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Knockdown of ALDH1A3 Reduces Breast Cancer Stem Cell Marker CD44 via miR-7-TGFBR2-Smad3-CD44 Regulatory Axis

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Abstract:

Purpose: Inhibition of ALDH1A3 leads to increase miR-7 expression and decrease CD44 expression significantly in breast cancer stem cells (BCSCs), but the mechanism is not clear. The aim of this study is to investigate the regulatory relationship between ALDH1A3, miR-7, and CD44 in BCSCs.

Methods: The expression of ALDH1A3 was inhibited by small interfering RNA(si RNA), and the expression of miR-7 was detected by RT-qPCR. Then, the ratio of CD44+cells was analyzed by flow cytometry in MDA-MB-231 cells. The dual-luciferase reporter system was used to prove that miR-7 binds to TGFBR2 3'UTR, and ChIP-PCR determines whether the transcription factor Smad3 binds to the upstream regulatory region of the CD44 promoter.

Results: The results showed that siALDH1A3 downregulated ALDH1A3 and promoted the miR-7 expression, which resulted in downregulation of CD44 expression. siALDH1A3 also downregulated the CD44 expression on the surface of MDA-MB-231 cells and inhibited the G2/M phase in BCSCs analyzed by flow cytometry. In addition, lenti-miR-7 cells transfected with TGF-β1 plus SB431542 showed that lenti-miR-7 inhibited the TGF-β1 pathway by inhibiting Smad2\3\4 expression and, thus, downregulated the CD44 expression. The dual-luciferase report demonstrated that miR-7 directly bound to TGFBR2 3'UTR, and ChIP-PCR proved that Smad3, a transcription factor, bound to the upstream region of the CD44 promoter. These results demonstrated the existence of the ALDH1A3-miR-7-TGFBR2-Smad3-CD44 axis in MDA-MB-231 cells. RT-qPCR results of 12 breast cancer surgical specimens and SK-BR-3, MCF-7, and LD cell lines further confirmed the presence of the regulatory axis.

Conclusions: The findings from the study demonstrates that the ALDH1A3-miR-7-TGFBR2-Smad3-CD44 regulatory axis is highly efficient in the inhibition of CD44 expression in BCSCs, and that the regulatory expression of ALDH1A3 and miR-7 may provide a strategy with the therapy of breast cancer.

Keywords: miR-7; Breast cancer ; cancer stem cells; ALDH1A3; CD44

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Introduction

Breast cancer is the most common malignant disease that mainly affects females. Recurrence and metastasis result in unfavorable prognoses for breast cancer patients; up to 30% of patients died of relapse and metastasis after having standard-of-care therapy[1]. Therefore, novel therapies are urgently needed. Cancer metastases can be attributed to multiple factors such as cancer cell biological programs that underlie the dissemination and metastatic outgrowth of cancer cells, cancer stem cells (CSCs)[2, 3]. MicroRNA (miR) is a class of small noncoding RNAs (19-22 nt) that are involved in biological processes such as proliferation, differentiation, apoptosis, and development [4, 5]. miR-based therapeutic strategies are promising for cancer therapy. MicroRNA-7 (miR-7) is an intronic microRNA that resides in the first intron of the heterogeneous ribonuclear protein K gene on chromosome 9 and is downregulated in different cancer types[6]. Our previous study had found that miR-7, which was downregulated in breast CSCs (BCSCs, EpCAM+CD44+CD24−/low) isolated from the human MCF-7 and MDA-MB-231 cell lines, inhibited cell invasion and metastasis, decreased the BCSC population, and partially reversed EMT in MDA-MB-231 cells by directly targeting the oncogene, SETDB1[7]. However, it is not clear what molecular mechanism by which miR-7 plays the role of the BCSC

subset downregulation. Because of the existing BCSC subset, cancer to chemotherapy, radiotherapy sensitivity, and therapeutic effects are obviously decreased. It is known that the quantity of BCSC subset are closely related to the survival of breast cancer patients. Therefore, it is of great significance to make clear the molecular mechanism of miR-7 to reduce the amount of BCSC subsets and to use miR-7 to target BCSC in the treatment of breast cancer.

Although our latest work investigated the relationship between miR-7 and ALDH1A3, a few questions remain unanswered[8]. For example, we do not know how to inhibit the ALDH1A3 activity and how to affect the expression of CD44 by miR-7 in MDA-MB-231 cells, which leads to a decrease of the BCSC subset. In this study we attempt to explore the mechanisms of siALDH1A3 downregulating CD44 via the TGF-β1 pathway.

Materials and Methods

Cell lines and Reagents

Human breast cancer cell lines MDA-MB-231, SK-BR-3, and MCF-7 were obtained from the Shanghai Institution of Biochemistry and Cell Biology in CAS, China. The LD cell line was established by our lab from a human breast cancer postsurgery sample[9]. All cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine serum (GibcoTM, Logan, Utah, USA) and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

Human breast cancer samples

Human breast cancer postsurgery samples, the data of 12 clinical specimens were recorded in the previous paper[9], were obtained from the Department of General Surgery of Zhongda Hospital at Southeast University in China. The investigation was approved by an ethics committee at Southeast University School of Medicine, and informed consent for the use of the postsurgery samples was obtained from the donors who were breast cancer patients.

MACS for BCSCs

CD44/CD24/CD326 antibodies conjugated to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were used to obtain the BCSCs from MDA-MB-231 cell lines, respectively. The isolation process followed the manufacturer's instructions.

Flow Cytometry (FCM)

The CD44 antibodies (eBioscience, Thermo Fisher Scientific) were applied on flow cytometry (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Quantitative Real-Time Polymerase Chain Reaction

To evaluate the expression of miR-7, Smad2, Smad3, Smad4, TGFBR2, and CD44 respectively, total RNAs were used for the reverse transcription (RT) reactions, and quantitative polymerase chain reaction (qPCR) was performed on a StepOnePlus™ System (AB Applied Biosystems, Carlsbad, CA, USA). GAPDH was used as an internal control.

Dual-Luciferase Reporter Assay

The full-length TGFBR2 3'UTR was amplified via PCR using PrimeSTAR® (Takara, Japan) from human genomic DNA, and the mutant TGFBR2 3'UTR was generated by Mut Express II Fast Mutagenesis Kit V2 (Vazyme, China). These DNA fragments were cloned into a psiCHECK™-2 Vector (Promega, San Luis Obispo, CA, USA). Plasmids were cut and connected by endonuclease QuickCut™ Not I, Xho I, T4 DNA Ligase (Takara, Japan). MDA-MB-231 cells were seeded in a 24-well plate and transfected, respectively, with the reporter constructs and miR-7 mimics for 48 hours. Then, the luciferase reporter assay was performed using a Dual-Luciferase Reporter System (Promega, San Luis Obispo, CA, USA).

Western Blot

Approximately 1×106 cells were harvested and lysed in radioimmunoprecipitation assay buffer, and the lysates were run on a Western blot as described previously[10]. The antibodies used for Western blot included CD44, Smad3, GAPDH, and TGFBR2 from Proteintech (Chicago, USA).

ChIP-PCR Assays

The ChIP assay was performed according to the Chromatin Immunoprecipitation Kit Instruction Manual (EZ-ChIP™ Catalog# 17-371, Merck Millipore, Germany). The Anti-Smad2+Smad3 antibody (ab207447) was used to precipitate the protein-DNA complexes (Abcam, UK)[11], and the DNA isolated through ChIP reactions was subjected to PCR using primers specific to the promoters of CD44, respectively.

Expression of Lentivirus Infected Cells and siRNA Silencing Gene

The three-plasmid system was made up of pSPAX2, pMD2G, and pHBLV-U6-ZsGreen. miR-7 fragment (245 bp) was inserted into the lentivirus vector and cultured in 293T cells, and the virus solution was obtained. (10 μL lenti-miR-7 virus solution infected 1×104 MDA-MB-231 cells and selecting miR-7 overexpression monoclonal cells.) siRNA and miR-7 mimic were synthesized by the RiboBio Company (Guangzhou, GD, China) and performed as described previously. 50 nM siRNA and miR-7mimic are used to transfect cells.

Statistical Analysis

SPSS Statistics 21.0 and GraphPad Prism 8.0 were used for data analysis and imaging. Values of interest were presented as the mean plus or minus standard deviation. Statistical analyses were performed using Student's t-test method and Spearman's correction analysis. Values shown are for one representative experiment, and the data are given as mean ± SD, *P<0.05, **P<0.01, and ***P<0.001. Differences were considered statistically significant if P<0.05.

Results

1. Inhibition of ALDH1A3 decreases CD44 expression Since miR-7 expression was detected by RT-qPCR after knocking down ALDH1A3 with siRNA, and it was found that miR-7 expression was significantly increased accidentally **(Figure 1A)**. The results confirmed that not only can miR-7 regulate ALDH1A3[8] but also ALDH1A3 can reversely regulate miR-7 expression. Using lentivirus to overexpress miR-7(lenti-miR-7) can significantly reduce the

expression of CD44 mRNA **(Figure 1B)**. Then, we examined the ratio of CD44+ cells in the MDA-MB-231 cells after knocking down ALDH1A3 with siRNA by FCM **(Figure 1C)**. **Figure 1D** shows the ratio of CD44+ cells was significantly decreased compared to the control group. In order to further evaluate the effect of siALDH1A3 on cell proliferation, we usded FCM to analyze the cell cycle of BCSC **(Figure 1E)**. Cell cycle analysis showed that BCSC-siALDH1A3 increased the S phase and reduced the G2/M phase compared to the BCSC-siNC cell population **(Figure 1F)**. Collectively, the knockdown of ALDH1A3 expression reduced the CD44 expression in MDA-MB-231 cells and their *in vitro* proliferation in BCSCs.

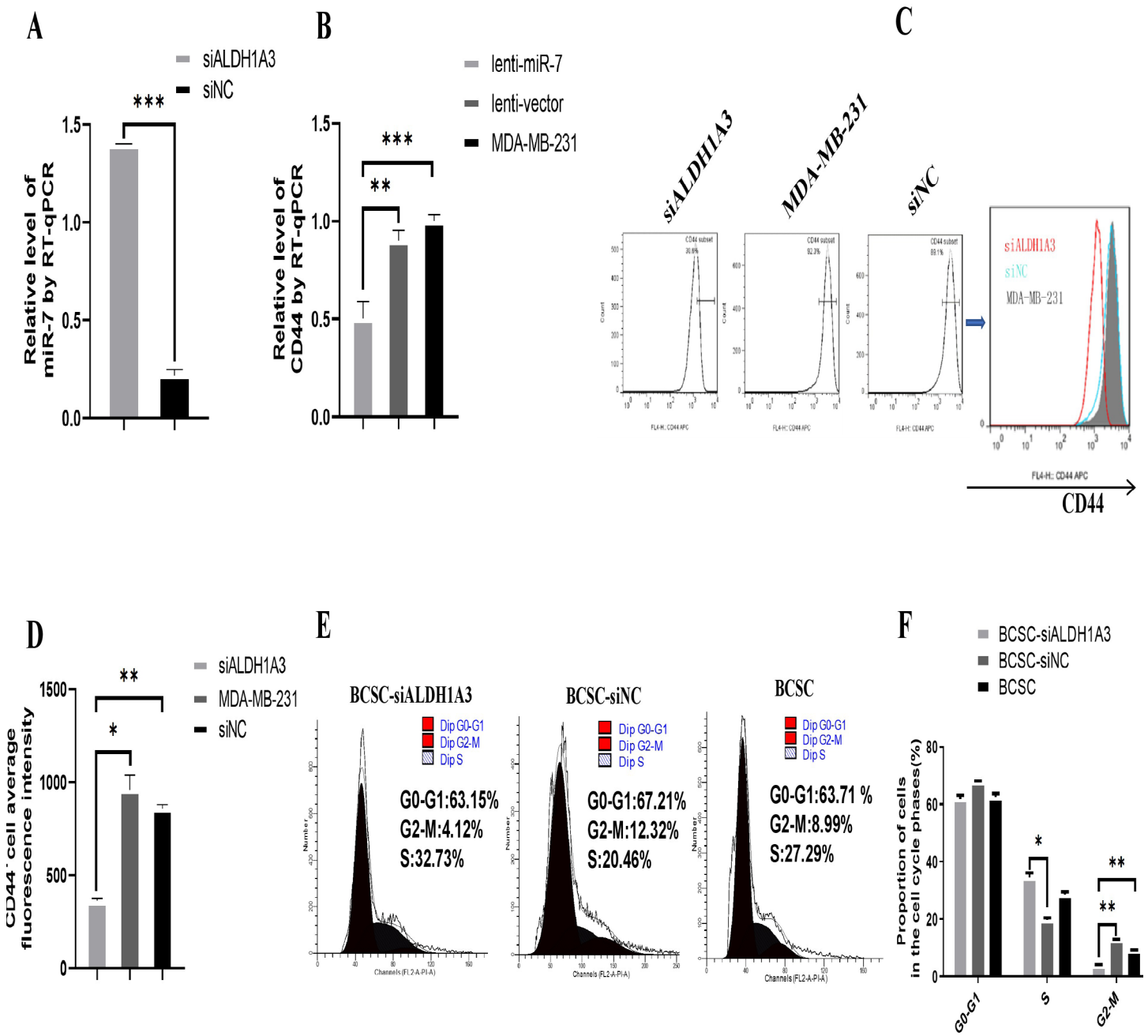

Figure 1: ALDH1A3 knockdown reduced the CD44 expression in MDA-MB-231 cells and their proliferation in BCSCs.

(A) RT-qPCR analysis of effectiveness of ALDH1A3 knockdown in MDA-MB-231 cells.

(B) Overexpression of miR-7 decreased CD44 gene expression.

(C) siALDH1A3 reduced the ratio of CD44+ cells analyzed by FCM.

(D) Statistical analysis shows that CD44+cell average fluorescence intensity was reduced.

(E) The proliferation of BCSC-siALDH1A3 cells was evaluated with FCM assay.

(F) Statistical analysis of cell cycle of BCSCs.

2.miR-7 overexpression inhibits TGF-β1 signaling pathway and downregulates CD44 expression

To prove whether miR-7 inhibits the TGF-β1 signaling pathway, Lenti-miR-7 and lentivector cells have been treated with 10 ng/mL TGF-β1 and 100 ng/mL TGF-β1 type I receptor antagonist SB431542. RT-qPCR results showed that miR-7 could obviously inhibit the effect of TGF-β1 on upregulating CD44, and that SB431542 could enhance the effect of miR-7 concomitantly, compared with the control group **(Figure 2A)**. Next, we adopted RT-qPCR to evaluate the effect of miR-7 on the main signaling molecules of the TGF-β1 signaling pathway. **Figure 2B** revealed that miR-7 downregulated the expression leves of Smad2, Smad3, and Smad4. **Figure 2C** indicated that miR-7 also downregulated the expression of TGFBR2

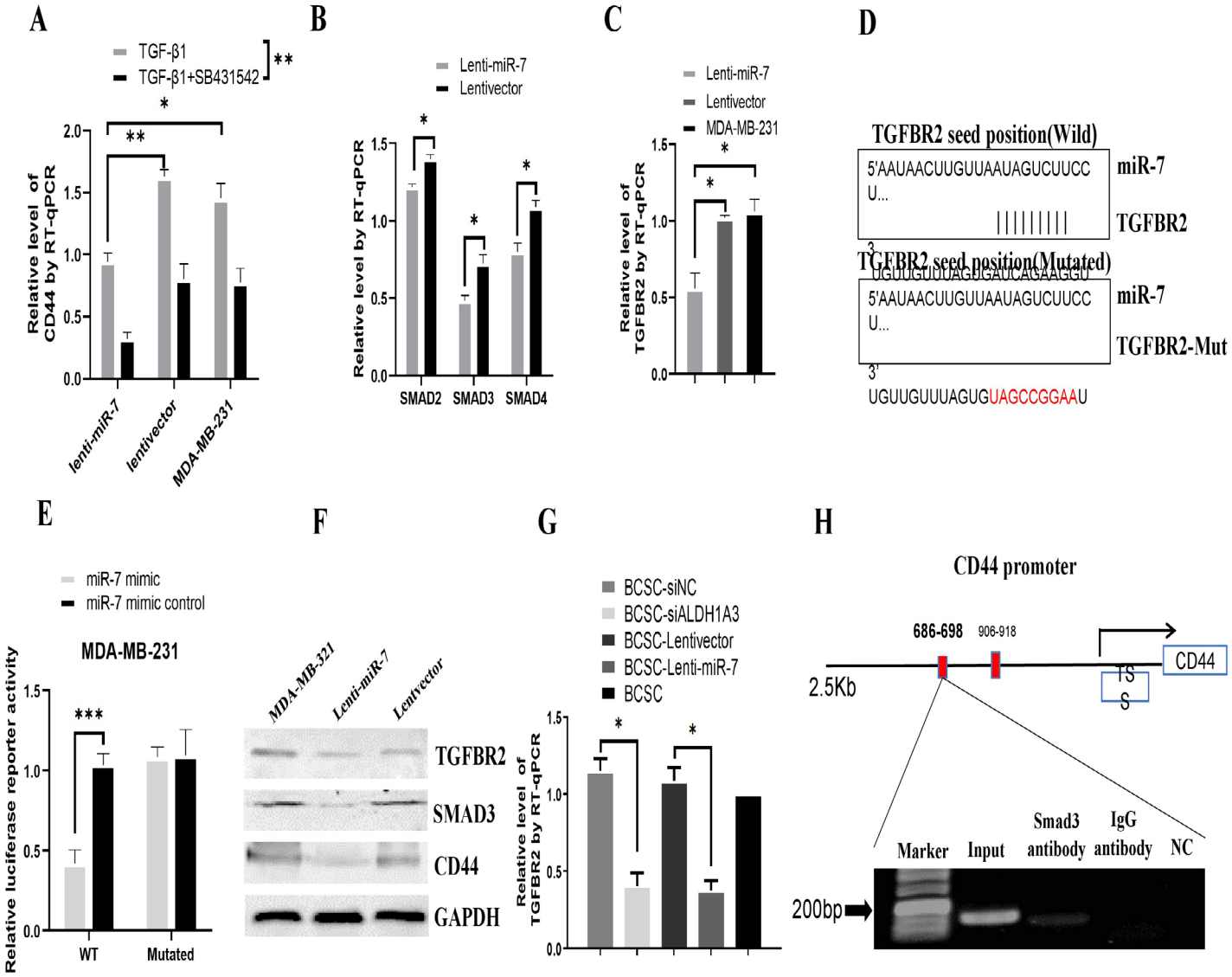

Figure 2: miR-7 directly targets TGFBR2 and Smad3 could indeed bind to the CD44 promoter region.

(A) Lenti-miR-7 and lentivector MDA-MB-231 cells were respectively incubated with TGF-β1 and TGF-β1+SB431542, and CD44 mRNA levels were measured by RT-qPCR.

(B) The expression levels of Smad2, Smad3, and Smad4 were measured by RT-qPCR in lenti-miR-7 and lentivector MDA-MB-231 cells.

(C) TGFBR2 mRNA levels were measured by RT-qPCR.

(D) TGFBR2 3'UTR with the miR-7 binding site was predicted, and complementary sequences of miR-7 to mutation TGFBR2 3'UTR are shaded red.

(E) Cells were harvested and luciferase activities were measured after 48-hour transfection.

(F) The expression levels of TGFBR2, Smad3, and CD44 analyzed by Western blotting.

(G) TGFBR2 mRNA levels were measured by RT-qPCR in BCSC-siALDH1A3, BCSC-lenti-miR-7, and control cells.

(H) ChIP-PCR assay in MDA-MB-231 cells.

(TGF-β receptor type 2). To demonstrate the targeted regulatory relationship between miR-7 and TGFBR2, the targeted binding sites of miR-7 and TGFBR2 3'UTR were predicted through the bioinformatics website (http://www.targetscan.org/vert_72/) [12] **(Figure 2D)**. For this reason, the MDA-MB-231 cells were transfected with psiCHECK-2-TGFBR2 and psiCHECK-2-TGFBR2-Mut dual-luciferase recombinant plasmids by liposomes 2000, respectively. **Figure 2E** shows that the luciferase activity of the dual-luciferase recombinant plasmid did not change in the TGFBR2 3'UTR mutation group, while the luciferase activity of the wild-type dual-luciferase recombinant plasmid showed a significant decrease, indicating that miR-7 mimic can effectively bind to TGFBR2 3'UTR and reduced the relative luciferase activity of the wild-type vector. Western blot results further proved that miR-7 downregulated TGFBR2, Smad3, and CD44 **(Figure 2F)**.Furthermore, siALDH1A3 and lenti-miR-7 were transfected with liposomes 2000 separately to verify the expression of TGFBR2 in the different treated BCSCs. Compared to the control, the expression of TGFBR2 in BCSC-lenti-miR-7 was decreased **(Figure 2G)**. Finally, the ChIP-PCR test was performed. Bioinformatics predicts that the transcription factor Smad3 binds to the upstream region of the CD44 gene promoter (http://jaspar.genereg.net/)[13, 14]. Immunoprecipitation was performed with the Smad3 antibody, and DNA fragments were eluted from the immunoprecipitation complex. The identification of a positive amplification product indicates that Smad3, a transcription factor, can regulate gene expression by binding the upstream region of the CD44 promoter **(Figure 2H)**.

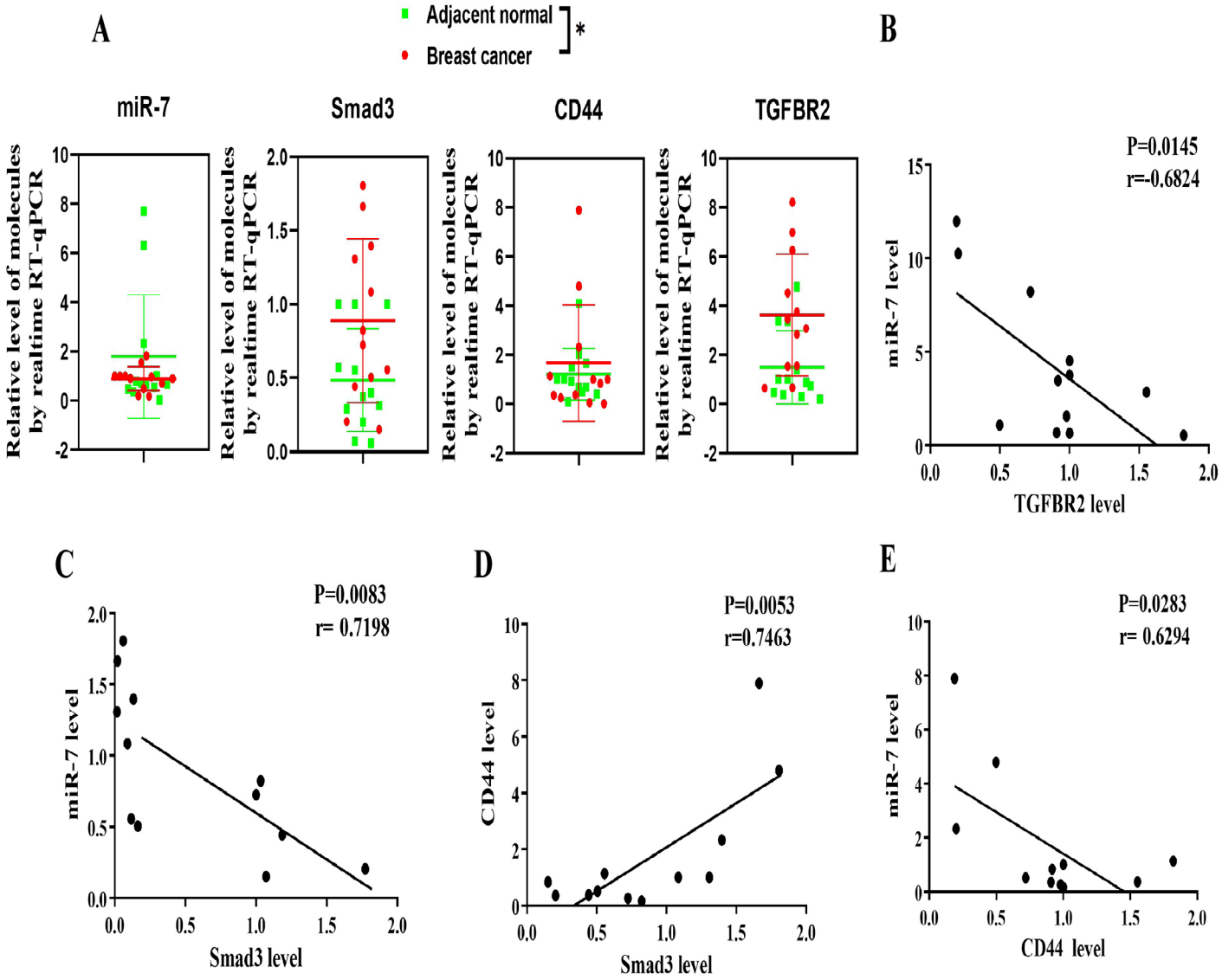

Figure 3: Detection of miR-7 and BCSC-related molecular expression in breast cancer surgical specimens.

(A) Relative expression levels of miR-7, Smad3, CD44, and TGFBR2 in breast cancer postsurgery samples analyzed by RT-qPCR.

(B–E) Relative expression levels of miR-7 and TGFBR2, miR-7 and Smad3, Smad3 and CD44, and miR-7 and CD44, respectively. The blue points represent adjacent noncancerous tissues; the red points represent tumor tissues (n=12).

3.Demonstration of miR-7-TGFBR2-Smad3-CD44 axis

We further evaluated whether there is miR-7-TGFBR2-Smad3-CD44 axis in both breast cancer cell lines and breast cancer surgical specimens. On the one hand, we obtained surgical tissues from 12 breast cancer patients. Comparing the cancerous tissues with adjacent tissues, RT-qPCR results showed that the relative expression level of miR-7 was low-expressed in breast cancer tissues; while the relative expression levels of TGFBR2, CD44, and Smad3 were significantly higher in breast cancer tissues than in adjacent noncancerous tissues, as is shown in **Figure 3A**. It was found that miR-7 was negatively correlated with TGFBR2 **(Figure 3B)**, CD44 **(Figure 3C)**, and Smad3 **(Figure 3D)**. Smad3 was positively correlated with CD44 **(Figure 3E)**. On the other hand, the relative expression levels of TGFBR2,Smad3, and CD44 were simaltaneously analyzed in breast cancer cell lines SK-BR-3, MCF-7, and LD using RT-qPCR after miR-7 mimic transfection. Figures 4A-AC show that the relative expression levels of TGFBR2, Smad3, and CD44 were all downregulated in the miR-7 mimic transfected cells. In contrast, the mRNA expressions of TGFBR2, Smad3, and CD44 were all upregulated after miR-7 inhibitor transfection that inhibited the miR-7 expression **(Figures 4D-4F)**. These results suggested that the miR-7-TGFBR2-Smad3-CD44 axis exists objectively in both breast cancer cell lines and breast cancer surgical specimens.

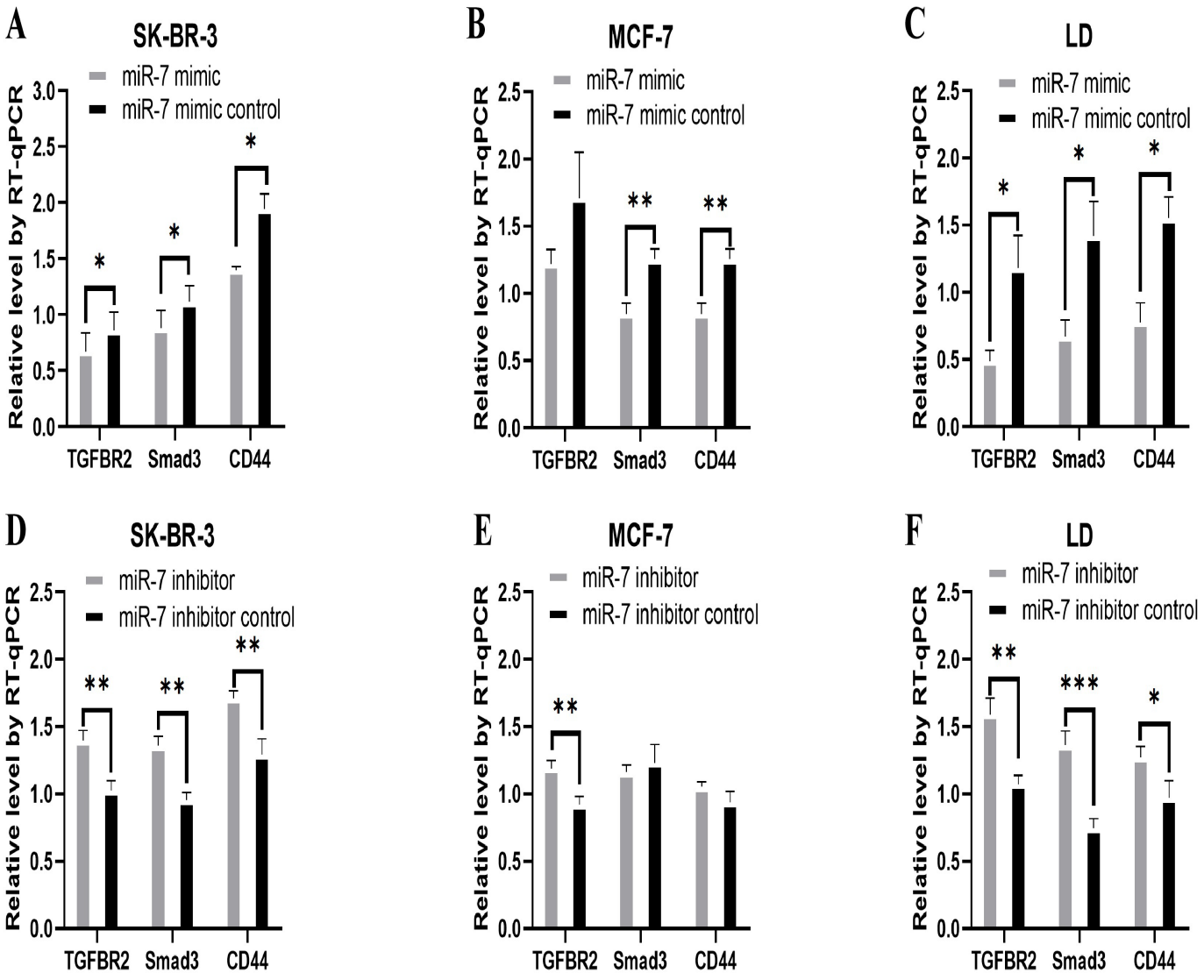

Figure 4: Detection of molecular expression in SK-BR-3, MCF-7, and LD breast cancer cell lines.

(A-C) TGFBR2, Smad3, and CD44 expressions in SK-BR-3, MCF-7, and LD cells were measured by RT-qPCR analysis after miR-7 mimic transfection.

(D-F) TGFBR2, Smad3, and CD44 relative expression levels in SK-BR-3, MCF-7, and LD cells were detected after the inhibitor transfection.

Discussion

The previous results of our research group found that the overexpression of miR-7 caused the downregulation of ALDH1A3 and CD44[8]. However, we did not explain well how miR-7 downregulates CD44 and what role of ALDH1A3 plays in impacting CD44 expression. Therefore, we attempt to explore the molecular mechanism that affects the expression of CD44 by regulating ALDH1A3 in this study.

ALDH1A3 is the major isozyme that contributes to ALDH enzyme activities in 58 human cancer cell lines. In breast cancer, glioma, and melanoma, ALDH1A3 expression is regulated by several mechanisms at epigenetic, transcriptional, and posttranslational levels[15-17]. Attenuation of ALDH1A3 expression by RNA interference (RNAi) significantly suppressed cell proliferation, reduced the number of persisted cells after anticancer drug treatment, and interfered with tumor growth in a mouse xenograft model[18]. Our previous studies have found that miR-7 expression can downregulate ALDH1A3 expression. RT-qPCR experiments showed that miR-7 expression was upregulated by downregulating ALDH1A3 by small interfering RNA (siRNA). This experiment results suggested that there is a mutual regulation between miR-7 and ALDH1A3. To further understand and uncovered the relationship between miR-7 and ALDH1A3, we carried out the investigation.

The cell surface protein CD44 has been widely used as a CSC marker in breast cancer and various other types of cancers[19, 20]. CD44 is important for tumor initiation in vivo and predominantly expressed in metastatic breast cancer cells. Previous research results have also shown that in these metastatic breast cancer cell lines, knockdown of CD44 significantly inhibits breast cancer metastasis[21, 22]. Therefore, it is of great significance to explore the mechanism of miR-7 inhibiting CD44 and reducing the tumorigenicity of BCSCs.

To better understand the relationship between miR-7 and CD44, we used a bioinformatics approach to predict that miR-7 may regulate the cell surface CD44 expression via the TGF-β1 signaling pathway. Therefore, we first need to confirm that the TGF-β1 signaling pathway is regulated by miR-7 and affects the CD44 gene expression. 10 ng/mL TGF-β1 and 100 ng/mL TGF-β1 type I receptor antagonist SB431542 were used to treat lenti-miR-7 and lentivector cells by liposomes, respectively[23]. After 72 hours, it was found that TGF-β1 can significantly increase the intracellular CD44 mRNA expression. On this basis, the inhibition of TGF-β1 by SB431542 can significantly reduce CD44 mRNA expression in MDA-MB-231 cells. Compared with lentivector cells, the expression of CD44 mRNA is lower in the case of miR-7 overexpression in lenti-miR-7 cells **(Figure 2A)**. The above results identified that the TGF-β1 signaling pathway is involved in the regulation of CD44. Next, we examined the expressions of Smad2, Smad3, and Smad4 in lenti-miR-7 cells. It was found that, in addition to the inhibitory factor, Smad2, Smad3, and Smad4 all showed decreased mRNA expression **(Figure 2B)**. This further suggests that TGF-β1 signaling is regulated by miR-7 overexpression.

To explain how miR-7 inhibits the TGF-β1 signaling pathway, we used bioinformatics to predict wether miR-7 has binding targets for TGFBR2 3'UTR **(Figure 2C)**. It is well known that TGF-β ligands assemble their corresponding receptors that contain two type 1 components and two type 2 components. Type 2 receptors serve as activators to phosphorylate type I receptors, whereas type 1 receptors function as propagators to transduce the downstream signal to cytoplasmic proteins. The components of both receptors are serine/threonine kinases. TGF-β type I receptors and activin type 1 receptors phosphorylate SMAD2/3[24, 25]. We first detected the expression of TGFBR2 by RT-qPCR and found that, in the case of miR-7 overexpression, TGFBR2 mRNA expression was downregulated **(Figure 2D)**. The results of RT-qPCR were further confirmed by Western blot **(Figure 2E)**, suggesting miR-7 affects TGFBR2 expression.

The psiCHECK-2-TGFBR2 and psiCHECK-2-TGFBR2-Mut dual-luciferase reporters were constructed, respectively. psiCHECK™-2 Vector is designed to provide a quantitative and rapid approach for the optimization of RNAi by Promega. The vectors enable the monitoring of changes in the expression of a target gene fused to the reporter gene, containing as the primary reporter gene the synthetic version of Renilla luciferase, hRluc[26]. This synthetic gene is engineered for more efficient expression in mammalian cells and for reduced anomalous transcription[27]. After transfecting miR-7 mimic and dual-luciferase reporters into MDA-MB-231 cells, the luciferase activity of wild-type cells decreased obviously, while the luciferase activity of mutant cells did not change significantly, indicating that miR-7 can bind to TGFBR2 3'UTR **(Figure 2F)**. At the same time, we also observed the mRNA expression of TGFBR2 in BCSCs that were isolated from MDA-MB-231 cells according to the phenotypes of CD44+CD24−ESA+ BCSCs [27]. The cDNA products of these BCSCs had been used again in published articles[8]. The results showed that the mRNA expression of TGFBR2 in BCSC in the siALDH1A3 and lenti-miR-7 groups was visibly lower than that in the control group **(Figure 2G)**.

Genome-wide identification of transcription factor binding sites (TFBSs) is a key to understanding transcriptional regulation. The genomic locations where TFs bind to DNA are typically short (6-20 bp) and exhibit sequence variability[28]. We obtained the DNA sequence of the 2500 bp region upstream of the CD44 promoter, used SMAD3 (Matrix ID: MA0795.1, GTCTAGAC) as a TF for motif binding prediction, and selected the TFBSs for research based on the motif conservation score[13]. The ChIP-PCR analysis strongly revealed that the Smad3 protein binds to the 686-698 position upstream of the CD44 promoter **(Figure 2H)**. The results prove that miR-7 affects the TGF-β1 signaling pathway molecule Smad3 by downregulating TGFBR2 and then inhibits CD44 gene transcription. Together, our work identifies miR-7-TGFBR2-Smad3-CD44 as a regulatory axis of BCSC marker CD44 expression.

We performed RT-qPCR experiments on cancer tissues and adjacent tissues from 12 clinical breast cancer surgical samples. It was found that, compared with adjacent tissues, miR-7 in cancer tissues showed low expression, and TGFBR2, Smad3, and CD44 showed high expression. We analyzed these molecular relationship, and found that miR-7 was negatively correlated with TGFBR2, Smad3 and CD44 repectively, and that CD44 and Smad3 were positively correlated **(Figures 3A-3E)**. Then, we used SK-BR-3, MCF-7, and LD breast cancer cell lines to analyze the effects of miR-7 on the miR-7-TGFBR2-Smad3-CD44 axis. In the case of miR-7 overexpression,

TGFBR2, Smad3, and CD44 were all downregulated **(Figures 4A-4C)**; while inhibiting miR-7 expression, all three molecules of TGFBR2, Smad3, and CD44 were upregulated in the breast cancer cell lines **(Figures 4D-4F)**.

Conclusions

Taken together, our findings show that the downregulation of ALDH1A3 can upregulate miR-7 and reduce the ratio of CD44+cells in breast cancer cells via the miR-7-TGFBR2-Smad3-CD44 axis. Significantly, these findings have potential clinical importance for understanding the multiple regulatory roles of miR-7. Inhibiting ALDH1A3 and/or miR-7 overexpression may be an important method for treating breast cancer.

Acknowledgments

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