Heliyon 9 (2023) e12654

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

# circAR-E2E4-miR-665-STAT3 axis is a potential regulatory network in triple-negative breast cancer

Hao Xu [a,b], Mengdie Fang [a], Bowen Zuo [a], Wanxin Yao [a], Juan Ren [a], Yanmei Zhang [a,*]

[a] School of Laboratory Medicine and Bioengineering, Hangzhou Medical College, Hangzhou 310013, China
[b] Department of Dermatology, Hangzhou Third People's Hospital, Hangzhou 310009, China

ARTICLE INFO

*Keywords:*
Circular RNA
microRNA
Androgen receptor
STAT3
Triple-negative breast cancer

ABSTRACT

Circular RNAs (circRNAs) are a novel type of endogenous non-coding RNAs (ncRNA). Many studies showed that circRNAs played different biological functions in triple-negative breast cancer (TNBC). However, the potential molecular mechanism of circRNAs in TNBC still remains to be investigated. In this study, circAR-E2E4 was defined as a novel circRNA involved in TNBC progression, derived from and regulated by androgen receptor (AR). CCK-8 assay showed circAR-E2E4 regulated TNBC cell proliferation. Potential binding miRNAs of circAR-E2E4 were predicted and miR-665 was identified to have a great prognosis value. Three databases were employed to predict target genes of miR-665, and STAT3 was regarded as the most potential downstream genes analyzed by protein-protein interaction (PPI), hub gene screening, correlation analysis, and survival analysis. Finally, knockdown of circAR-E2E4 led to the decrease of STAT3 expression. Collectively, the regulatory network circAR-E2E4-miR-665-STAT3 axis we constructed was associated with TNBC progression, providing a promising diagnostic, prognostic, and therapeutic target in future treatment for TNBC.

## 1. Introduction

Circular RNAs (circRNAs) are a class of non-coding RNAs (ncRNAs) with covalently closed loops, and without both a 5′ cap or a 3' poly-A tail. CircRNAs are more stable than linear RNAs, and not easily degraded by exonuclease [1]. CircRNAs have multiple microRNA (miRNA) binding sites and perform the function of miRNA sponge, which can relieve the inhibition of miRNA on target genes and then regulate target genes at the transcriptional level, affecting the progression of the tumor [2]. Besides, circRNAs are expressed widely in tissues and various body fluids. The copy number of certain circRNA is much higher in single cells than their parental genes because circRNAs are transcribed continuously. CircRNAs are abundant, stable, and have higher detection sensitivity, so they are ideal differential diagnostic markers for diseases and are expected to be used for diagnoses [1, 3]. CircRNAs are likely to affect diseases [4], so it is potential for circRNAs to serve as candidate diagnostic, prognostic, and therapeutic targets.

Breast cancer (BRCA) has a serious impact on the life and health of patients around the world. The current differential diagnosis of BRCA is usually based on the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and ki67 in BRCA tissues after puncture or surgery. They are classified into luminal A, luminal B, triple-negative, and

* Corresponding author.
*E-mail address:* yanmeizhang@hmc.edu.cn (Y. Zhang).

https://doi.org/10.1016/j.heliyon.2022.e12654
Received 3 February 2022; Received in revised form 13 May 2022; Accepted 19 December 2022
Available online 23 December 2022

HER2-positive subtypes [5, 6]. However, triple-negative breast cancer (TNBC) lacks effective therapeutic targets. The rapid growth of the tumor, poor prognosis, and relatively high incidence rate of recurrence and metastasis all make it essential to find a novel molecular target and regulatory network to overcome TNBC [7]. Androgen receptor (AR) plays an important role in the development of BRCA [8]. Compared to ER and PR, AR is expressed most widely in BRCA, especially in about 70%–90% of TNBC [9, 10, 11]. The safety and efficacy of AR antagonists are confirmed by numerous studies and some preclinical trials in TNBC are evaluating [12, 13, 14], and AR may be a potential target for the treatment of TNBC.

Several circRNAs were proved to be related to different subtypes and the progression of BRCA [15]. The expression of circRNAs in ER-positive BRCA tissues was correlated with factors affecting BRCA proliferation [16, 17]. CircDCUN1D4 affects the progression of TNBC through Wnt/β-catenin signaling pathway, and is a potential marker for TNBC [18]. *Li et al.* found a novel circRNA named circ-HER2 generated from *HER2* gene, which encoded HER2-103 protein and promoted EGFR/HER3 interaction and activation in parts of TNBC [19]. All the evidence suggested that circRNAs are associated with the development of BRCA, especially TNBC, and can be used to indicate the progression of it, nevertheless, the regulatory network remains poor.

In this study, we detected a novel circRNA (circAR-E2E4) generated from *AR* gene, and found it correlated with AR in BRCA. Thus, this circRNA was selected for further research. Knockdown of circAR-E2E4 inhibited cell proliferation of TNBC, and RNA pull-down assay as well as qRT-PCR assay showed circAR-E2E4 sponged miR-665 and miR-671-5p. Next, we constructed a potential circRNA/miRNA/mRNA axis regulatory network in TNBC through a serious bioinformatics and experimental analysis. These findings may provide evidence that this regulatory network plays an important role in seeking potential diagnostic and therapeutic targets in TNBC.

## 2. Materials and methods

### 2.1. Cell culture and transfection

Human VCap, SK-BR-3, ZR-75-1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gemini, USA). Human MDA-MB-468, MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, USA) supplemented with 10% FBS. Human MDA-MB-231 cells were cultured in Leibovitz's L-15 medium (BioSharp, China) supplemented with 10% FBS. Human MDA-MB-361 cells were cultured in Leibovitz's L-15 medium supplemented with 20% FBS. VCap, SK-BR-3, ZR-75-1, MDA-MB-468, and MCF-7 cells were grown at 37 °C in a 5% $CO_2$ atmosphere. MDA-MB-231 and MDA-MB-361 cells were grown at 37 °C. siRNA transfection was carried out using Lipofectamine RNAiMAX Reagent (Life Technologies, USA).

### 2.2. RNA extraction, RT-PCR, qRT-PCR, and PCR

Total RNA was isolated using Trizol reagent according to the manufacturer's protocol. The RNA was then reverse transcribed to cDNAs using the Goldenstar™ RT6 cDNA Synthesis Kit (Tsingke, China) and subjected to qPCR and PCR analysis. qRT-PCR was performed using the 2×T5 Fast qPCR Mix (SYBR Green I, Tsingke, China) and a CFX Connect Real-Time PCR detection system (BIO-RAD, USA), according to the manufacturer's instructions. Hairpin-it™ miRNAs qPCR Quantitation Kit (GenePharma, China) was used to define the expression of miRNAs. The expression of mRNA and circRNA was normalized by *GAPDH*, and *U6* was used as an internal control for miRNA expression normalization. The relative quantity of mRNA, circRNA, and miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are provided in Table S1.

### 2.3. CCK-8 assay

Cell proliferation rate was evaluated by CCK-8 assay (BioSharp, China). Cells (at a density of $3\times10^3$) were placed into 96-well plates and transfected 48 h before CCK-8 solution (10μL) was added. The absorbance at 450 nm was detected in SpectraMax iD5 multi-mode microplate reader after incubation at 37 °C for 1 h.

### 2.4. RNA pull-down assay

The biotinylated probes were designed to be the same sequence as miR-665 and miR-671-5p, while the oligo probe was taken as a control. Approximately $1 \times 10^7$ cells were harvested and lysed. Probes (Youkang, China) were incubated with Dynabeads™ MyOne™ Streptavidin C1 (Invitrogen, USA) respectively at room temperature for 30 min to generate probe-coated beads. The cell lysates were incubated with probe-coated beads at 4 °C overnight. The beads were washed and the bound RNA in the pull-down materials were extracted using Trizol reagent and analyzed by qRT-PCR assay.

### 2.5. Animal experiment

Eight SCID mice were divided equally into two groups. $1\times10^6$ VCap cells were subcutaneously injected into the nude mice. Mice were castrated when tumor volumes were about 50–100 $mm^3$ under sterile conditions. When the tumor volume of the model control group reached 1000 $mm^3$, all the mice were killed and the tumors were collected for further experiments.

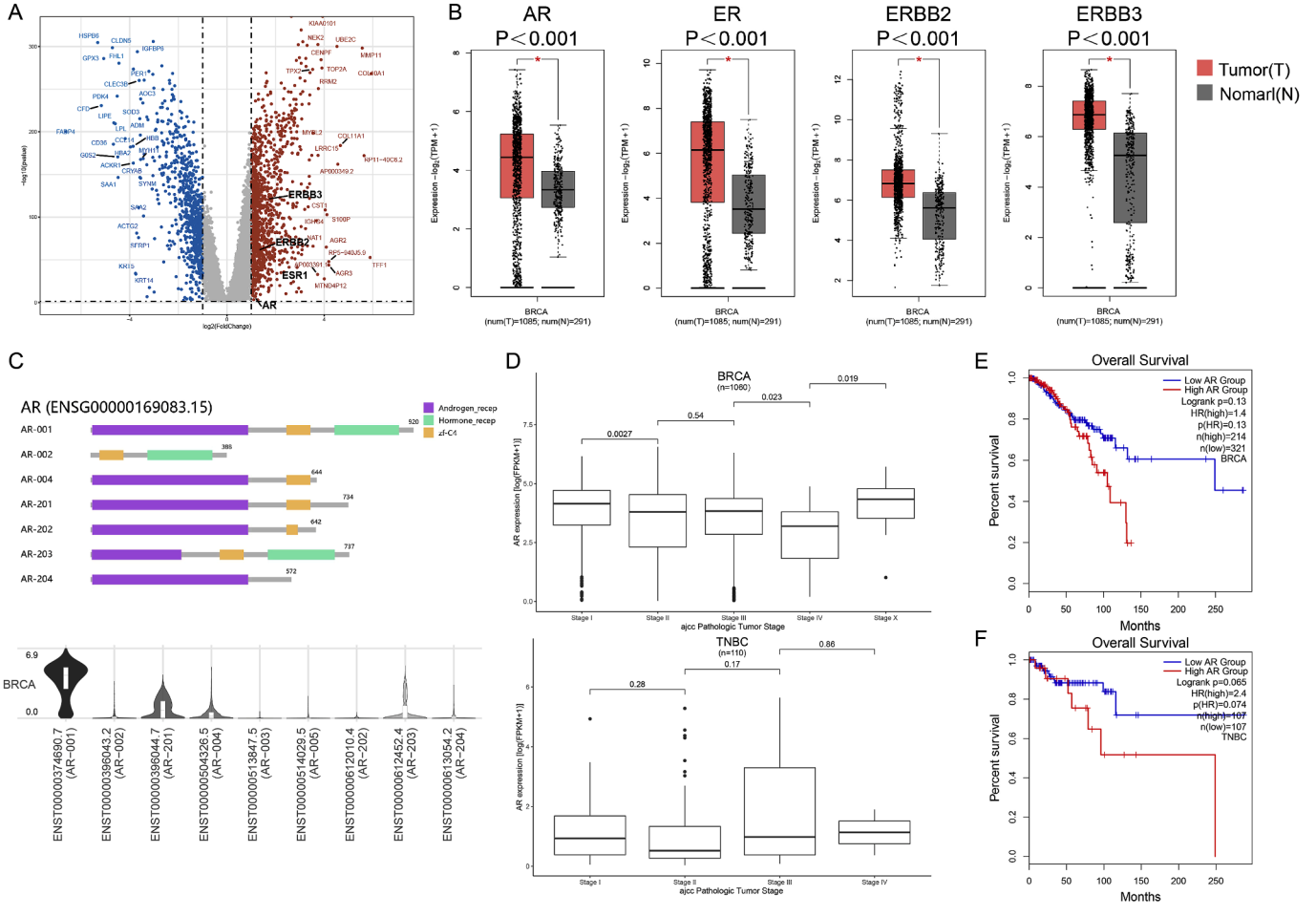


**Figure 1.** Identification for the role of AR in BRCA. (A) The volcano plot of differentially expressed genes between BRCA and normal tissues. (B) The expression of AR, ER, ERBB2, and ERBB3 in BRCA. (C) Different isoforms of AR and their expression levels in different stages. (D) AR expression in pathological stages for BRCA and TNBC patients. (E) Overall survival curve for BRCA patients with high or low AR expression. (F) Overall survival curve for TNBC patients with high or low AR expression.

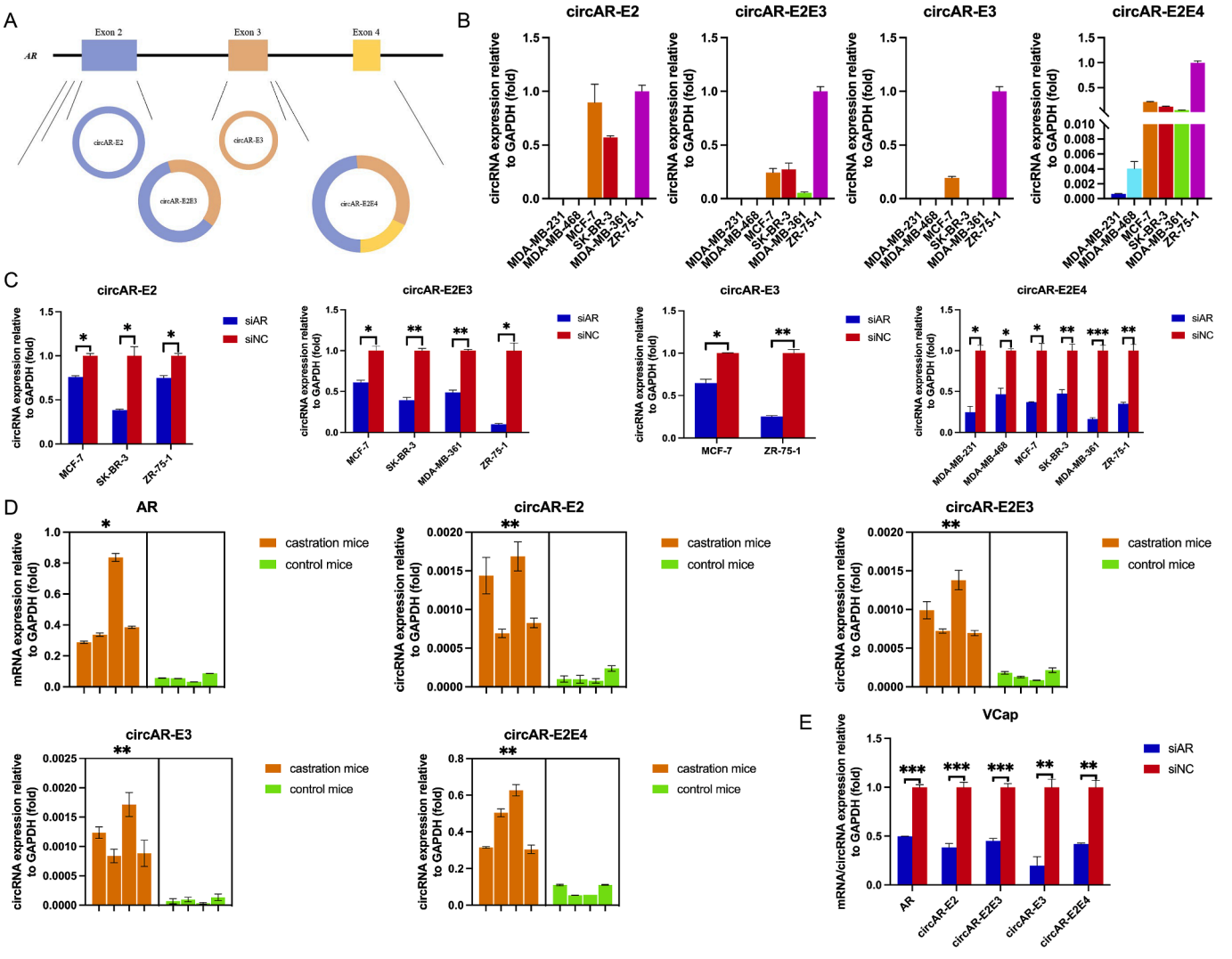


**Figure 2.** circAR-E2E4 is regulated by AR and expresses higher in TNBC. (A) The structure of four circAR isoforms. (B) qRT-PCR analysis was performed to determine circRNAs expression in BRCA cells. (C) qRT-PCR analysis was performed to determine circRNAs expression in BRCA cells treated with siAR. (D) qRT-PCR analysis was performed to determine mRNA and circRNAs expression in two groups of mice tumors. (E) qRT-PCR analysis was performed to determine mRNA and circRNAs expression in VCap cells treated with siAR. Each value is the mean ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

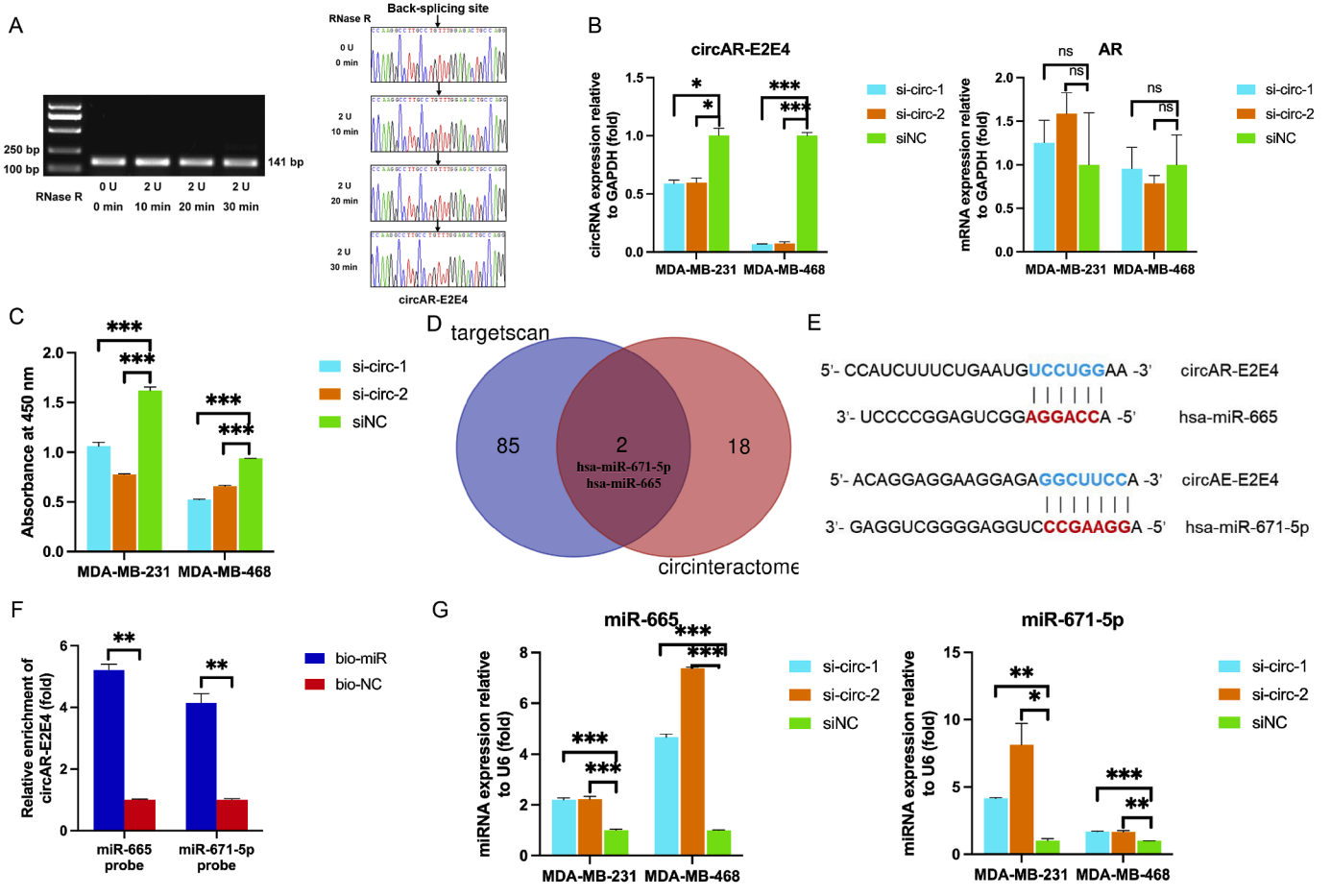


**Figure 3.** circAR-E2E4 knockdown inhibits TNBC cell proliferation and is a sponge of miR-665 and miR-671-5p. (A) Gel image of circAR-E2E4 (141bp) amplified from the total RNA untreated or treated by RNase R and its sequence of back-splicing sites. For the original non-adjusted image of gel, refer to supplementary figure S1. (B) qRT-PCR analysis was performed to determine circAR-E2E4 and AR expression in TNBC cells treated with si-circAR-E2E4. (C) CCK-8 assay indicated that knockdown of circAR-E2E4 inhibited cell proliferation in MDA-MB-231 and MDA-MB-468 TNBC cells. (D) Venn diagrams showing the number of potential binding miRNAs of circAR-E2E4. (E) Potential binding sites of circAR-E2E4 and miR-665, miR-671-5p. (F) qRT-PCR analysis was performed to determine the binding levels of circAR-E2E4 binding with miR-probe. (G) qRT-PCR analysis was performed to determine miR-665 and miR-671-5p expression in TNBC cells treated with si-circAR-E2E4. Each value is the mean ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

### 2.6. Western blotting

Cells were collected and lysed 48 h after transfection to extract total proteins. The cells were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) using a Bioruptor sonicator. Proteins were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, blocked for 1 h in TBS containing 0.1% Tween 20 surfactant and 5% (wt/vol) nonfat milk with rocking at room temperature, and then incubated overnight at 4 °C with the following primary antibodies: anti-STAT3 rabbit monoclonal antibody (1: 1000) (CST), and anti-GAPDH polyclonal antibody produced in rabbit (1: 5000) (Sigma-Aldrich). The secondary antibodies used were goat anti-rabbit (H + L) HRP (Dawen Biotec).

### 2.7. Bioinformatics analysis

GEPIA 2 database [20] was performed to obtain differential genes, expression levels of mRNA, gene isoform details, gene overall survival. The databases Targetscan [21], and Circinteractome [22] were used to predict binding miRNAs of circRNA. StarBase database [23] was employed to analyze co-expression for RNA-RNA. Kaplan-Meier plotter database [24] was utilized to evaluate the prognostic values of miRNAs. The databases MiRMAP [25], MiRWalk [26], and Targetscan were conducted to predict potential target genes of miRNAs. STRING database was introduced to analyze the protein-protein interaction (PPI) network, enrichment of target genes of miRNAs. Gene Ontology (GO) functional annotation, and KEGG pathway enrichment were analyzed by g:Profiler [27]. Cytoscape software was used to calculate hub genes. The information and details of samples was all obtained from TCGA database, and the bioinformatics analyses were carried out using R.

### 2.8. Statistical analysis

GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. The nested t-test and student's t-test were used whenever appropriate. Variance was assessed by calculating the standard error of the mean in each group. *P*-values less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Selection of the potential gene in BRCA

To find some potential regulatory genes associated with BRCA, differential genes expression profile in BRCA was analyzed by GEPIA 2. As shown in Figure 1A, hormone receptors AR and ER (ESR1), growth factor receptors HER2 (ERBB2) and HER3 (ERBB3) as well were all significantly up-regulated ($\log_2$FC $> 1$) in BRCA tissues when compared with normal tissues. The expression of four receptors in BRCA tissues was significantly higher than that in normal tissues (Figure 1B). AR can be transcribed into different types of isoforms in BRCA, and isoform AR-001 contains domains of androgen receptor, hormone receptor, and zinc finger completely (Figure 1C). Expression distribution of AR-001 was also shown in Figure 1C. AR expression is involved in BRCA and TNBC progression, and the expression of AR existed in different pathological stages (Figure 1D). Survival analysis presented that AR upregulation was correlated with poor survival rates in BRCA (Figure 1E). Overall survival curve also showed that high AR expression was associated with poor clinical outcomes in patients with TNBC (Figure 1F). In general, AR plays an essential role in BRCA, especially in TNBC.

### 3.2. CircAR-E2E4 is regulated by AR in TNBC

Previous research identified many circRNAs generated from AR, and circARs may serve as surrogate circulating biomarkers for AR expression [28]. CircARC1 was proved to be regulated by AR, and affected the progression of both prostate cancer and bladder cancer [29]. To define circARs expression in BRCA, different subtypes of BRCA cells were used. Figure 2A showed the detailed structure of circARs (circAR-E2, circAR-E2E3, circAR-E3, circAR-E2E4). Though AR was expressed in 6 BRCA cells, circAR-E2E4 was the only circAR expressed in MDA-MB-231 and MDA-MB-468 TNBC cells, and circAR-E2E4 could be detected easier than AR in TNBC cells (Figure 2B). Then AR was knocked down in BRCA cells, finding that knockdown of AR reduced all four circARs expression, including circAR-E2E4 (Figure 2C). AR was expressed highly in prostate cancer, so VCap cells were used to confirm the relationship between AR and circARs *in vivo* and *in vitro*. The VCap tumors shrank to some extent after castration, but relapsed a week later, forming castration-resistant prostate cancer (CRPC). The expression of AR and related circARs was examined in subcutaneous prostate cancer transplantation. Not only AR, but also circARs were upregulated in CRPC tumors when compared with normal tumors (Figure 2D). AR was also knockdown in VCap cells to investigate the relationship between AR and circARs, finding that circARs decreased together with AR (Figure 2E). Thus, circAR-E2E4 was regulated by AR, and may replace AR to be a candidate biomarker in TNBC.

### 3.3. circAR-E2E4 regulates TNBC progression, and sponges miR-665 and miR-671-5p

CircAR-E2E4 was derived from exon 2–4 of *AR* gene (Figure 2A). Total RNA was treated by RNase R and divergent primers were used for PCR, and Sanger sequencing for PCR products proved the predicted circular junction of circAR-E2E4 (Figure 3A). CircAR-E2E4 expression was downregulated by using siRNA (si-circ-1: CAAGGCCUUGCCUGUUUGGTT, si-circ-2: CUUGCCUGUUUGGAGACUGTT)

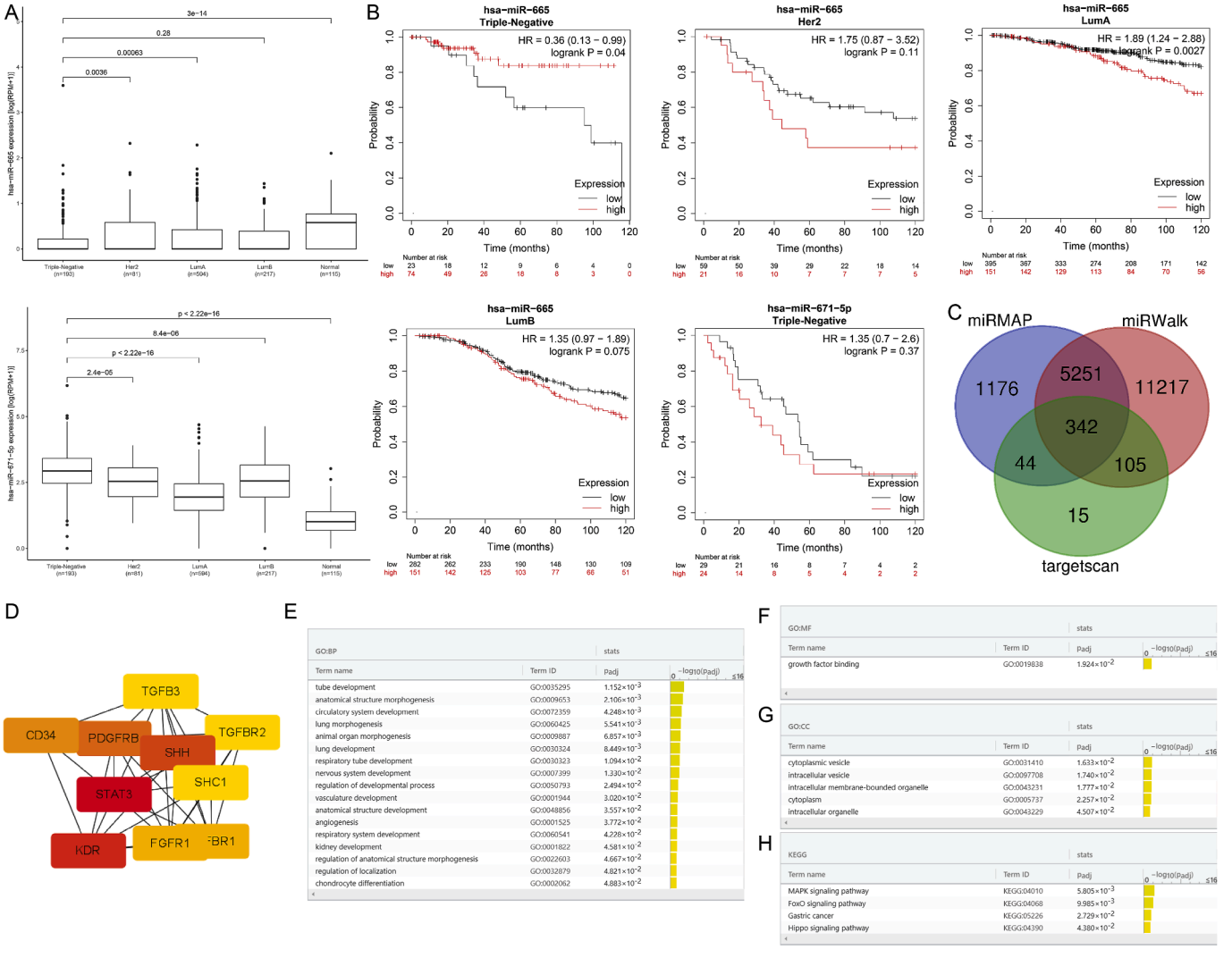


**Figure 4.** Analysis of expressions and prognostic values for mi-665 and miR-671-5p and their target genes. (A) The expression of miR-665 and miR-671-5p in different subtypes of BRCA samples and normal samples compared with TNBC patient samples. (B) The prognostic values of miR-665 and miR-671-5p in different subtypes of BRCA. (C) Venn diagrams showing the number of potential target genes of miR-665. (D) The PPI analysis for target genes of miR-665. (E) The GO biological process items for target genes of miR-665. (F) The GO molecular function items for target genes of miR-665. (G) The GO cellular component items for target genes of miR-665. (H)The KEGG pathways for target genes of miR-665.

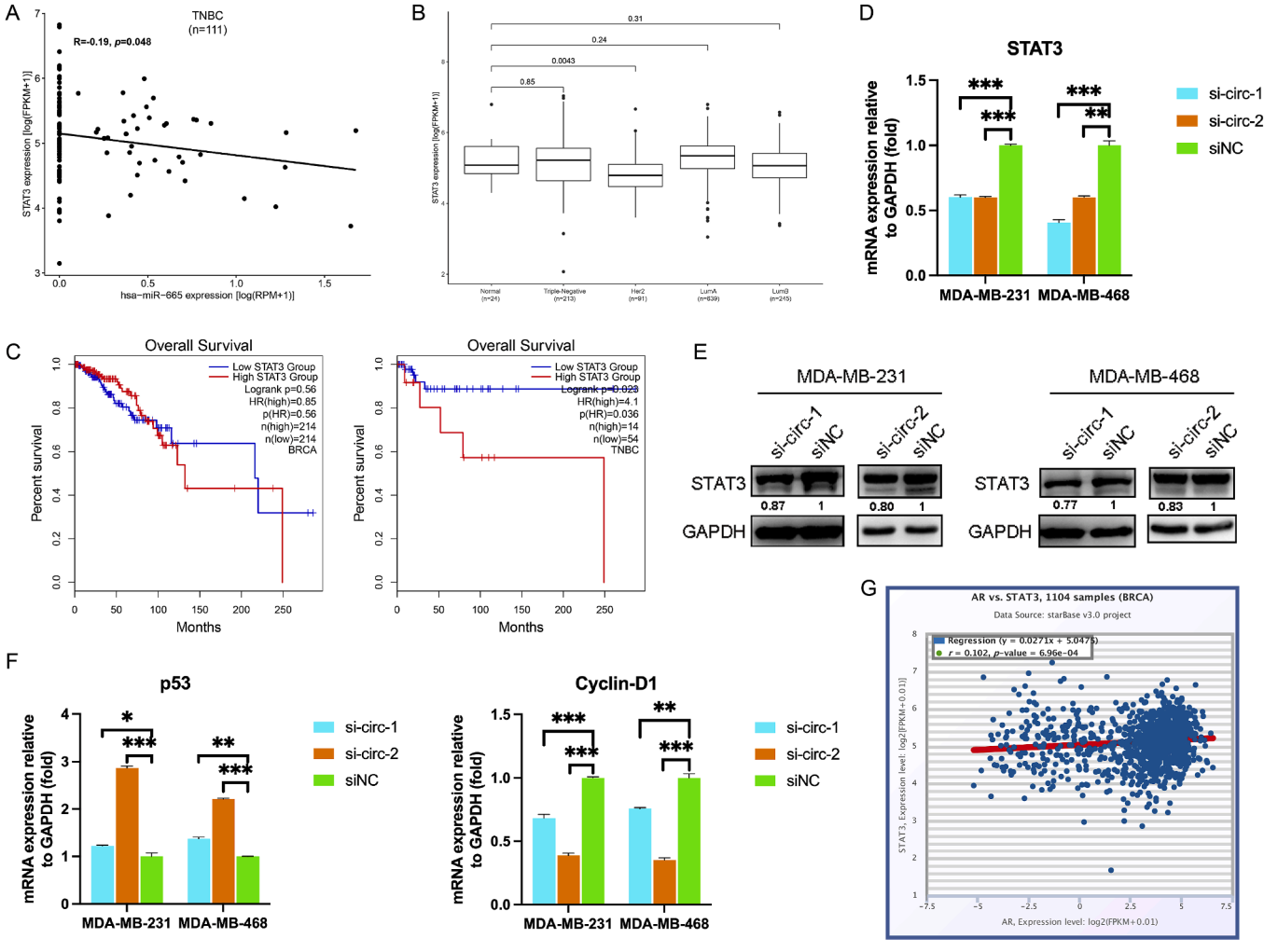


**Figure 5.** CircAR-E2E4-miR-665-STAT3 is a potential axis in TNBC. (A) The expression correlation of miR-665 with STAT3 in TNBC. (B) The mRNA expression level of STAT3 in different types of BRCA. (C) Overall survival curve for BRCA (left) and TNBC (right) patients with high or low STAT3 expression. (D) qRT-PCR analysis was performed to determine STAT3 expression levels in TNBC cells treated with si-circAR-E2E4. (E) Representative western blots of STAT3 and GAPDH expression in MDA-MB-231 and MDA-MB-468 TNBC cells treated with si-circAR-E2E4. Numbers represent gray intensity analysis of STAT3/GAPDH. For the original non-adjusted images of blots and total protein, refer to supplementary figure S2. (F) qRT-PCR analysis was performed to determine p53 and Cyclin-D1 expression in TNBC cells treated with si-circAR-E2E4. (G) The expression correlation of AR with STAT3 in BRCA. Each value is the mean ± SEM of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

which had no effects on AR expression to explore its biological functions in TNBC (Figure 3B). The results showed that circAR-E2E4 downregulation significantly inhibited cell proliferation through CCK8 assay in MDA-MB-231 and MDA-MB-468 TNBC cells (Figure 3C). Most of circRNAs can act as miRNA sponges [30]. Thus, the databases targetscan and circinteractome were utilized to predict potential binding miRNAs of circAR-E2E4. MiR-665 and miR-671-5p were commonly predicted by 2 databases, and they were selected for the following investigation, considered as candidate miRNAs (Figure 3D). The sequence of circAR-E2E4 was found to have potential binding sites of miR-665 and miR-671-5p (Figure 3E). To further verify the binding of circAR-E2E4 with miR-665 and miR-671-5p, we performed a miRNA pull-down assay with biotin-labeled miRNA probes, and enrichment of circAR-E2E4 was detected by qRT-PCR in the miRNA probe group compared with the control probe (Figure 3F). The knockdown of circAR-E2E4 promoted the expression of miR-665 and miR-671-5p in TNBC cells (Figure 3G). Our results indicated that knockdown of circAR-E2E4 restrained TNBC cell proliferation, and this biological function may be performed through sponging miR-665 and miR-671-5p.

### *3.4. Analysis of miR-665 and miR-671-5p and their target genes in TNBC*

To further research biological functions of miR-665 and miR-671-5p in BRCA and TNBC, the expression of miRNAs and prognostic values of them were accessed. The expression of miR-665 and miR-671-5p was analyzed, we found that miR-665 was downregulated while miR-671-5p was upregulated significantly in TNBC compared with normal tissues (Figure 4A). Subsequently, the prognostic values of these 2 miRNAs were analyzed via Kaplan-Meier plotter (Figure 4B). As a result, miR-665 was downregulated in TNBC significantly, and upregulation of miR-665 was correlated with positive survival time of TNBC patients. Then we analyzed target genes of miR-665 using 3 databases including miRMAP, miRWalk, and targetscan, and 342 target genes were predicted (Figure 4C). PPI network analysis was performed by STRING database to help us better understand the interaction relationship of 342 target genes. The top 10 hub genes (STAT3, KDR, SHH, PDGFRB, CD34, TGFBR1, FGFR1, TGFB3, SHC1, TGFBR2) were shown in Figure 4D. Furthermore, GO functional annotation for 342 target genes of miR-665 was conducted. In the biological process (BP) category, enriched items contained tube development, circulatory system development, and regulation of developmental process (Figure 4E); in the cellular component (CC) category, enriched items included cytoplasmic vesicle, intracellular vesicle, and intracellular membrane-bounded organelle (Figure 4F); in the molecular function (MF) category, enriched item comprised growth factor binding (Figure 4G). Then, KEGG pathway enrichment analysis illustrated that MAPK signaling pathway, FoxO signaling pathway, and Hippo signaling pathway were top enriched pathways (Figure 4H). Collectively, bioinformatics analysis indicated that miR-665 and its target genes can be downstream regulatory pairs of circAR-E2E4.

### *3.5. CircAR-E2E4-miR-665-STAT3 is a potential regulatory network in TNBC*

Signal transducer and activator of transcription 3 (STAT3) was reported to be an oncogene in many cancers, including BRCA and TNBC [31, 32]. miR-665 was proved to bind with 3' UTR of STAT3, which was a direct target gene of miR-665. miR-665 inhibited the expression and activation of STAT3 [33]. As shown in Figure 5A, miR-665-STAT3 pairs presented a negative co-expression relationship significantly in TNBC. The expression level of STAT3 mRNA was not changed significantly in TNBC samples compared with normal samples (Figure 5B). However, overall survival analysis determined that high expression level of STAT3 in TNBC had a positive relationship with poor survival rate (Figure 5C). Knockdown of circAR-E2E4 reduced STAT3 expression in MDA-MB-231 and MDA-MB-468 TNBC cells (Figure 5D). We also found that knockdown of circAR-E2E4 inhibited the protein expression of STAT3 in TNBC cells (Figure 5E). STAT3 is a DNA-binding transcription factor. The broad influence STAT3 has on cellular function can be attributed to the numerous gene targets. STAT3 can indirectly regulate genes by mediating expression of other transcription factors or physical association with other transcription factors to enhance or suppress their function in gene regulation [34]. STAT3 could directly associate with one of promoter sites (−128 bp) to suppress p53 gene expression, and active form of STAT3 could induce cyclin D1. The expression of p53 and cyclin-D1 was used to further confirm the effects of circAR-E2E4 on STAT3 (Figure 5F). RNA-RNA co-expression in BRCA showed that AR had a positive correlation with STAT3 (Figure 5G). Therefore, such effects suggested circAR-E2E4 may have the potential to act as a diagnostic and therapeutic target, and AR-circAR-E2E4-miR-665-STAT3 axis can be a potential novel regulatory network in TNBC.

## 4. Discussion

Although many studies indicated the important role of AR in TNBC [8, 9, 10, 11], potential regulatory networks related to AR in TNBC have not been completely uncovered. So, the detailed molecular regulatory network needs further investigation for improving the diagnosis and therapy of patients with TNBC.

In this study, bioinformatics analysis including differential expression analysis, stage analysis, and overall survival analysis showed that the key role of AR in BRCA, as well in TNBC. These data indicated that further investigation on AR may be reliable. Then we used animal and cell experiments to reveal the relationship between AR and circARs. Interestingly, we found knockdown of AR reduced the expression of circARs in VCap cells and BRCA cells, and circAR-E2E4 was the only circAR expressed in TNBC cells. Thus, the biological function of circAR-E2E4 in TNBC cell proliferation was confirmed by CCK-8 assay.

circRNAs were reported to be full of miRNA binding sites, and sponge miRNAs to get rid of their inhibition on target genes [1]. Many recent studies revealed circRNAs could regulate the progression of TNBC by different biological functions, such as sponging miRNAs [18, 19]. Therefore, potential binding miRNAs were predicted by 2 databases, namely, targetscan and circinteractome. RNA pull-down assay was employed to confirm that circAR-E2E4 could bind with miR-665 and miR-671-5p. Using expression analysis and

survival analysis, miR-665 was indicated to have better prognosis value in TNBC. Previous study revealed that miR-665 targeted nuclear receptor subfamily 4 group A member 3 (NR4A3) and regulated the progression of BRCA [35]. Therefore, 3 databases were used to predict target genes of miR-665, and these genes were used for further PPI analysis, GO and KEGG analysis.

STAT3 was calculated to be the best hub gene, which was an oncogene and researched to affect BRCA development [31, 32]. In TNBC, miR-665 had a negative expression relationship with STAT3. Also, expression and survival analysis confirmed that high STAT3 expression led to poor survival rate in TNBC. At the end, the evidence of knockdown of circAR-E2E4 cutting down STAT3 expression, affecting downstream genes of STAT3, and AR having a positive expression relationship with STAT3 also made this study more solid.

Much further research can be studied continuously. CircRNAs are enriched and stable in exosomes [36]. Nowadays, neoadjuvant chemotherapy for breast cancer is an important means to alleviate or cure breast cancer. If noninvasive molecular testing is used to monitor and guide neoadjuvant chemotherapy, the efficacy of neoadjuvant chemotherapy can be improved to prolong the survival time of patients. If circAR-E2E4 exists in body fluid, it can be a potential candidate biomarker to help us learn more about TNBC progression.

In conclusion, we elucidated a novel regulatory network circAR-E2E4-miR-665-STAT3 axis in TNBC using a series of bioinformatics analysis and some experiments. AR knockdown could lead to circAR-E2E4 decrease. CircAR-E2E4 inhibited TNBC cell progression by sponging miR-665, and then reduced the expression of STAT3. This regulatory network may contribute to the development of effective therapeutic targets for treating TNBC. However, the current findings need to be further confirmed by clinical trials in the future.

## 5. Conclusions

Molecular functional roles in treating TNBC have not been revealed completely. In this study, we provided a novel insight into the roles circRNAs played in TNBC progression. Overall, AR was found to be a key acceptor in TNBC and regulate circAR-E2E4 expression. Knockdown of circAR-E2E4 inhibited cell proliferation of TNBC. Then, potential binding miRNAs were predicted, and the prognostic value of miR-665 stood out. Finally, miR-665 targeting genes were analyzed by bioinformatics, and it regulated STAT3 expression negatively. Indeed, circAR-E2E4 may control the balance in circAR-E2E4-miR-665-STAT3 axis, and inhibition of circAR-E2E4 may promote the treatment of TNBC. Therefore, this study highlights the importance of circAR-E2E4-miR-665-STAT3 regulatory network, and circAR-E2E4 has the potential to be a promising diagnostic, prognostic, and therapeutic target in TNBC.

## Declarations

### *Author contribution statement*

Hao Xu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Mengdie Fang: Conceived and designed the experiments; Performed the experiments.
Shuhui Ye: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Wanxin Yao: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Juan Ren: Performed the experiments; Analyzed and interpreted the data.
Yanmei Zhang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

### *Ethics approval and consent to participate*

All animal experiments were approved by the Medical Ethics Committee of Hangzhou Medical College and conducted according to the Guidelines for the Care and Use of Animals for Scientific Research.

### *Institutional review board statement*

Not applicable.

### *Informed consent statement*

Not applicable.

### *Funding statement*

This work was supported by the Huadong Medicine Joint Funds of the Zhejiang Provincial Natural Science Foundation of China [NO. HDMY22H318024] and [NO. LHDMY23H160003], Medical and Health Science and Technology Project of Zhejiang Province [NO. 2022RC128], Foundation of Zhejiang Academy of Medical Sciences [NO. YS2022005], and Scientific Research Fund of Zhejiang Provincial Education Department [NO. Y202146054].

*Data availability statement*

Data will be made available on request.

*Declaration of interests statement*

The authors declare no conflict of interest.

*Additional information*

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e12654.

## References


[1] E. Lasda, R. Parker, Circular RNAs: diversity of form and function, RNA 20 (2014) 1829–1842.
[2] L. Salmena, L. Poliseno, Y. Tay, L. Kats, P.P. Pandolfi, A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell 146 (2011) 353–358.
[3] E. Arnaiz, et al., CircRNAs and cancer: biomarkers and master regulators, Semin. Cancer Biol. 58 (2019) 90–99.
[4] Y. Yin, et al., Emerging roles of circRNA in formation and progression of cancer, J. Cancer 10 (2019) 5015–5021.
[5] M.C. Cheang, et al., Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer, J. Natl. Cancer Inst. 101 (2009) 736–750.
[6] D.C. Koboldt, et al., Comprehensive molecular portraits of human breast tumours, Nature 490 (2012) 61–70.
[7] M. De Laurentiis, et al., Treatment of triple negative breast cancer (TNBC): current options and future perspectives, Cancer Treat Rev. 36 (2010) S80–S86.
[8] D.G. Powe, et al., Alpha- and beta-adrenergic receptor (AR) protein expression is associated with poor clinical outcome in breast cancer: an immunohistochemical study, Breast Cancer Res. Treat. 130 (2011) 457–463.
[9] B.D. Lehmann, J.A. Pietenpol, Identification and use of biomarkers in treatment strategies for triple-negative breast cancer subtypes, J. Pathol. 232 (2014) 142–150.
[10] T.E. Hickey, J.L.L. Robinson, J.S. Carroll, W.D. Tilley, The androgen receptor in breast tissues: growth inhibitor, tumor suppressor, oncogene? Mol. Endocrinol. 26 (2012) 1252–1267.
[11] L. Zhang, et al., Androgen receptor, EGFR, and BRCA1 as biomarkers in triple-negative breast cancer: a meta-analysis, BioMed Res. Int. 12 (2015), 2015.
[12] V.N. Barton, et al., Multiple molecular subtypes of triple-negative breast cancer critically rely on androgen receptor and respond to enzalutamide in vivo, Mol. Cancer Therapeut. 14 (2015) 769–778.
[13] B.D. Lehmann, et al., PIK3CA mutations in androgen receptor-positive triple negative breast cancer confer sensitivity to the combination of PI3K and androgen receptor inhibitors, Cancer Res. 75 (2015) 2.
[14] T.A. Traina, et al., Enzalutamide for the treatment of androgen receptor-expressing triple-negative breast cancer, J. Clin. Oncol. 36 (2018) 884.
[15] A.A. Nair, et al., Circular RNAs and their associations with breast cancer subtypes, Oncotarget 7 (2016) 80967–80979.
[16] T.O. Nielsen, et al., A comparison of PAM50 intrinsic subtyping with immunohistochemistry and clinical prognostic factors in tamoxifen-treated estrogen receptor-positive breast cancer, Clin. Cancer Res. 16 (2010) 5222–5232.
[17] L. Coscujuela Tarrero, et al., Luminal breast cancer-specific circular RNAs uncovered by a novel tool for data analysis, Oncotarget 9 (2018) 14580–14596.
[18] J. Zhang, et al., CircRNA _ 069718 promotes cell proliferation and invasion in triple-negative breast cancer by activating Wnt/beta-catenin pathway, Eur. Rev. Med. Pharmacol. Sci. 23 (2019) 5315–5322.
[19] J. Li, et al., Circular HER2 RNA positive triple negative breast cancer is sensitive to Pertuzumab, Mol. Cancer 19 (2020) 142.
[20] Z.F. Tang, B.X. Kang, C.W. Li, T.X. Chen, Z.M. Zhang, GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis, Nucleic Acids Res. 47 (2019) W556–W560.
[21] S.E. McGeary, et al., The biochemical basis of microRNA targeting efficacy, Science 366 (2019) 1470.
[22] D.B. Dudekulay, et al., CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs, RNA Biol. 13 (2016) 34–42.
[23] J.H. Li, S. Liu, H. Zhou, L.H. Qu, Yang, J. H. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data, Nucleic Acids Res. 42 (2014) D92–D97.
[24] B. Gyorffy, Survival analysis across the entire transcriptome identifies biomarkers with the highest prognostic power in breast cancer, Comput. Struct. Biotechnol. J. 19 (2021) 4101–4109.
[25] C.E. Vejnar, E.M. Zdobnov, miRmap: comprehensive prediction of microRNA target repression strength, Nucleic Acids Res. 40 (2012) 11673–11683.
[26] H. Dweep, C. Sticht, P. Pandey, N. Gretz, miRWalk - database: Prediction of possible miRNA binding sites by "walking" the genes of three genomes, J. Biomed. Inf. 44 (2011) 839–847.
[27] J. Reimand, M. Kull, H. Peterson, J. Hansen, J. g Vilo, Profiler - a web-based toolset for functional profiling of gene lists from large-scale experiments, Nucleic Acids Res. 35 (2007) W193–W200.
[28] S. Cao, et al., Circular RNAs add diversity to androgen receptor isoform repertoire in castration-resistant prostate cancer, Oncogene 38 (2019) 7060–7072.
[29] G. Deng, et al., Targeting androgen receptor (AR) with antiandrogen Enzalutamide increases prostate cancer cell invasion yet decreases bladder cancer cell invasion via differentially altering the AR/circRNA-ARC1/miR-125b-2-3p or miR-4736/PPARγ/MMP-9 signals, Cell Death Differ. 28 (2021) 2145–2159.
[30] X. Li, L. Yang, L.L. Chen, The biogenesis, functions, and challenges of circular RNAs, Mol. Cell 71 (2018) 428–442.
[31] J.H. Ma, L. Qin, X. Li, Role of STAT3 signaling pathway in breast cancer, Cell Commun. Signal. 18 (2020) 13.
[32] X. Song, Z.Y. Liu, Z.Y. Yu, EGFR promotes the development of triple negative breast cancer through JAK/STAT3 signaling, Cancer Manag. Res. 12 (2020) 703–717.
[33] S.R. Ouyang, et al., LncRNA BCAR4, targeting to miR-665/STAT3 signaling, maintains cancer stem cells stemness and promotes tumorigenicity in colorectal cancer, Cancer Cell Int. 19 (2019) 12.
[34] R.L. Carpenter, H.W. Lo, STAT3 target genes relevant to human cancers, Cancers 6 (2014) 897–925.
[35] X.G. Zhao, et al., miR-665 expression predicts poor survival and promotes tumor metastasis by targeting NR4A3 in breast cancer, Cell Death Dis. 10 (2019) 21.
[36] Y. Li, et al., Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis, Cell Res. 25 (2015) 981–984.