC curve (AUC) of miR-301b-3p was 0.785, and that of miR-301b-5p was 0.752. According to the analysis results, both miR-301b-3p and miR-301b-5p had good accuracy in the diagnosis of breast cancer (Fig. 3A–B). Although the expression level of miR-301b-5p is much lower than that of miR-301b-3p, it has similar diagnostic efficacy to miR-301b-3p. From the clinical point of view, miR-301b-3p has a higher expression level in the same breast cancer sample type and it may be more easily detected than miR-301b-5p. Therefore, as a potential diagnostic marker, miR-301b-3p is superior to miR-301b-5p. According to the analysis of time-related ROC curve [9], the diagnostic efficacy of miR-301b-3p is mainly in the first year, especially in the prediction of OS and DSS, but this advantage gradually disappearred as time went by, and miR-301b-3p had no significant predictive value for PFI of breast cancer (Fig. 3 C-E).

3.5. Prognostic significance of miR-301b-3p/5p in breast cancer

We analyzed the expression and prognosis of miR-301b-3p/5p in breast cancer [9] and then made KM curve. The original follow-up lasted

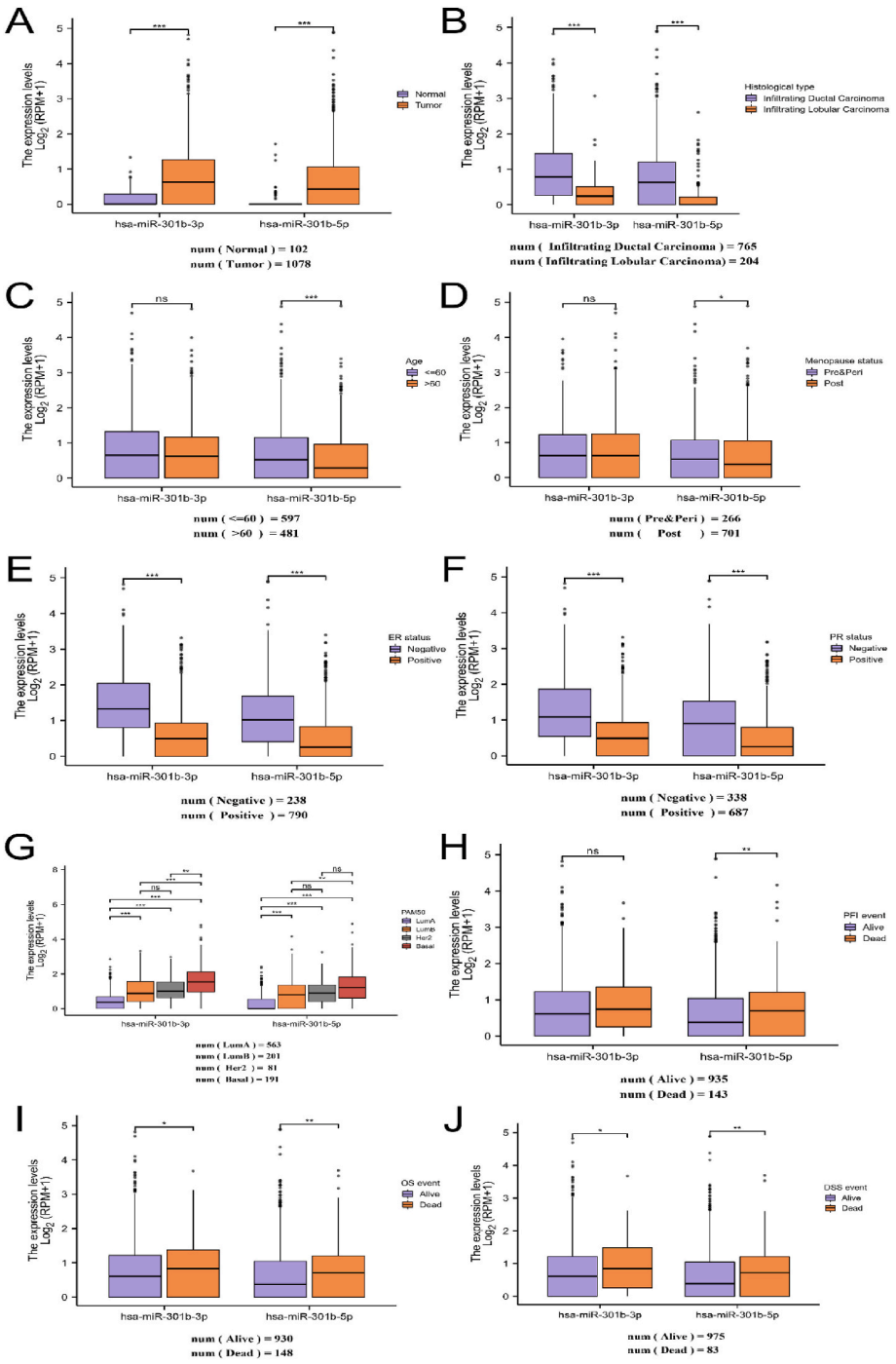

Fig. 2. Expression of different clinical factors in breast cancer (TCGA) .

for about 25 years, including 538 cases with miR-301b-3p low expression and 539 cases with miR-301b-3p high expression, while median survival time was 142.2 months and 115.7 months respectively; 537 patients with low miR-301b-5p expression and 540 patients with high miR-301b-5p expression, while the median survival time was 219.8 months and 115.4 months respectively.

Cox regression analysis showed that the difference in survival time distribution between miR-301b-5p group with high expression and low expression can be considered statistically, the prognosis of miR-301b-5p group with high expression was worse (Fig. 4A).

Combined with different clinical factors of breast cancer, subtype prognostic analysis showed that from the perspective of age, all patients with high expression of miR-301b-5p had a poorer prognosis, and prognosis of patients over 60 years old with high expression of miR-301b-5p was worse. miR-301b-5p was originally low expressed in postmenopausal and ER-positive or PR-positive breast cancer patients. In other words, once miR-301b-5p overexpressed in this case, it would have a worse prognosis. However, in premenopausal and perimenopausal breast cancer patients high expression of miR-301b-5p had no statistical significance (Fig. 4B)

3.6. Prediction of target genes and GO, KEGG enrichment, immune infiltration analysis

Prediction of miR-301b-3p and miR-301b-5p target genes was referred to miRDB, Targetscan and miRwalk databases, the intersection

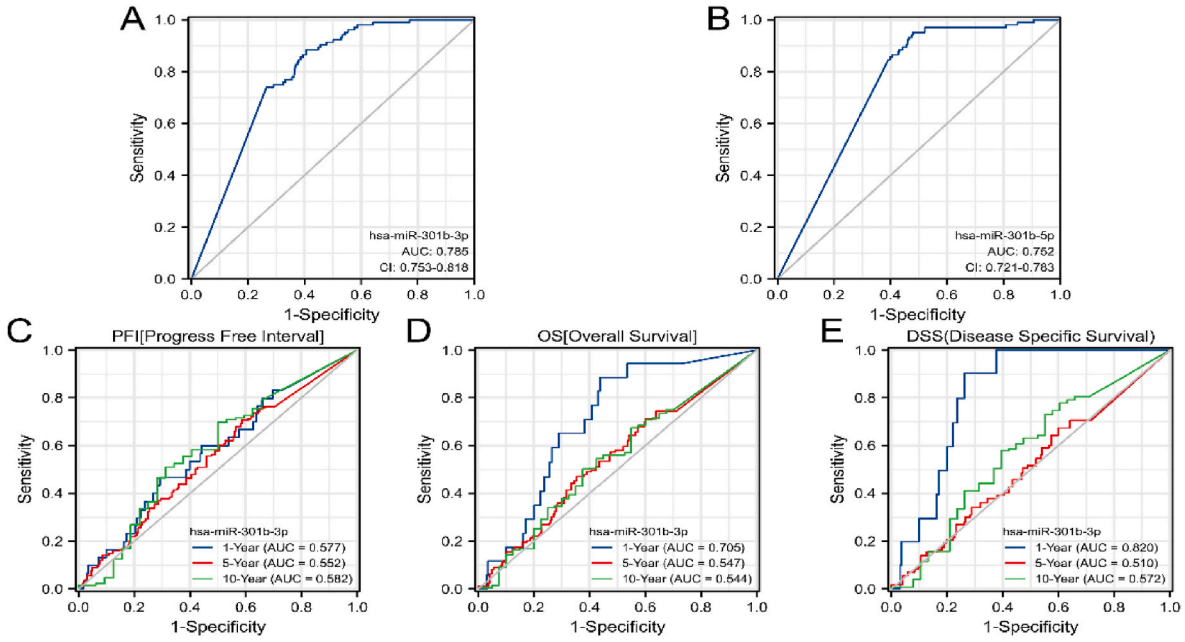

Fig. 3. A.B. ROC of miR-301b-3p/5p in breast cancer C-E. Time dependent ROC curve of miR-301b-3p in breast cancer (Normal = 102 , Tumor = 1078 , TCGA) .

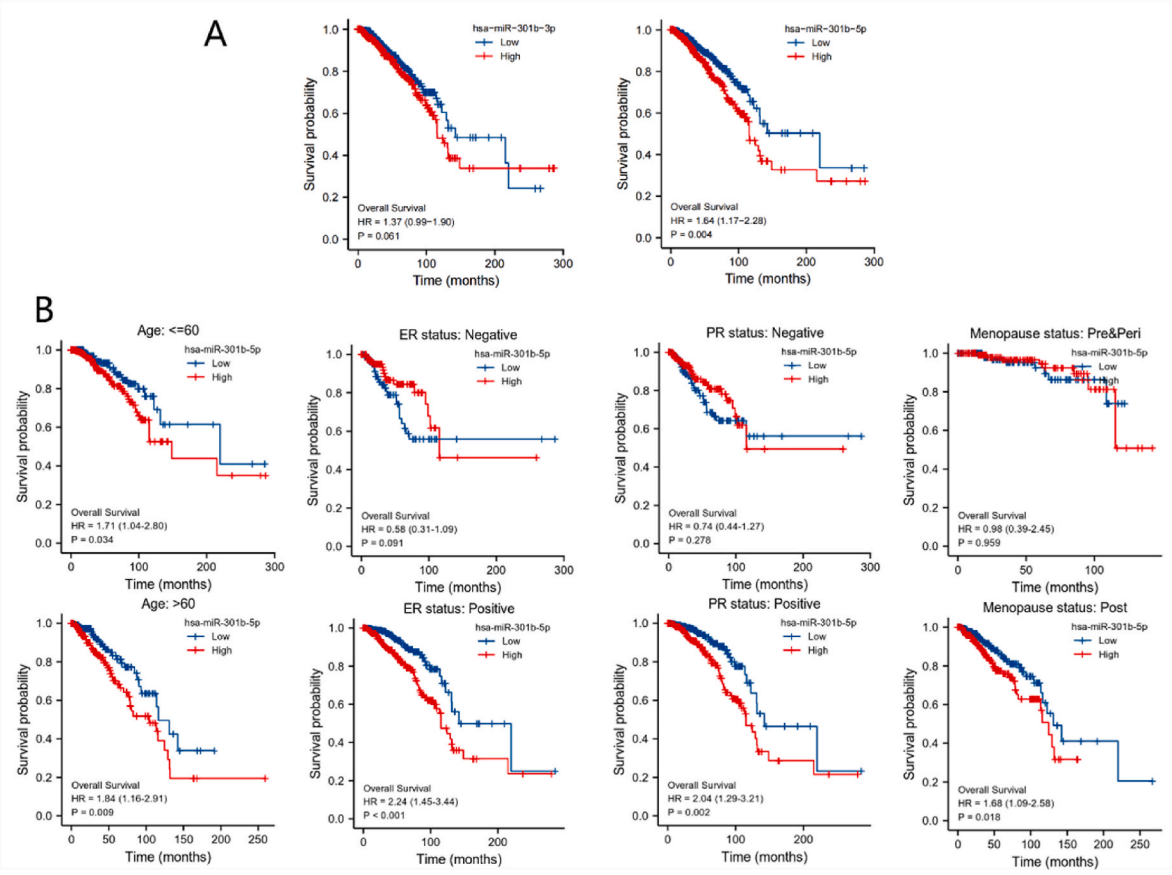

Fig. 4. Prognostic analysis curve of miR-301b-3p or miR-301b-5p in breast cancer (Cox analysis , low = 539, high = 538, TCGA).

of target genes of miR-301b-3p and miR-301b-5p was calculated respectively. Results showed that there were 17 common target genes of miR-301b-3p in these three databases, and 47 of miR-301b-5p (Fig. 5A–B). GO enrichment and KEGG analysis were performed using the predicted target genes of miR-301b-3p and miR-301b-5p respectively. According to the results of miR-301b-3p target gene enrichment analysis, GO analysis of it was mainly focused on MF, including translation repressor activity, mRNA regulatory element binding; translation regulator activity; nucleic acid binding; steroid hormone receptor activity; ribosome binding. KEGG enrichment showed

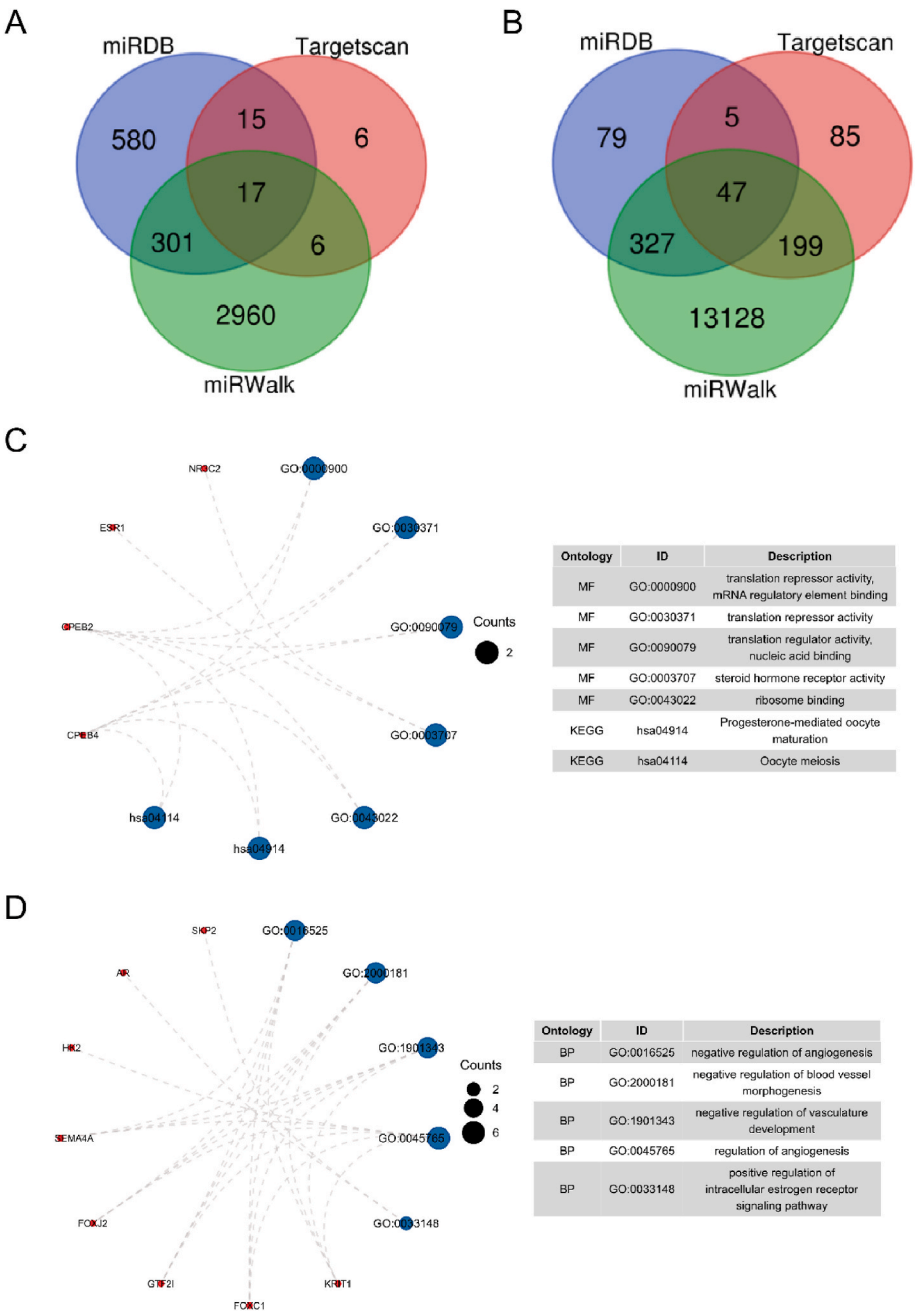

Fig. 5. A. Venn diagram of target genes of miR-301b-3p B. Venn diagram of target genes of miR-301b-5p C. GO and KEGG analysis of miR-301b-3p D. GO analysis of miR-301b-5p.

that main pathway of miR-301b-3p was progesterone mediated oocyte maturation and oocyte meiosis. The results showed that miR-301b-3p may play a role in progesterone mediated oocyte maturation and oocyte meiosis during embryo development (Fig. 5C).

According to the results of miR-301b-5p target gene enrichment analysis, GO analysis of it was mainly focused on BP, including negative regulation of angiogenesis, negative regulation of blood vessel morphogenesis, negative regulation of vasculature development, regulation of angiogenesis, positive regulation of intracellular estrogen receptor signaling pathway. There was no KEGG pathway enrichment of miR-301b-5p with these predicted target genes (Fig. 5D).

Angiogenesis is one of the characteristics of cancer, the angiogenesis of tumor cell supply oxygen and nutrients, and it maybe link with the immune system, especially the immune cells called T cells in the tumor microenvironment [15]. We analyzed miR-301-5p and immune infiltration of immune cell in breast cancer [13]. Results showed that activated dendritic cells, Gamma Delta T cells, Th1 and Th2 cells, and regulatory T cells increased when miR-301b-5p was overexpressed, while local infiltration of CD8 T cells, cytotoxic cells, eosinophils, immature dendritic cells, mast cells, neutrophils, NK CD56 (bright) cells, NK cells, plasmacytoid dendritic cells and Th17 cells decreased. Next, we analyzed correlation between miR-301b-5p and local immune cells, results showed that miR-301b-5p was positively correlated with local infiltration of Th2 cells, regulatory T cells, Th1 cells, activated dendritic cells, Gamma Delta T cells, NK CD56 (DIM) cells, B cells, macrophages and helper T cells in breast cancer, while negatively correlated with mast cells, NK cells, eosinophils, NK CD56 (bright) cells, plasmacytoid dendritic cells, CD8 T cells, Th17 cells, immature dendritic cells, cytotoxic cells, and neutrophils (Fig. 6)

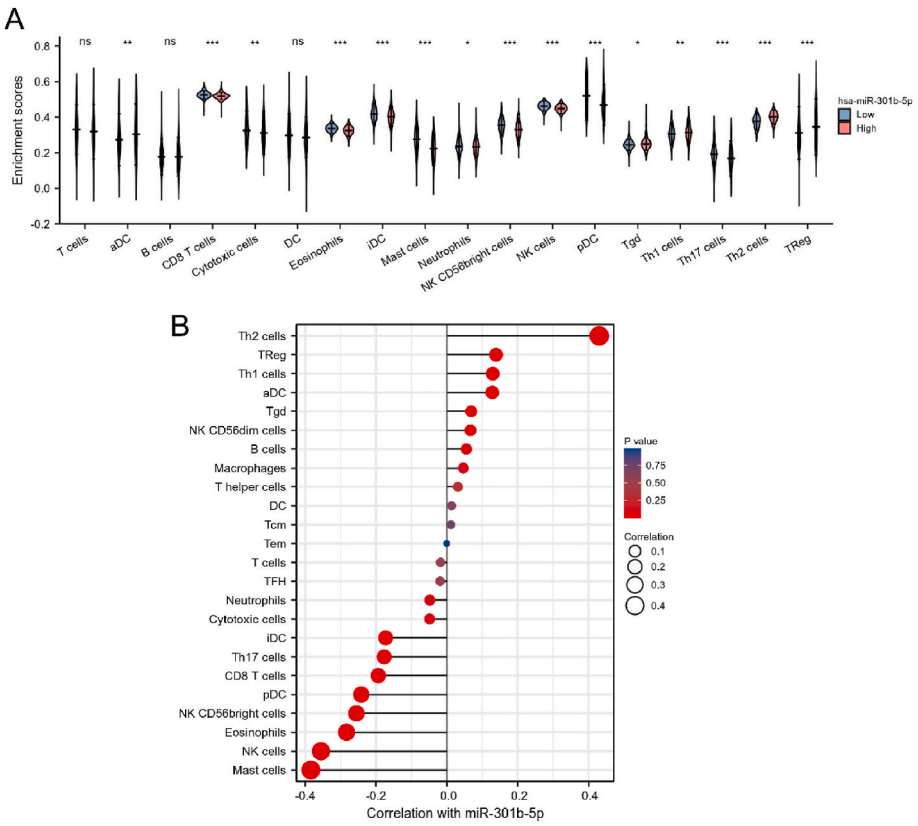

Fig. 6. A. Immunoinfiltration analysis of miR-301b-5p in breast cancer B. Correlation analysis of local immune cells with miR-301b-5p in breast cancer (High = 538 , Low = 534 , TCGA) .

4. Conclusion

miR-301b-3p and miR-301b-5p are both tumor promoter in breast cancer, miR-301b-3p can be used as a potential diagnostic marker, while miR-301b-5p can be used as a prognostic marker and an underlying therapy target. Although miR-301b-3p/5p is a pair of miRNAs from two arms of the same pre-miRNA, they have different clinical meanings and may promote the progression of breast cancer together through different pathway.

5. Discussion

Dysfunction of microRNA is one of the manifestations of breast cancer, leading to dysregulation of tumor suppressors and oncogenes in the progression of it. Dysfunction of microRNA leads to breast cancer progression, a key step in miRNA processing is the miRNA-5p and miRNA-3p arm selection preference. It is a complex mechanism that regulates the biological function of miRNAs. Although the -5p/-3p arms of miRNAs are generated by the same pre-miRNA during maturation, miRNA expression varies in different tissues, different clinical stages and different species. The selection of miRNA-5p/-3p arms is a mechanism that improves the regulation of miRNA biological functions and complicates the regulatory network of human cancer types [16–18].

As a pair derived from the same pre-miRNA, miR-301b-3p and miR-301b-5p have not been reported for joint analysis. Sequence of hsa-miR-301b-3p mature miRNA is CAGUGCAAUGAUAUUGUCAAAGC and that of hsa-miR-301b-5p mature miRNA is GCUCUGACGAGGUUGCACUACU, they have the same hairpins: hsa-mir-301b.

Previous studies mainly focused on the function of miR-301b-3p, it has been found that miR-301b-3p can participate in the transport of cholesterol-lipoprotein and regulate cholesterol and triglyceride homeostasis [19]; in liver cancer, expression level of miR-301b-3p is triggered by tumor-associated neutrophils (TANs), subsequently inhibit the gene expression of limbic system-associated membrane protein (LSAMP) and CYLD, and increase the stem cell characteristics of HCC cells [20]. In prostate, down-regulation of miR-130b/301b clusters mediated by abnormal promoter methylation can activate cellular senescence in prostate cancer [21]; in bladder cancer, USP13 is target gene of both miR-130b-3p and miR-301b-3p [22], activation of NF-KB can induce overexpression of miR-130b/301b, and the decreased expression of USP13 leads to down-regulation of PTEN protein, thereby promoting the occurrence of bladder cancer.In lung cancer, miR-301b can be induced by hypoxia and regulate the apoptosis of lung cancer cells by targeting Bim [23]. In NSCLC, downregulation of miR-301b-3p expression can be the basis of tumor inhibition by long non-coding RNA MBNL1-AS1 [24].In breast cancer, miR-301b and NR3C2 regulate the malignant characteristics of cells, and may be an independent prognostic factor of breast cancer [25]. In terms of diagnosis, circulating miR-301b combined with miR-625 can be used as markers for early diagnosis of breast cancer [26]. In terms of function, miR-301b-3p promotes the development of breast cancer cells by inhibiting target genes such as HOXA547 [27], NR3C2 [25,28] or CYLD [29]. On the other hand, there are few studies on miR-301b-5p in tumor, and only one high-throughput sequencing analysis of miR-301b showed that it may promote tumor progression by inhibiting target gene CCNG2 in triple-negative breast cancer [30].

In our study, with the breast cancer clinical analysis of miR-301b-3p and miR-301b-5p, we know that although miR-301b-3p/5p are from two arms of the same pre-miRNA, their expression and clinical significance in breast cancer are different. miR-301b-5p had a stronger prognostic value in breast cancer, high expression of miR-301b-5p had a worse prognosis, it can be used as a prognostic predictor, as well as a potential target for breast cancer treatment. miR-301b-3p/5p have a significant positive correlation. Both miR-301b-3p and miR-301b-5p can

act on hormone receptors. The difference is that miR-301b-3p focuses on molecular functions, including inhibiting and regulating the activity of transcription factors, which is the function mode of traditional miRNA. KEGG results showed the main pathway of miR-301b-3p was progesterone mediated oocyte maturation and oocyte meiosis. The function of miR-301b-5p focuses on vascular regulation of the organism, while immune infiltration analysis suggested that miR-301b-5p was correlated with a variety of immune cells. High expression of miR-301b-5p was connected with enrichment of variety immune cells, meanwhile KEGG analysis of miR-301b-5p did not enrich any pathways, therefore, miR-301b-5p may promote the progress of breast cancer by changing its local microenvironment, including invasion of immune cells. As we know, immune checkpoint inhibitors (ICIs) has revolutionized anti-tumor therapy, programmed cell death ligand 1 (PD-L1) and programmed cell death receptor 1 (PD-1) are among the most promising outcomes for encouraging the immune system of cancer patients , they have made clinical remission for solid tumors, including TNBC [31–33]. These results are helpful for us to explore the biogenesis and function of miRNAs further.

Although miR-301b-3p/5p is a pair of miRNAs from two arms of the same precursor, they may promote progression of breast cancer through different ways. It is necessary for us to study further to understand more about the arm selection preference of miRNAs, the relationship between miRNAs and immune invasion in tumor microenvironment, and it will provide a new perspective for finding new therapeutic strategies in the future.

Ethical approval and consent to participate

Not applicable.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

Data in this study was got from TCGA (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) pan-cancer project and invasive breast cancer project.

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Not applicable.

CRediT authorship contribution statement

Qian Zhou: Conceptualization, design, Formal analysis, interpretation of data, Manuscript drafting and reviewing. **Fengliang Wang:** Formal analysis, interpretation of data, Writing – original draft, reviewing. **Erhu Sun:** Formal analysis, interpretation of data, Writing – original draft, reviewing. **Xiaofeng Liu:** Formal analysis, interpretation of data, Writing – original draft, reviewing. **Cheng Lu:** is responsible for the overall content, Conceptualization, design, Formal analysis, interpretation of data, Writing – original draft, reviewing.

Declaration of competing interest

The authors have no financial disclosures or conflicts of interest.

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