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Original Articles

A tRNA fragment, 5′-tiRNA^{Val}, suppresses the Wnt/β-catenin signaling pathway by targeting *FZD3* in breast cancer

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ABSTRACT

tRNA-derived fragments offer a recently identified group of non-coding single-stranded RNAs that are often as abundant as microRNAs in cancer cells and play important roles in carcinogenesis. However, the biological functions of them in breast cancer are still unclear. Hence, we focused on investigating whether tiRNAs could play a key role in the progression of breast cancer. We have identified 5′-tiRNA^{Val} with significantly low expression in breast cancer tissues. The down-regulation of serum 5′-tiRNA^{Val} was positively correlated with stage progression and lymph node metastasis. Overexpression of 5′-tiRNA^{Val} suppressed cells malignant activities. *FZD3* was confirmed to be a direct target of 5′-tiRNA^{Val} in breast cancer. In addition, FZD3, β-Catenin, c-myc and cyclinD1 levels in 5′-tiRNA^{Val} overexpressing cells were downregulated while APC was inversely upregulated. Moreover, 5′-tiRNA^{Val} inhibited the *FZD3*-mediated Wnt/β-Catenin signaling pathway in breast cancer cells. Finally, 5′-tiRNA^{Val} levels differentiated breast cancer from healthy controls with a sensitivity of 90.0% and specificity of 62.7%. This is the first study to show that 5′-tiRNA^{Val} as a new tumor-suppressor through inhibition of FZD3/Wnt/β-Catenin signaling pathway, which could be as a potential diagnostic biomarker for breast cancer.

1. Introduction

Breast cancer is the most common cancer type among women and the second most common cancer overall. Each year, about 1.7 million females worldwide are diagnosed with breast cancer, and over half of them (53%) occur in less-developed regions [1]. Effective diagnostic and prognostic markers are essential for the detection and treatment of breast cancer. Although decades of research have provided considerable insights into the multistep metastatic process, the molecular and cellular mechanisms of breast cancer is still poorly understood. Given this scenario, further directions are aimed at not only deepening our understanding of the mechanisms involved in the tumorigenesis of breast cancer, but also focusing on potential therapeutic strategies.

In recent years, several studies have demonstrated a close relationship between small non-coding RNAs (e.g. miRNAs, piRNAs and circRNAs) and a variety of cancers [2–4]. Furthermore, due to increased utilization of high throughput sequencing technologies more types of non-coding RNAs are being identified [5,6]. One group of recently identified non-coding RNAs implicated in cancer biology was the tRNA derivatives, including tRNA-derived RNA fragment (tRFs) [7], tRNA-derived stress-induced RNA (tiRNAs) [8,9], and tRNA-derived small RNAs (tsRNAs) [10]. These tRNA derivatives were generated by cleavage of mature or precursor tRNAs in the particular environment [11,12]. tiRNAs, including 5′-tiRNAs and 3′-tiRNAs, were generated as a consequence of the anticodon loop by a ribonuclease, angiogenin (ANG), to produce 30–35 nt 5′-tiRNAs and 40–50 nt 3′-tiRNAs [13]. 5′-tiRNAs bound to tRNase Z^{L} (tRNA endonuclease) which cleaved the target gene complementary to the 5′-tiRNAs sequence where 5′-tiRNAs served as a small guide RNA, thereby down-regulating the expression of target genes [14,15]. Some studies have also demonstrated that

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complementary between some tRNA derivatives and their target mRNAs is indispensable for efficient silencing, and tRNA derivatives are found associated with Argonaute proteins which are essential for target recognition in an RNAi manner [16,17]. Further study have revealed that tRNA derivatives may play a major role in RNA silencing [18]. Generally, increasing evidence showed that the dysregulation of a series of tRNA derivatives is associated with several types of human disease and could be new diagnostic biomarkers or therapeutic targets [19].

In this study, we focused on investigating whether tiRNAs could play a key role in the progression of breast cancer. Based on the tRFs & tiRNAs sequencing analysis, we identified a group of tRFs & tiRNAs that were differentially expressed in breast cancer. Among them, a 5′ fragment of tRNA-Val-CAC (5′-tiRNA^{Val}) attracted our attention as we demonstrated that 5′-tiRNA^{Val} was capable of directly binding a predicted target, the human Frizzled homolog 3 (*FZD3*), in breast cancer cells. Human frizzed-3 protein is mapped to chromosome 8p21, which is one of the major components of the Wnt signaling pathway and plays a crucial role in regulating early neurodevelopmental processes [20,21]. However, regulatory mechanisms governing the expression of *FZD3* in breast cancer remain unclear. To identify the function and regulation of 5′-tiRNA^{Val} and *FZD3* in breast cancer, we demonstrated that 5′-tiRNA^{Val} mediated *FZD3* downregulation inhibited the malignant activities of breast cancer cells, suggesting that 5′-tiRNA^{Val} plays an important role in breast carcinogenesis through regulation of *FZD3*.

2. Materials and methods

2.1. Clinical samples

The study was approved by the Clinical Research Ethics Committee of Nanjing Medical University. Written informed consent was obtained from all study patients. Breast cancer tissue samples and matched non-tumor adjacent tissues (NATs) were obtained from patients who underwent surgical resection in Jiangsu Cancer Hospital and stored at −80 °C until further processing. Meanwhile, we collected 60 serum samples from patients with breast cancer. Pathological classification, grading, and staging were made on WHO Classification of Breast Tumor, 2012, differentiation status of cancer cell, and TNM system [22]. Furthermore, serum from 20 healthy controls were also obtained. Serum samples were extracted from whole blood after centrifugation (12000 g, 10 min) and stored at −80 °C until further processing.

2.2. tRFs&tiRNAs sequences processing and expression analysis

Total RNA was pretreated to remove some RNA modifications that interfere with small RNA-seq library construction by rtStar™ tRF&tiRNA Pretreatment Kit (Cat#: AS–FS–005, Arraystar, MD, USA). The kit removes 3′- aminoacyl and 3′-cP for 3′ adaptor ligation, phosphorylates 5′-OH for 5′-adaptor ligation, and demethylates m1A, m1G, and m3C for efficient cDNA reverse transcription [23,24]. These modifications include terminal modifications that block adaptor ligation to the RNA ends and internal methylations that hider reverse transcription for cDNA synthesis [25]. Each sample was taken for tRF&tiRNA-seq library preparation by rtStar™ First-Strand cDNA Synthesis Kit (3′ and 5′ adaptors) (Cat#: AS–FS–003, Arraystar, MD, USA). Library preparation procedures included: 1) 3′-adapter ligation; 2) 5′-adapter ligation; 3) cDNA synthesis; 4) PCR amplification; 5) size selection of ~135-160bp PCR amplified fragments (corresponding to 15–40 nt small RNAs). The completed libraries were quantified by Agilent 2100 Bioanalyzer (Agilent, California, USA). Illumina NextSeq 500 raw sequencing reads that passed the Illmina chastity filter were used for the sequence analysis. After quality control, trimmed reads (with 5′, 3′-adaptor based removed) were aligned to mature-tRNA and pre-tRNA reference sequences and filtered for ≥ 15 nt using cutadapt software (http://doi, org/10.14806/ej,17.1.200) [26]. Then the sequencing reads were aligned to mature-tRNA on the entire genome using MINTbase v2.0 (http://cm.jefferson.edu/MINTbase/) [27]. The tRFs&tiRNAs-Seq data focused on alignment of all kinds of tRNA derivatives.

2.3. RNA isolation, reverse transcription, and quantitative real-time RT-PCR

Total RNA was extracted from cells and tissue using Trizol reagent (Life Technologies, USA). While total RNA from patients' serums were isolated with Trizol LS reagent (Life Technologies, USA), following the manufacturer's protocol. The OD 260/280 absorbance ratios of all the samples were between 1.8 and 2.0 and stored at −80 °C in RNase-free water. And then both miRNA and mRNA were reverse transcribed to cDNA. The Bulge-Loop miRNA Stater Kit (Ribobio, China) with specific stem-loop RT primers was used to quantify the expression of 5′-tiRNA^{Val}, following the manufacturer's protocol. RT reaction conditions were 60 min at 42 °C and 10 min at 70 °C. Then, qPCR was performed using the SYBR Green Mix containing Taq enzyme, dNTP mix, PCR Buffer and SYBR Green I [23]. After adding forward primer and reverse primer, the mixtures were incubated at 95 °C for 10 min, followed by 40 cycle of 95 °C for 10 s, 60 °C for 20 s, and 70 °C for 10 s (5′-tiRNA^{Val}). Quantitative real-time PCR for *FZD3* and other genes were performed using the SYBR® Select Master Mix (Takara). RNU6B was used for 5′-tiRNA^{Val} template normalization and beta actin (β-actin) for mRNA template normalization. The levels of miRNA and mRNA were calculated using $2^{-\Delta\Delta Ct}$ and $2^{-\Delta Ct}$ method for relative quantification of gene expression, in which $\Delta Ct = Ct_{5'\text{-tiRNA}^{Val}} - Ct_{U6}$ or $\Delta Ct = Ct_{\text{genes of interest}} - Ct_{\beta\text{-actin}}$, and $\Delta\Delta Ct = \Delta Ct_{case} - \Delta Ct_{control}$ [28]. The primers sequences were listed in Additional file 1: Table S1.

2.4. Cell culture

The human breast cancer cell lines MDA-MB-231, MCF-7 and human breast cells HBL-100 were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). These cells were maintained in DMEM medium and supplemented with 10% (v/v) fetal bovine serum (Gibco Invitrogen, Carlsbad, CA) in a 37 °C 5% CO_2 incubator.

2.5. Cell transfection

Cells were seeded into 6-well plates 24 h before transfection and transfected with lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. The tiRNA synthetic single-strand mimics and corresponding negative control were transfected using Lipofectamine 2000. A final concentration of 100 nM mimics for 5′-tiRNA^{Val} and the negative controls (Ribobio, Guangzhou, China) were optimized for each well. Short interfering (si)RNAs targeting *FZD3* were obtained from GenePharma Co., Ltd (Shanghai, China). Three different siRNAs against *FZD3* were chosen (si776 sense strand: GCACUCUACGCUCCUAUUUTT, and anti-sense strand: AAAUAGGAGCGUAGAGUGCTT; si1260 sense strand: GCUACAUGAUGGUAUCCUUTT, and anti-sense strand: AAGG AUACCAUCAUGUAGCTT; si1876 sense strand: GGAAACAACGUGGAU ACAATT, and anti-sense strand: UUGUAUCCACGUUGUUUCCTT).

2.6. Gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment analyses of target genes

For GO mapping, the GO terms for 5′-tiRNA^{Val} target genes based on homologies were extracted (GO; http://www.geneontology.org). Fisher's exact test was used to find if there was more overlap between the differentially expressed genes (DEGs) list and the GO annotation list than expected. GO terms with corrected $P < 0.05$ were considered to be significantly enriched. KEGG pathways for target genes were retrieved form KEGG database (http://www.genome.jp/kegg/). A pathway with an FDR of < 0.05 was defined as an enrichment pathway.

2.7. Luciferase assay and constructs

The plasmid reporters which contained the predicted 3′-UTR sites in *FZD3* for 5′-tiRNA^{Val} were analyzed using a Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). The *FZD3* 3′-UTR wild-type and a *FZD3* 3′-mutant were synthesized and inserted into the pmirGLO luciferase reporter vector to produce pmirGLO-FZD3-wt and pmirGLO-FZD3-mut constructs, respectively. The cells were co-transfected with wild-type or mutant reporter plasmid vector and 5′-tiRNA^{Val} mimics or negative control. After 48 h, cells were harvested for analysis.

2.8. Western blot

Total protein was extracted from cells and the protein concentration determined using a BCA protein assay kit (Servicebio, #G2026). Equal amount of protein from each sample were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes and incubated with primary antibodies directed against target proteins: FZD3 (#ab75233; Abcam), APC (#ab40778; Abcam), c-myc (#ab32072; Abcam), cyclinD1 (#ab134175; Abcam), β-Catenin (#GB11015, Servicebio). The ECL method (Millipore) was used to detect the expression of proteins.

2.9. Cell proliferation assay

Cell viability and proliferation were determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Laboratories, Japan) according to the manufacturer's instructions. Cells were seeded in 96-well plates at an initial density of 3×10^3 cells/well. After 24 h, 48 h, and 72 h of culture, 10 μl of CCK-8 solution was added and the plates were incubated for 3 h at 37 °C. Absorbance at 450 nm was determined by a microplate reader, and the absorbance of the cultured medium plus CCK-8 in the absence of cells was used as a background control. Experiments were performed in triplicate.

2.10. Colony formation assay

For colony formation assay, after transfection with mimics or siRNA for 48 h, cells were plated in 6-well plates (1×10^3 cells/well) and incubated for 7 days. Subsequently, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and then stained with 1% crystal violet. The number of colonies was detected and counted under a light microscope. The percentage of the colony formation was measured by adjusting control to 100%.

2.11. Transwell assay

After 24 h transfection, cells were digested with trypsin. Then cells were seeded into Transwell upper chamber (Corning Inc costar®, USA). The upper chamber was added with serum-free medium, and 500 μl medium containing 10% (v/v) FBS in the lower chamber. Cells were cultured in an incubator for 24 h, and then non-migrated cells in the upper chamber were removed with cotton swabs, and the cells on the lower surface of the inserts were fixed and stained. The numbers of cells across the polycarbonate membrane was counted, and served as the migration ability of cells. As for cell invasion capability, the cells were seeded into Transwell upper chamber (Corning® Matrigel® invasion chamber, USA). After incubated with 4×10^4 cell suspension into the upper chamber, the upper chamber was added with serum-free medium, and lower chamber was added with culture medium containing 10% FBS. The number of cells in each group across the Matrigel was counted and used as the invasion ability of cells. Assay were conducted in triplicate in three independent experiments.

2.12. Wound healing assay

Transfected cells were plated in the 6-well plate and the cell monolayer was scraped using a 10 μl pipette tip. Floating cells and debris were carefully removed with PBS. The initial (0 h) and residual gap width of 24 h after wounding were calculated from photomicrographs. The percentage of the wound healing was calculated as (the width of wound at 0 h–the width of wound at 24 h)/the width of wound at 0 h.

2.13. Statistical analysis

Statistical analysis was conducted using SPSS Statistics Version 20.0 and GraphPad Prism v6.0. Data were presented as mean ± standard error on the mean (SEM). Differences were analyzed by Student's *t*-test between two groups. The Mann–Whitney *U* test or the Wilcoxon match pairs test was used to evaluate continuous data. Pearson's Chi squared test and Fisher's exact test were used to assess the correlation between 5′-tiRNA^{Val} levels and clinicopathological factor in breast cancer. The efficacy of 5′-tiRNA^{Val} was evaluated by sensitivity, specificity, and area under receiver operating characteristic (ROC) curve (AUC). The sensitivity-specificity relationship was determined using Youden's index. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of tRFs&tiRNAs in breast cancer tumor tissues and serum

We used a high-throughput sequencing technique to determine differential expression of tRFs&tiRNAs in 6 pairs of breast cancer patients and matched non-tumor adjacent tissues specimens. According to the tRF&tiRNA-Seq data, we identified 30 tRFs&tiRNAs between the two groups, of which 17 were up-regulated (fold change > 2.6, $P < 0.05$) and 13 were down-regulated (fold change > 2.0, $P < 0.05$) in tumor tissues relative to NATs (Fig. 1A). Among the 13 types of low-expressing tRNA derivatives, derivatives from tRNAs-Val and tRNAs-Asp were most prevalent in tumor tissues, comprising of 37.93% and 20.69% respectively (Fig. 1B). To validate the results of tRF&tiRNA-Seq data, we further collected 16 pairs of breast cancer samples and detected these tRFs&tiRNAs expression levels. Intriguingly, significantly lower levels of AS-tDR-001430 were detected in tumor tissues than NATs ($P = 0.0007$, Fig. 1C). Meanwhile, we detected the expression level of AS-tDR-001430 in serum from 60 patients and found that AS-tDR-001430 levels were significantly decreased in breast cancer patients as compared to healthy controls ($P = 0.0011$, Fig. 1D). Lower expression of AS-tDR-001430 was also observed in patients with higher TNM stages (stage I-II *vs.* stage III-IV: $P = 0.0358$) and lymph node metastasis ($P = 0.0311$). Consistently, lower levels of serum AS-tDR-001430 were significantly correlated with higher TNM stage ($P = 0.035$) and lymph node metastasis in breast cancer patients ($P = 0.018$) (Table 1). Given that no studies have reported this novel tRNA derivative, we reasoned that AS-tDR-001430 maybe act as a tumor suppressor to inhibit breast cancer progression.

3.2. AS-tDR-001430 is a type of tRNA halves

AS-tDR-001430 was mapped to chromosome 6p22.1 with coordinates of 27,248,049–27,248,121 and a length of 73 bp using the UCSC Genome Browser database (Fig. 2A). We detected the product of qRT-PCR with agarose gel electrophoresis, and showed a single electrophoresis band about 73 bp in size. After recovering and cloning, they were confirmed by sequencing (Fig. 2B). In the MINTbase v2.0 (http://cm.jefferson.edu/MINTbase/.), AS-tDR-001430 was a 5′-half fragment of tRNA-Val-CAC, which was processed from the mature tRNA-Val-CAC-2-1 (Fig. 2C). Based on the mature tRNA sequencing, the fragment was 32 nt (5′-GCTTCTGTAGTGTAGTGGTTATCACGTTCGCC-3′) long

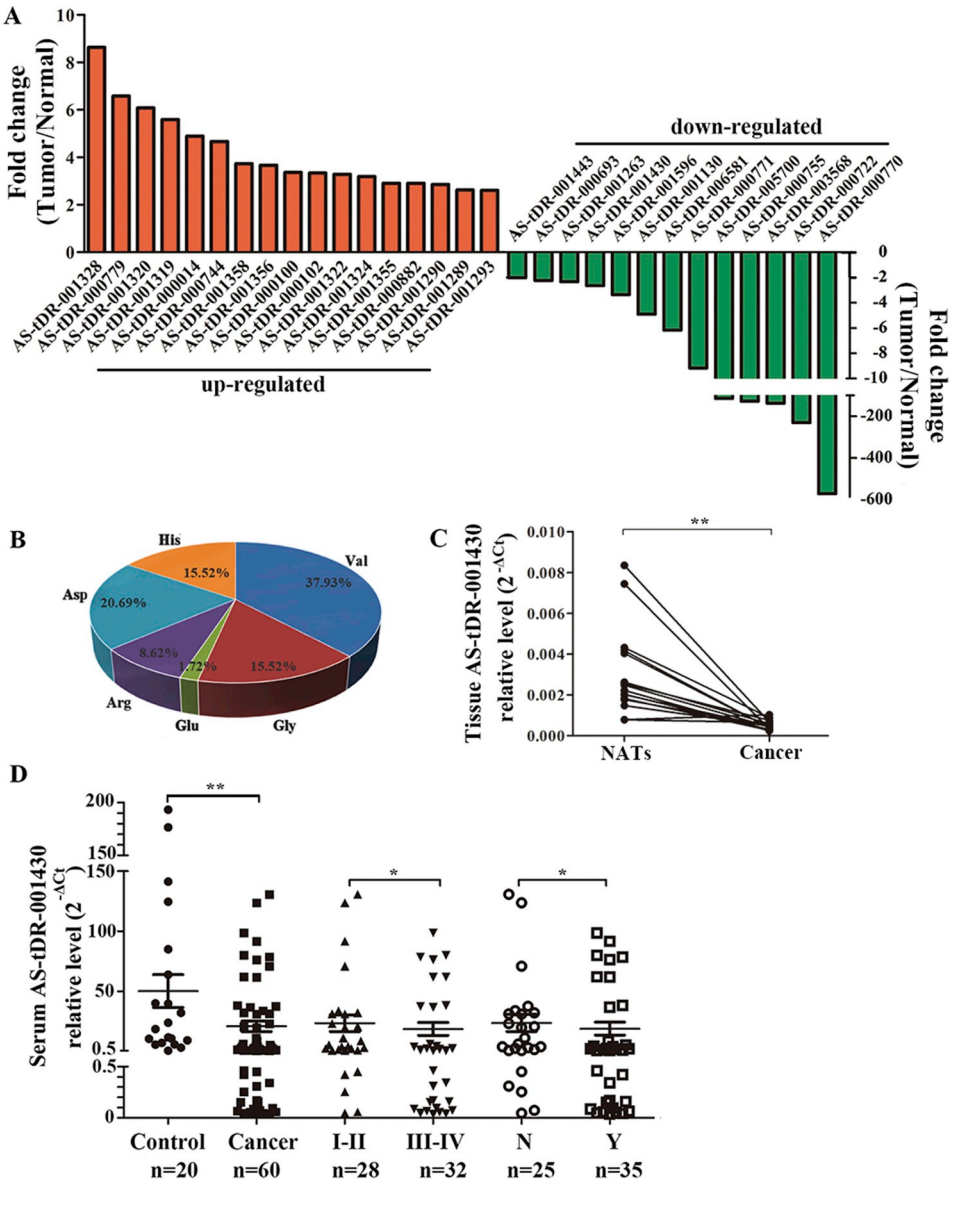

Fig. 1. Expression of tRFs&tiRNAs in breast cancer tumor tissues and serum

(A) Thirty tRFs&tiRNAs between the two groups, of which 17 were up-regulated and 13 were down-regulated in tumor tissues relative to NATs. (B) As two of the most prevalent tRNA derivatives, tRNAs-Val and tRNAs-Asp occupied 37.93% and 20.69% of the 30 kinds of derivatives. (C) Expression of AS-tDR-001430 was quantified by qRT-PCR in tissues and NATs, and its expression is normalized by U6 RNA in each sample. (D) Scatter plot representation of serum AS-tDR-001430 levels in healthy controls and breast cancer patients. U6 was used normalization. $*P < 0.05$; $**P < 0.01$, statistically significant. NATs, non-tumor adjacent tissues.

Table 1
Correlation between AS-tDR-001430 and clinicopathological characteristics in serum.

Characteristics	N	AS-tDR-001430 low expression (≤median)	AS-tDR-001430 high expression (> median)	χ^2	*P*-value
Number	60	30	30		
Age (years)				1.071	0.219
≤50	32	14	18		
> 50	28	16	12		
Tumor stage					
I-II	28	10	18	4.286	0.035*
III- IV	32	20	12		
Lymph node metastasis					
No	25	8	17	5.554	0.018*
Yes	35	22	13		

* Statistical significance ($P < 0.05$).

and the cleavage site was located on the anticodon loop (CTCACAC) (Fig .2D). According to the naming method of Lyons et al. [29], we named AS-tDR-001430 as 5′-tiRNA^{Val}.

3.3. 5′-tiRNA^{Val} regulates proliferation, migration and invasion of breast cancer cells

qRT-PCR analysis showed that 5′-tiRNA^{Val} levels were significantly lower in MDA-MB-231 and MCF-7 cells than in HBL-100 cells (Fig. 3A). The significantly reduced expression of 5′-tiRNA^{Val} in breast cancer cells compared with corresponding controls inspired us to investigate the biological function of 5′-tiRNA^{Val} in breast cancer. To assess the potential involvement of 5′-tiRNA^{Val} in breast cancer cells, we applied experiments of gain-of function of 5′-tiRNA^{Val} in MDA-MB-231 and MCF-7 cells, respectively. The 5′-tiRNA^{Val} mimics or negative controls were transfected into cells, and the relative expression of 5′-tiRNA^{Val} was shown in Additional file 2: Fig. S1. CCK-8 assay and colony formation assay demonstrated that 5′-tiRNA^{Val} overexpression significantly suppressed cell viability and colony formation in MDA-MB-231 and MCF-7 cell lines (Fig. 3B–C). Meanwhile, we found that 5′-tiRNA^{Val} overexpression markedly reduced the migration and invasion of breast cancer cells (Fig. 3D–E). Taken together, these results suggest that 5′-tiRNA^{Val} acts as a potential tumor-suppressor in the progression of breast cancer.

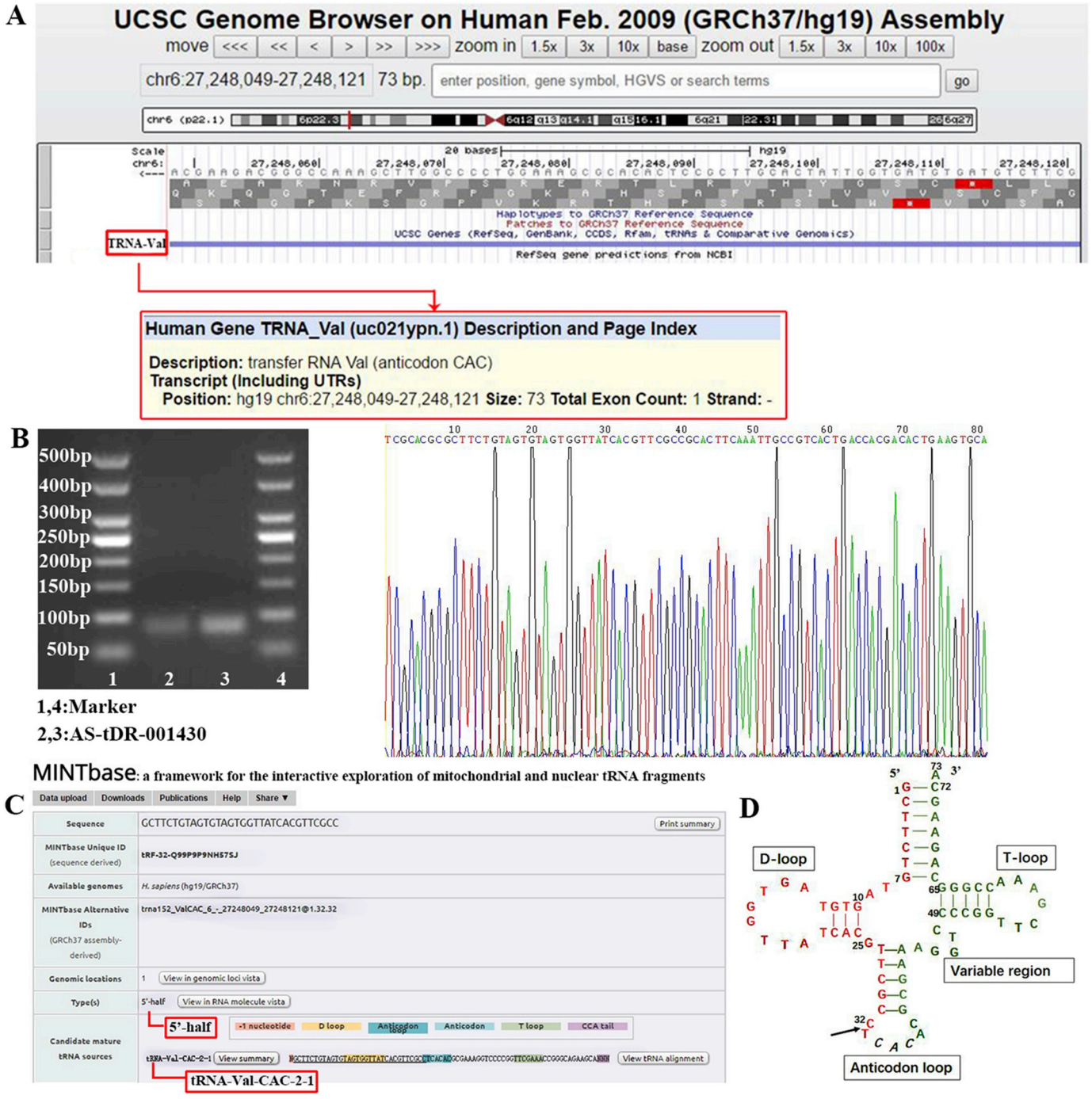

Fig. 2. AS-tDR-001430 is a type of tRNA halves

(A) AS-tDR-001430 is located on chromosome 6p22.1, with the length of 73bp using the UCSC Genome Browser database. Details item can be displayed by clicking on it, as pointed out by the red arrows. (B) The product of RT-PCR was run on 2% agarose gel and confirmed by sequencing. (C) AS-tDR-001430 is a 5′-half fragment of tRNA-Val-CAC, which is processed from the mature tRNA-Val-CAC-2-1. (D) 5′-tiRNA^{Val} was derived from ends of tRNA-Val-CAC with the length of 32 nt. The cleavage site is located on the anticodon loop. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. GO and KEGG enrichment analysis of 5′-tiRNA^{Val} target genes

To explore the molecular mechanisms of 5′-tiRNA^{Val} in breast cancer, potential target genes of 5′-tiRNA^{Val} were analyzed by GO and KEGG analyses. The results of the GO analysis of the DEGs-5′-tiRNA^{Val} target genes are shown in Fig. 4A. The significantly enriched GO were mainly distributed in the Biological Process (BP) categories, Cellular Component (CC) and Molecular Function Classification (MF). The target genes enriched in the BP category were mainly involved the following terms: biological regulation, metabolic process, regulation of biological process, regulation of cellular process and metabolic process. In the CCs, the main differences were involved in the following terms: cell, cell part, intracellular, intracellular part organelle part, membrane-bounded organelle and cytoplasm. In the MF, the main differences included: binding, protein binding, ion binding and organic cyclic compound binding. The KEGG pathway enrichment analyses showed that 10 pathways changed significantly ($P < 0.05$) in breast cancer tissues in the current study (Fig. 4B).

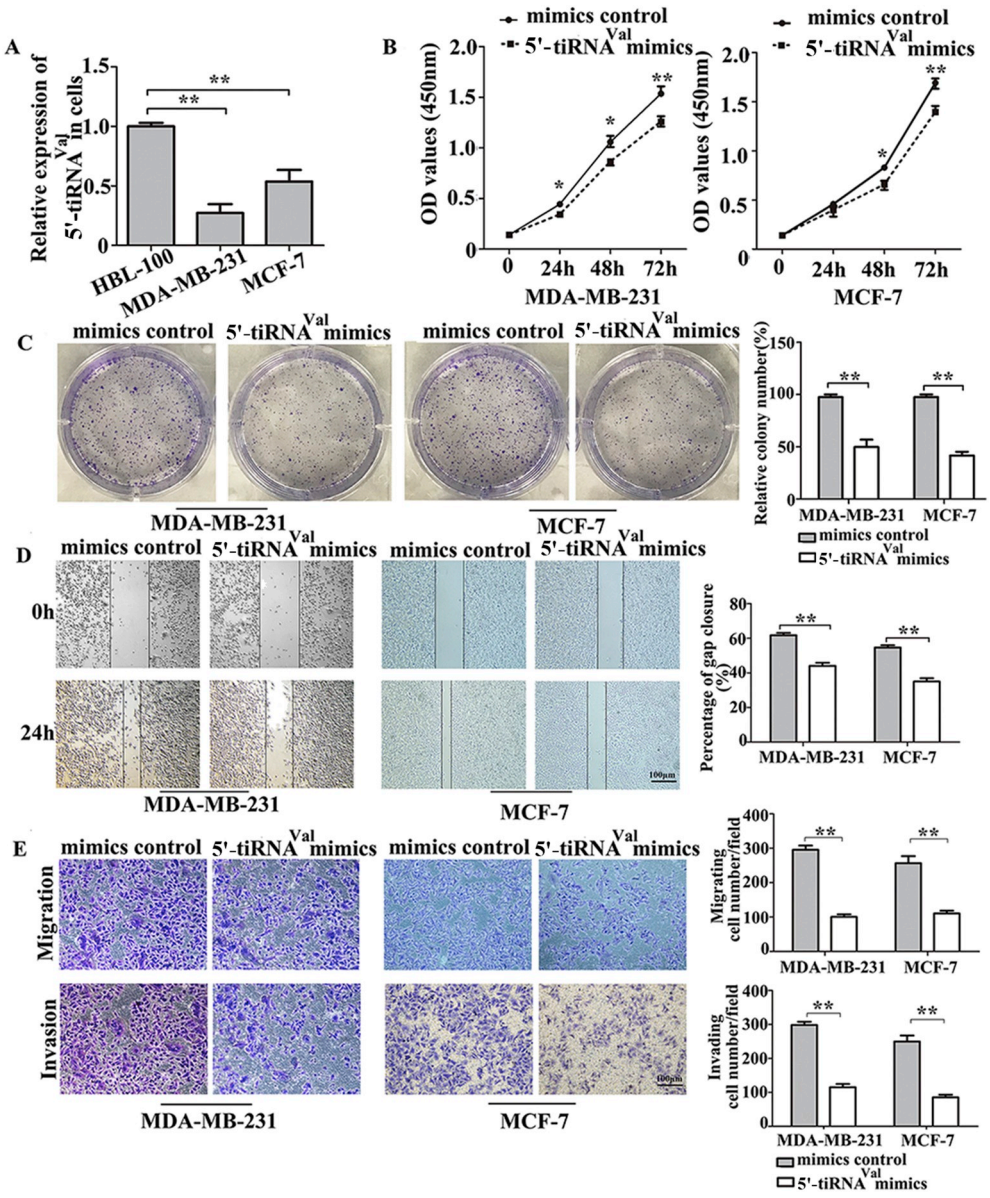

Fig. 3. The effect of 5′-tiRNA^{Val} on breast cancer cells proliferation, migration and invasion.

(A) 5′-tiRNA^{Val} expression levels in HBL-100, MDA-MB-231 and MCF-7 cells were detected by qRT-PCR. (B) Cell viability of breast cancer cell lines after transfection with 5′-tiRNA^{Val} mimics or negative control was detected by CCK-8 assay. (C) Colony formation assays in the breast cancer cell lines overexpressing 5′-tiRNA^{Val}. (D) The wound healing assays were performed to detected the cell migration in breast cancer cell lines transfected with 5′-tiRNA^{Val} mimics or negative control. And numbers of cells invaded were counted in five fields. (E) Representative images and bar graphs were depicted to investigate the migration and invasion ability of breast cancer cells after the 24 h transfection mimics or the negative control. $^{**}P < 0.01$, $^{*}P < 0.05$, statistically significant.

Meanwhile, we found that a common pathway for GO and KEGG enrichment analysis was Axon guidance pathway (GO ID: 0007411; Pathway ID: hsa04360). Axon guidance pathway genes have been shown to have aberrations in pancreatic cancer, breast cancer, lung cancer and leukemia [30,31]. From the intersection of the Axon guidance pathway with pathways in cancer (Pathway ID: has05200) in the KEGG enrichment analysis, we obtained 4 candidate genes (*FZD3*, *PIK3CB*, *PRKCA* and *PTCH1*) (Fig. 4C).

3.5. 5′-tiRNA^{Val} directly regulates FZD3 expression in breast cancer cells

We performed qRT-PCR to screen the genes downregulated by 5′-tiRNA^{Val} and found that *FZD3* mRNA levels were most downregulated by 5′-tiRNA^{Val} in the MDA-MB-231 and MCF-7 cells when compared with the controls (Fig. 5A). In addition, Western-blot analysis showed that upregulation of 5′-tiRNA^{Val} induced a significant decrease of FZD3 levels in cells (Fig. 5B). These results suggest that *FZD3* expression is downregulated by 5′-tiRNA^{Val} in breast cancer.

Analysis of the *FZD3* 3′-UTR sequence using TargetScan (http://www.targetscan.org/) and microRNA.org (http://www.microrna.org/microrna/) revealed a possible binding site for 5′-tiRNA^{Val}, implying that the *FZD3* gene transcript may be a direct target of 5′-tiRNA^{Val}. Luciferase-reporter plasmids containing the wild type and mutant 3′-UTR of *FZD3* were constructed (Fig. 5C). The wild type or mutant constructs were co-transfected with 5′-tiRNA^{Val} mimics or negative control into MDA-MB-231 and MCF-7 cells, respectively. As shown in Fig. 5D, the mimics of 5′-tiRNA^{Val} significantly reduced the related luciferase activity when co-transfected with the wide-type reporter plasmid. However, 5′-tiRNA^{Val} mimics had no effect on the luciferase activity of the mutant reporter plasmid. Taken together, these results indicated that 5′-tiRNA^{Val} inhibits the translation of *FZD3* by directly targeting its 3′-UTR.

3.6. Silence of FZD3 inhibits migration and proliferation of breast cancer cells

Next, we transfected breast cancer cells with three different siRNAs to *FZD3* or scramble controls and demonstrated that si1260 was the most effective siRNA in inhibition of *FZD3* mRNA expression in MDA-MB-231 and MCF-7 cells (68% and 83%, respectively; Fig. 6A). Moreover, the protein levels of FZD3 were also reduced by 64% and 35%, respectively, in these breast cancer cells (Fig. 6B). We further measured

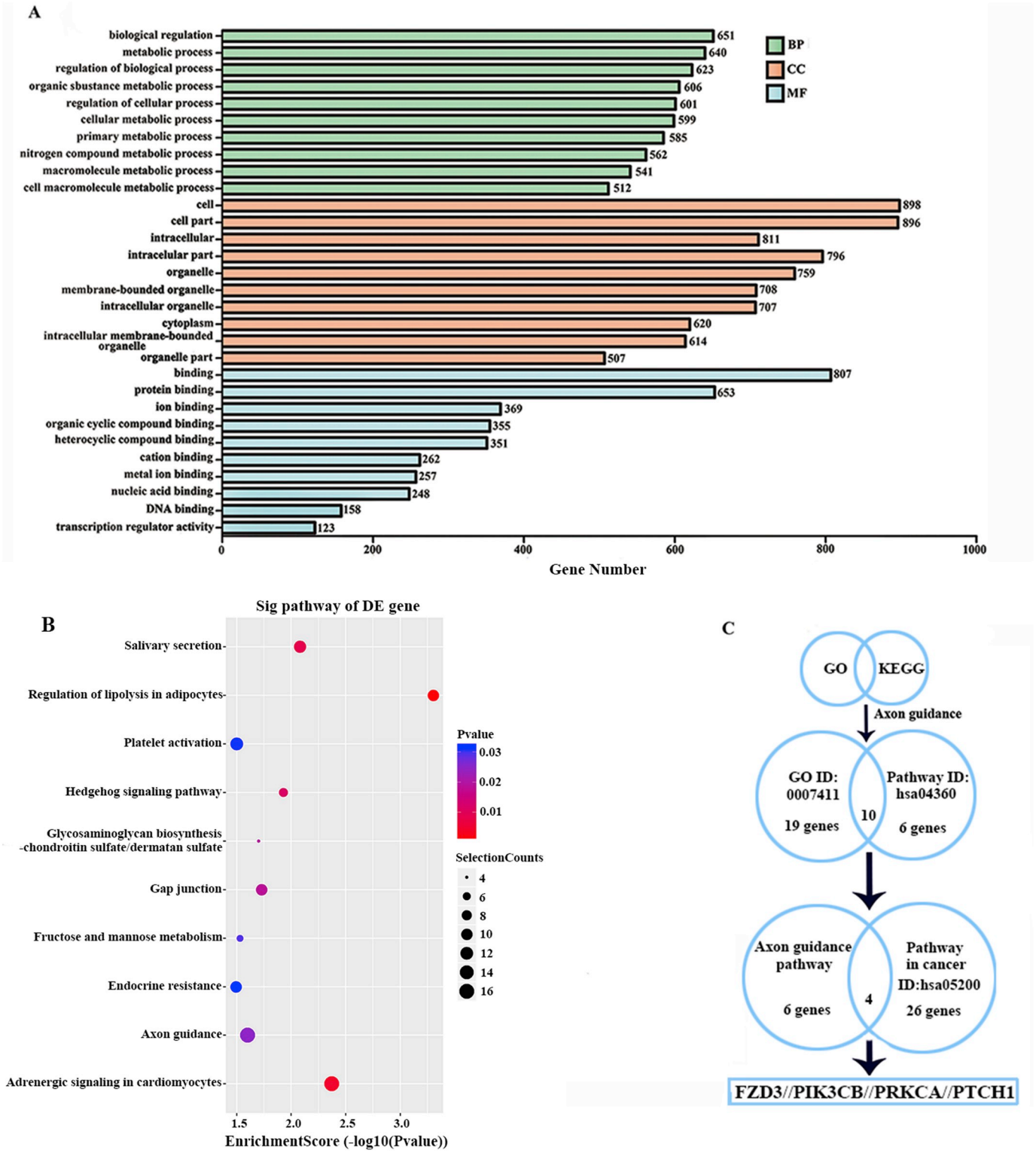

Fig. 4. GO and KEGG enrichment analysis of DEGs-5′-tiRNA^{Val}'s target genes

(A) Bar graph of the most enriched GO terms of target genes (the y-axis shows the percentages of differentially expressed associated with each term, and the x-axis shows the BP, CC, and MF terms). (B) KEGG pathway analysis showed that the 10 related pathways changed significantly in breast cancer tissues. (C) Initial screening of 5′-tiRNA^{Val} target genes in breast cancer using GO and KEGG enrichment analysis predictions and literature review. A total of four genes were selected. DEGs: differentially expressed genes.

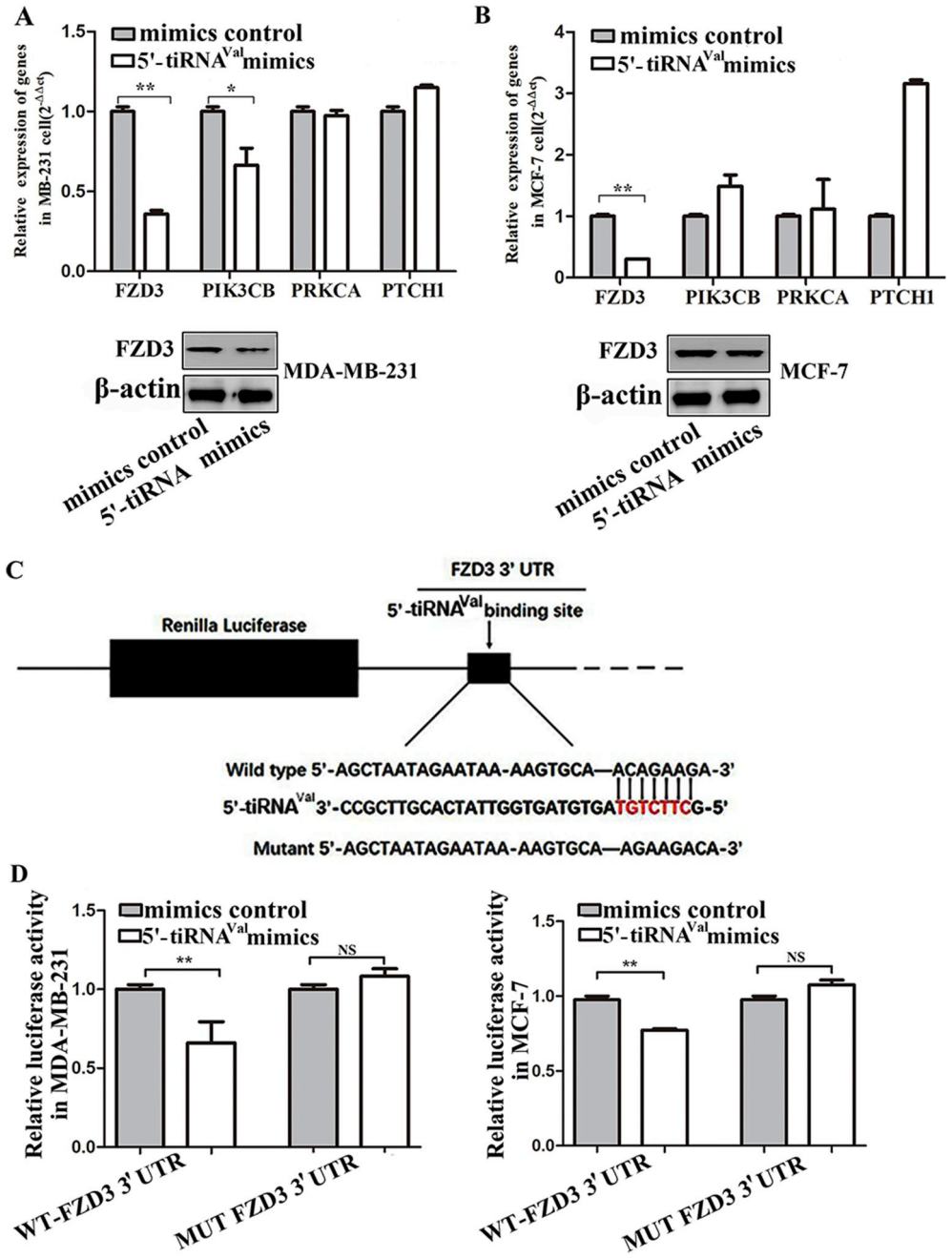

Fig. 5. 5′-tiRNA^{Val} directly regulates *FZD3* expression in breast cancer cells.
(A and B) *FZD3* mRNA expression and protein levels in transfected MD-MB-231 cells (A) and MCF-7 cells (B), respectively. (C) 3′-UTR fragment of wide-type (wt) and mutated which disrupted interaction with 5′-tiRNA^{Val}. (D) The wt or mutated reporter plasmid was co-transfected into MD-MB-231 and MCF-7 cells with 5′-tiRNA^{Val} mimics or negative control. Luciferase activity of wt *FZD3* 3′-UTR was significantly decreased by 5′-tiRNA^{Val} in cells. $^{**}P < 0.01$, statistically significant. NS: no significance.

cell proliferation, migration and invasion in response to *FZD3* knockdown with si1260. As shown in Fig. 6C–D, CCK-8 assay and colony formation assay showed that silencing of *FZD3* significantly inhibited cell proliferation. Similar to the effect of 5′-tiRNA^{Val}, *FZD3* knockdown markedly reduced the migration and invasion of breast cancer cells in *trans*-well studies (Fig. 6E–F).

3.7. Attenuation of FZD3 expression rescues the 5′-tiRNA^{Val}-mediated inhibitory effects on breast cancer cells

To further determine whether 5′-tiRNA^{Val} regulates the biological function of breast cancer cells via regulation of *FZD3* expression, we performed a “rescue” experiment to investigate the effect of 5′-tiRNA^{Val} with or without *FZD3* silencing using MCF-7 cells. As shown in Fig. 7A, co-transfection with *FZD3* siRNA markedly abolished the elevated levels of *FZD3* induced by the 5′-tiRNA^{Val} inhibitor in MCF-7 cells. Furthermore, attenuation of *FZD3* expression also partially reversed the effect of 5′-tiRNA^{Val} inhibition on MCF-7 cell viability, migration and invasion compared to these in the control group (Fig. 7B–D). Taken together, these data suggest that 5′-tiRNA^{Val} exerts its effects through inhibition of *FZD3* in breast cancer cells.

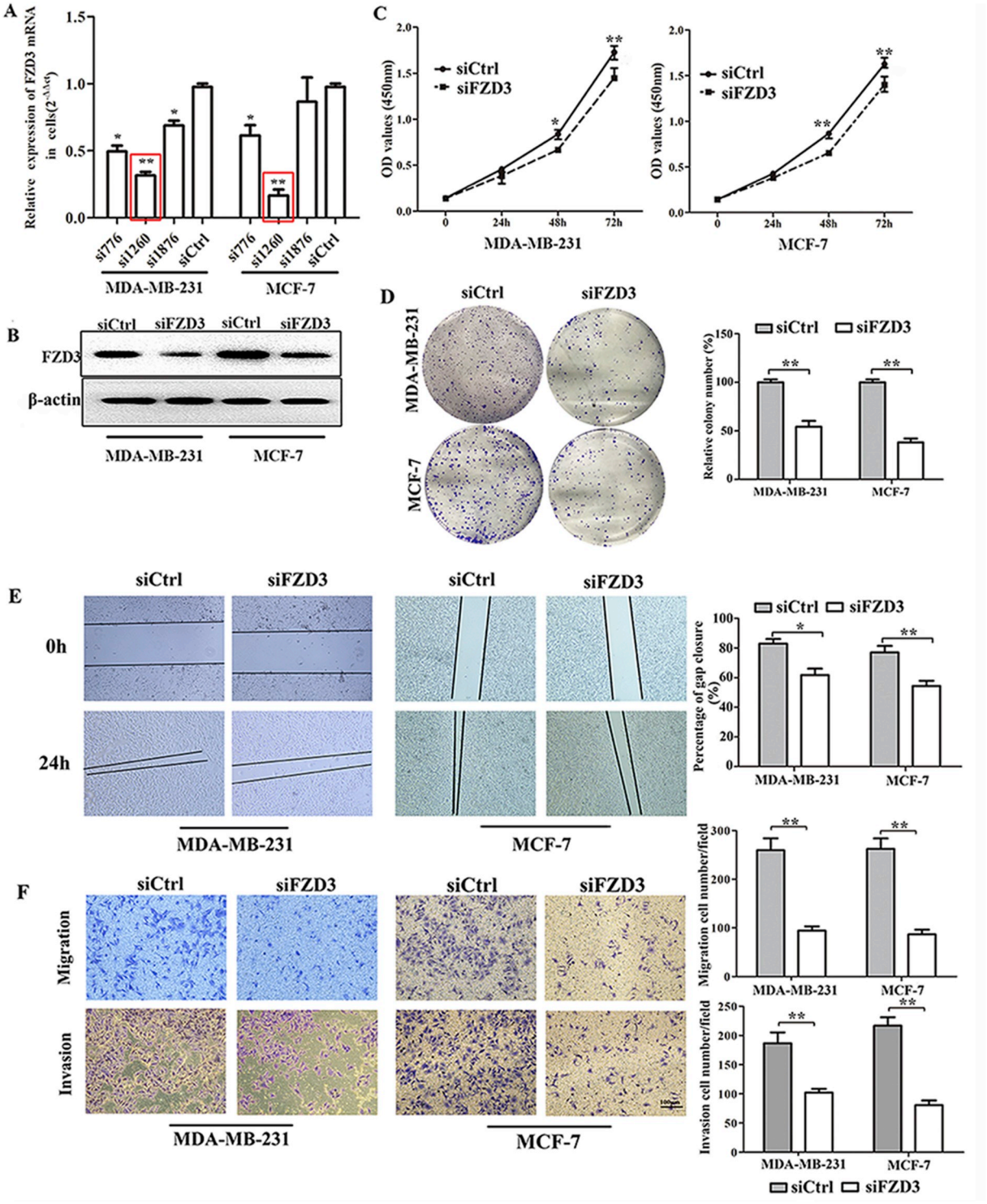

Fig. 6. Silence of *FZD3* inhibited cell malignant activities.
(A and B) *FZD3* mRNA expression (A) and protein levels (B) in MD-MB-231 and MCF-7 cells transfected with *FZD3* siRNA or siRNA negative control. (C) Cell viability of breast cancer cell lines after transfection with *FZD3* siRNA or siRNA negative control was detected by CCK-8 assay. (D) Colony formation assays in the breast cancer cell lines silencing *FZD3*. (E) The wound healing assays were applied to assess the cell migration in the silencing *FZD3* cells. And numbers of cells invaded were counted in five fields. (F) Representative images and bar graphs were depicted to investigate the migration and invasion ability of breast cancer cells after the 24 h transfection *FZD3* siRNA or the negative control. $^{**}P < 0.01$, $^{*}P < 0.05$, statistically significant.

3.8. 5′-tiRNA^{Val} suppresses Wnt/β-catenin signaling pathway by targeting FZD3

To determine the possible mechanisms underlying the tumor-suppression effect of 5′-tiRNA^{Val}, we further explored signaling pathway downstream of *FZD3*. As *FZD3* was one of the major components of the Wnt signaling pathway, we hypothesized that 5′-tiRNA^{Val}-mediated *FZD3* inhibition may lead to the inactivation of Wnt/β-Catenin signaling in breast cancer cells (Fig. 8A). We measured the expression of β-Catenin, APC, c-myc and cyclinD1 in breast cancer cells transfected

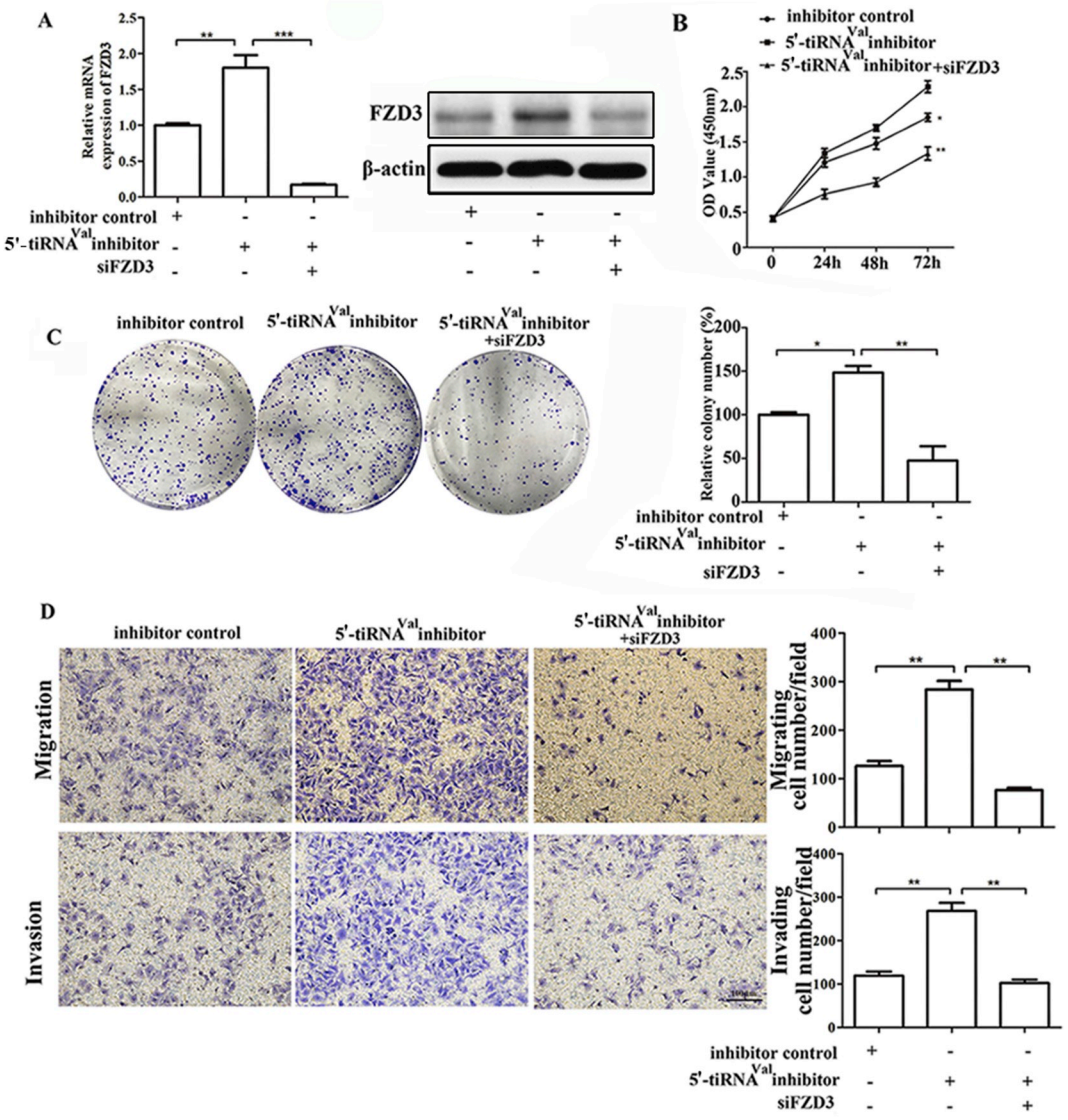

Fig. 7. Rescue experiment was performed the relationship between 5′-tiRNA^{Val} and *FZD3*.
(A) MCF-7 cells co-transfected with 5′-tiRNA^{Val} inhibitor and siFZD3, and the FZD3 protein levels and mRNA expression were evaluated. (B) Cell viability of MCF-7 cells after co-transfected with 5′-tiRNA^{Val} inhibitor and siFZD3 was detected by CCK-8 assay. (C) Colony formation assays in the MCF-7 cells after co-transfected with 5′-tiRNA^{Val} inhibitor and siFZD3. (D) Representative images and bar graphs were depicted to investigate the migration and invasion ability of breast cancer cells after the 24 h co-transfection. And numbers of cells invaded were counted in five fields. $^{**}P < 0.01$, $^{*}P < 0.05$, statistically significant.

with 5′-tiRNA^{Val} mimics or controls. As shown in Fig. 8B, β-Catenin, c-myc and cyclinD1 levels were downregulated, while the APC was inversely upregulated, in breast cancer cells overexpressing 5′-tiRNA^{Val}, suggesting that 5′-tiRNA^{Val} may inhibit the Wnt/β-Catenin pathway. To further verify the influence of 5′-tiRNA^{Val} on regulation of the Wnt/β-Catenin signaling pathway through *FZD3*, 5′-tiRNA^{Val} inhibitor and si-FZD3 were co-transfected in breast cancer cells. Our results showed that silence of FZD3 expression apparently decreased β-catenin, cyclin D1 and c-myc, but restored APC expression in both breast cancer cell lines (Fig. 8C). All the data suggest that 5′-tiRNA^{Val} suppresses the Wnt/β-Catenin signaling pathway by targeting *FZD3* in breast cancer cells.

3.9. Diagnostic value of 5′-tiRNA^{Val} for patients with breast cancer

In order to evaluate the discriminative power of 5′-tiRNA^{Val} between controls and breast cancer, the area under the ROC-AUC was performed. As shown in Fig. 9A, the ROC-AUC for 5′-tiRNA^{Val} was 0.756 (95% confidence interval (CI) [0.647–0.865]) for differentiating all breast cancer patients from healthy control, with a cut-off value of 5.433 (sensitivity: 90.0%, specificity 62.7%). To differentiate different TNM stage of breast cancer from healthy control, a cut-off value of 5.433 yielded a sensitivity of 85.0% and a specificity of 51.9% in the early stages, and a cut-off value of yielded a sensitivity of 90.0% and a specificity of 75.9% in the advanced stages (Fig. 9B–C). Furthermore, the ROC-AUC of 0.785 was specified for lymph node metastasis of breast cancer form healthy control with 89.0% sensitivity and 70.6% specificity at a cut-off value of 5.433 (Fig. 9D). While a ROC-AUC of 5′-tiRNA^{Val} for differentiating non-lymph node metastasis of breast cancer form healthy control was 0.669 (95% CI [0.503–0.836]) but wasn't statistically different from the non-discriminant bisector ($P = 0.0641$) (Fig. 9E). The results demonstrated that 5′-tiRNA^{Val} levels maybe as a potential diagnostic marker for breast cancer.

4. Discussion

As the most common malignancy in women, breast cancer causes a high mortality due to multiple factors, including late diagnosis, lack of treatment options and cancer heterogeneity. Next-generation

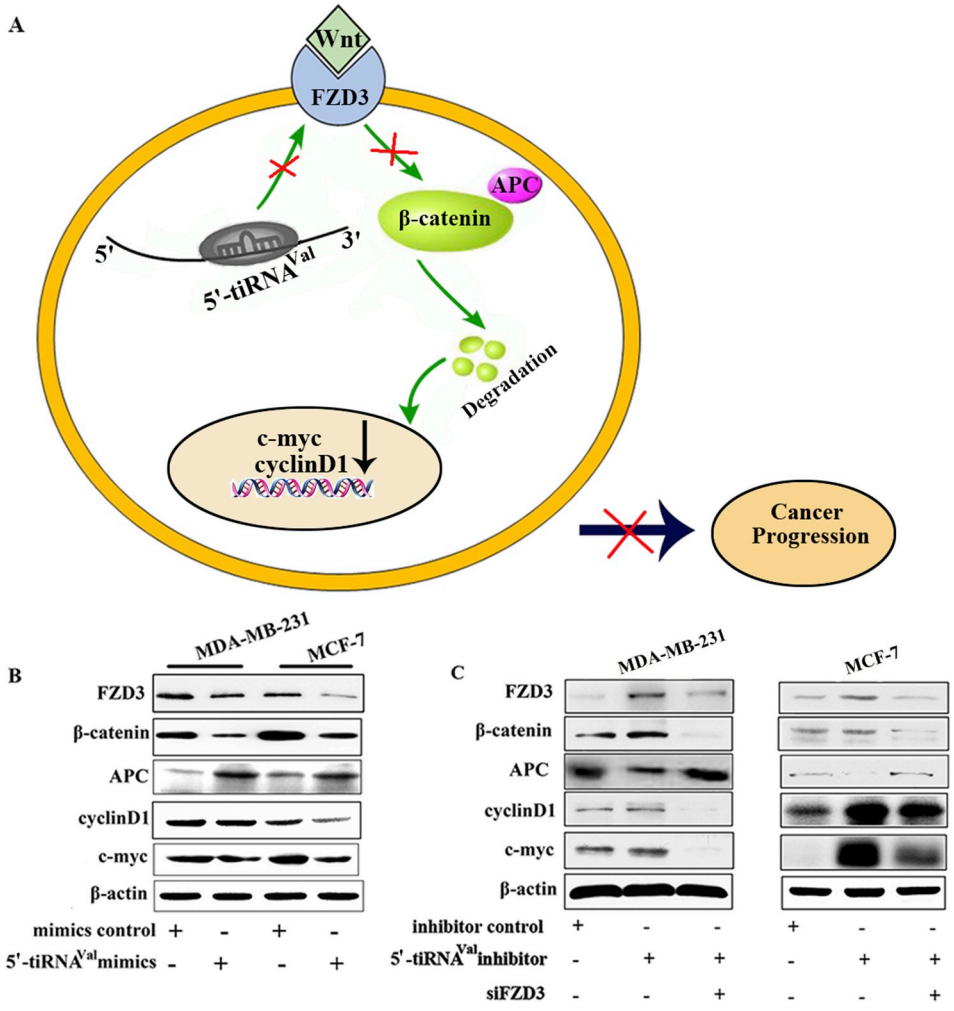

Fig. 8. 5′-tiRNA^{Val} suppresses Wnt/β-Catenin signaling pathway by targeting *FZD3*.
(A) Proposed working model. 5′-tiRNA^{Val} fragment binds the *FZD3* mRNA 3′-UTR and induced its downregulation, leading to decreased β-Catenin protein, which inhibits the activation of the Wnt/β-Catenin in breast cancer cells. (B) Western blot analysis of FZD3, β-Catenin, APC, cyclinD1 and c-Myc in breast cancer cells transfected with 5′-tiRNA^{Val} mimics or negative control. (C) MD-MB-231 and MCF-7 cells were co-transfected with 5′-tiRNA^{Val} inhibitor and siFZD3, and the related protein levels of Wnt/β-Catenin signaling pathway were evaluated by Western blot, and β-actin was used as a loading control.

sequencing is becoming routine in clinical settings and adds to our understanding of molecular mechanisms of tumorigenesis [32]. With the development of sequencing technology, new types of small non-coding RNAs are identified in different cells and tissues [33]. Recently, increased evidence indicates that tRNAs and their derivatives are dysregulated in cancer and involved in the pathogenic process of cancer, including non-small cell lung cancer [34], ovarian cancer [35], colorectal cancer [36]. Although received significant attention, the biological function of tRNAs and tRNA derivatives in different disease settings remains largely unknown [37,38].

Many literature reported that tRNA derivatives were dysregulated in breast cancer [39–41]. Clinical characteristics of breast cancer were related with changes in abundance of specific tiRNA [42]. Honda et al. manifested that 5′-tiRNA^{Asp} and 5′-tiRNA^{His} exhibited elevated expression in breast cancer tissues and cells [32]. Evidence also showed that specific knockdown of 5′-tiRNA impaired cell proliferation, indicating that tiRNAs are not nonfunctionally accumulated but enhanced the cell proliferation [41]. In the current study, our data demonstrated that 5′-tiRNA^{Val} was markedly downregulated in breast cancer cells and serum, and showed a negative correlation with cancer TNM stage and lymph node metastasis. Meanwhile, the experiment of gain function of 5′-tiRNA^{Val} in breast cancer cells demonstrated that 5′-tiRNA^{Val} overexpression significantly suppresses breast tumor cell proliferation, migration and invasion. The results suggest that 5′-tiRNA^{Val} maybe act as a tumor suppressor in breast cancer.

tRNA derivatives have been confirmed to function in stress responses by regulating stress granule formation, targeting RNA binding protein (e.g. YBX1) [40]. Two tRNA derivatives are known to directly bind mRNA targets in a manner similar to canonical microRNAs: one is tRF-3 class, designated CU1276 in B cell lymphoma [17] and the other is tRF5-Glu in ovarian cancer [35]. Interestingly, we also found that 5′-tiRNA^{Val} could regulate downstream target genes. When the expression of 5′-tiRNA^{Val} was upregulated, the expression of FZD3 was suppressed at the protein, as well as the mRNA level. Thus, we assumed that *FZD3* may be an important target of 5′-tiRNA^{Val} in breast cancer. Using the luciferase assay to verify the target of 5′-tiRNA^{Val}, we demonstrated that the predicted site in the 3′-UTR of *FZD3* was capable of binding mimics of 5′-tiRNA^{Val} and inducing downregulation. Moreover, inhibition of *FZD3* expression by siRNA could reduce breast cancer cells proliferation, colony formation, migration and invasion. In other words, silencing of *FZD3* inhibited breast cancer cell aggressiveness similar to the effects of 5′-tiRNA^{Val} overexpression. Importantly, rescue experiments and functional assays demonstrated that attenuation of *FZD3* partially abolished the inhibitory effect of 5′-tiRNA^{Val} on proliferation and metastasis of breast cancer cells.

The downstream molecular signaling involved in the regulation of 5′-tiRNA^{Val}/FZD3 axis on breast cancer progression still remains unknown. A previous study showed that *FZD3* activated the Wnt/β-Catenin signaling pathway [43]. The Wnt/β-Catenin pathway controls the intracellular levels of β-Catenin. In the absence of Wnt signals, free β-Catenin is targeted by a cytoplasmic protein complex known as the β-Catenin destruction complex [44,45]. Wnt/β-Catenin signaling pathway abnormal activation in a series of epithelia is linked to generation or progression of the colon, breast, liver, pancreas and others [46–48]. In this study, we speculated that 5′-tiRNA^{Val} might contribute to breast cancer progression via the FZD3/Wnt/β-Catenin pathway. Our

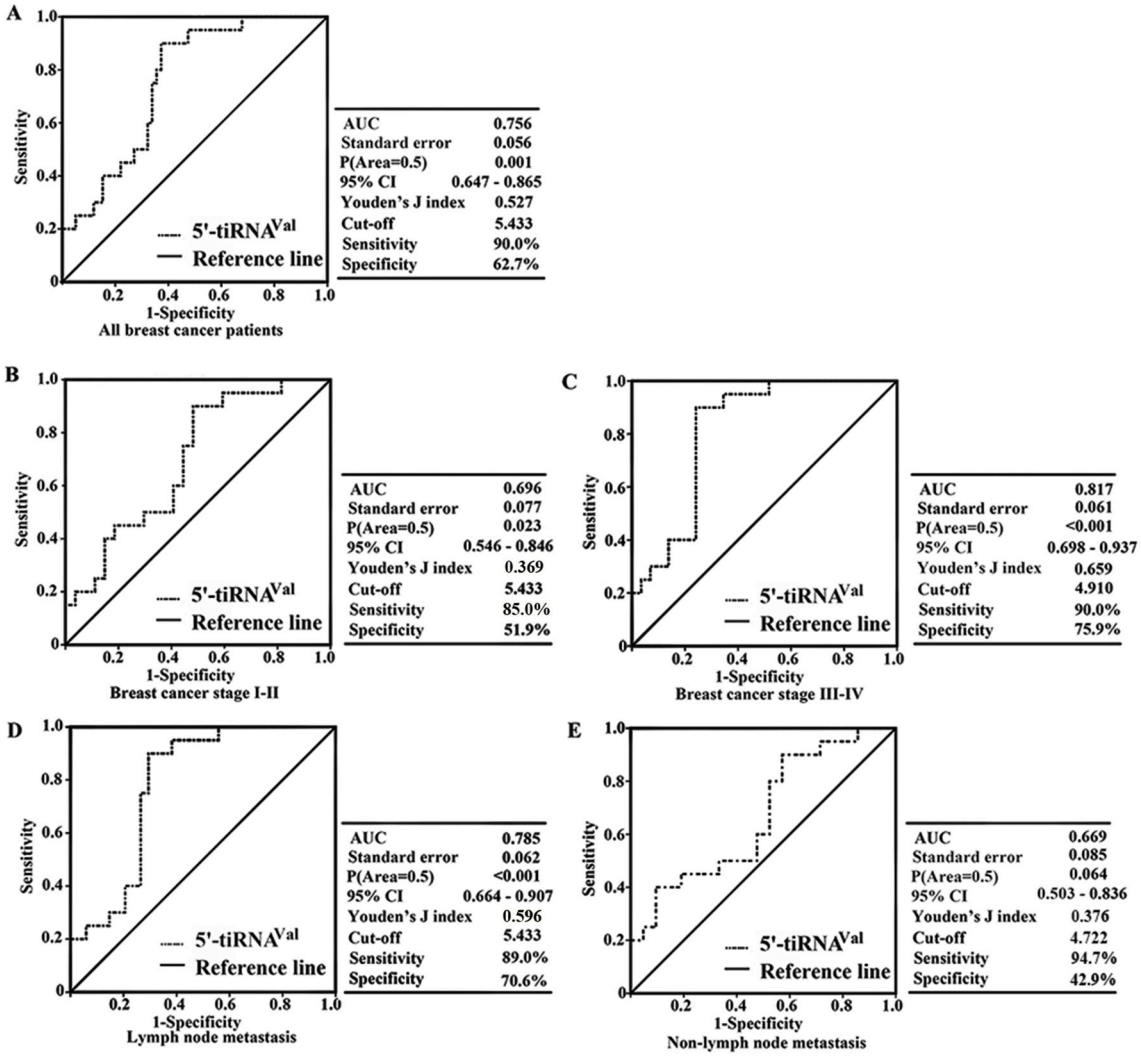

Fig. 9. ROC curve analysis of serum 5′-tiRNA^{Val}.
(A) Differentiating breast cancer form healthy controls. (B and C) TNM stage I-II (B) and stage III-IV (C) in breast cancer from healthy controls. (D and E) Lymph node metastasis (D) and Non-lymph node metastasis (E) in breast cancer from healthy controls. TNM: Tumor-Node-Metastasis, ROC: receiver operating characteristic, CI: confidence interval.

data suggest that 5′-tiRNA^{Val} inhibits the Wnt/β-Catenin pathway and increases the APC through downregulating FZD3 expression. Meanwhile, 5′-tiRNA^{Val} inhibition reverses the negative effect of reduced FZD3 on Wnt/β-Catenin signaling. Finally, Using ROC analysis, we found that 5′-tiRNA^{Val} for differentiating breast cancer patients form healthy control was 0.756 (95% CI, [0.647–0.865]; $P = 0.001$), with a sensitivity of 90.0% and a specificity of 62.7%. The results indicated that serum level of 5′-tiRNA^{Val} maybe as a potential diagnostic marker for breast cancer.

5. Conclusions

In conclusion, we identified a new 5′-half fragment of tRNA, which was named as 5′-tiRNA^{Val} in breast cancer patients. We not only demonstrated a molecular connection between 5′-tiRNA^{Val} and *FZD3*, but also identified 5′-tiRNA^{Val} as a new tumor-suppressor through inhibition of FZD3/Wnt/β-Catenin signaling in breast cancer. Thus, as a new group of non-coding RNAs, tRNA derivatives may provide a novel therapeutic strategy for treatment of breast cancer and other malignant diseases.

Additional files

Additional file 1: Table S1. Primers used in the polymerase chain reaction.

Additional file 2: Fig. S1. Transfection efficiency of 5′-tiRNA^{Val} mimics in breast cancer cells. (A) Representative figures of MDA-MB-231 and MCF-7 transfected by 5′-tiRNA^{Val} mimics under fluorescent and light scope. (B) 5′-tiRNA^{Val} expression levels were detected by qRT-PCR in each group. The transfection of 5′-tiRNA^{Val} mimics led to about 300-fold upregulation of 5′-tiRNA^{Val} expression. $^{**}P < 0.01$, statistically significant.

Abbreviations

FZD3: Frizzled homolog 3; *APC*: Adenomatous polyposis coli; DMEM: Dulbecco's modified Eagle's medium; TNM: Tumor-Node-Metastasis; qRT-PCR: Quantitative real time-PCR; AUC: Area under curve.

Author's contributions

FY and DPM made conception and design this study. YNY, XLM and

XMW collected the acquisition of data. DPM and XT analyzed and interpret the data. DW and XYT provided the study materials of patients. DPM, PJ and LT performed writing the manuscript. FY and BL provide administrative, technical support. All authors reviewed the manuscript. All authors read and approved the final manuscript.

Ethical approval

All procedures performed in studies involving human participants were in accordance with ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflicts of interest

These authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.05.007.

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