ZW10 Binding Factor (ZWINT), a Direct Target of Mir-204, Predicts Poor Survival and Promotes Proliferation in Breast Cancer

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Background: ZW10 binding factor (ZWINT) has been reported to be upregulated in various human cancers and predict worse survival. However, the expression profile, clinical significance, and biological role of ZWINT remains unclear in breast cancer.

Material/Methods: In this study, we investigated messenger RNA (mRNA) and protein expression levels of ZWINT in breast cancer tissues, and the prognostic value of ZWINT protein expression was validated in a cohort of breast cancer patients using immunohistochemistry analysis. Then, different bioinformatic analyses were combined to explore the potential cancer-related hallmark underlying ZWINT in breast cancer, and a series of experiments in vitro were performed to reveal the oncogenic role of ZWINT in breast cancer.

Results: Significant upregulation of ZWINT was observed in breast cancer tissues compared to normal and para-tumor tissues and upregulation of ZWINT predicts poor prognosis in breast cancer patients. Additionally, ZWINT could promote breast cancer proliferation via cell cycle regulation, especially by influencing the expression of some critical cell cycle regulators involved in G1 phase and G1/S transition. Finally, miR-204 was identified as a tumor suppressor microRNA which directly targets a specific site in 3'-UTR of ZWINT.

Conclusions: Overall, our results indicated that miR-204/ZWINT/cell cycle process might play an important role in breast cancer progression.

MeSH Keywords: Breast Neoplasms • Cell Cycle • Cell Proliferation

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Background

Breast cancer is the most diagnosed malignancy in women worldwide, which comprises 25.2% of all newly diagnosed cancers [1]. Its high mortality rate also makes breast cancer the leading cause of death, with 358 989 estimated deaths in 2018 worldwide [2,3]. Despite rapid advances in anti-cancer therapies such as surgery, chemotherapy, radiotherapy, and endocrine therapy, breast cancer remains the cause of a vast number of deaths [4–6]. In recent years, due to the combination of improved detection and earlier diagnosis, and more effective treatment regimens, the overall survival of breast cancer patients has made step wise improvements. These achievements encourage us to identify more reliable biomarkers and targets to improve the diagnosis and treatments of breast cancer.

The ZW10 binding factor (ZWINT) was first identified as a centromere-complex component that is required for the mitotic spindle checkpoint, which is related to the function of the centromere [7]. In addition, ZWINT could interact with an E3 ubiquitin ligase and contributes to cell proliferation [8]. Recent studies suggest that overexpression of ZWINT widely occurs in various cancers and is associated with cancer progression. For example, a bioinformatic analysis indicated that overexpression of ZWINT correlates with poor prognosis in lung cancer [9], and ZWINT might also serve as a potential target [10]. Moreover, overexpression of ZWINT in hepatocellular carcinoma predicts poor survival and promotes cancer cell proliferation [11]. These evidences encourage further investigation of potential functions of ZWINT in breast cancer.

Because the expression profile, clinical significance, and biological function of ZWINT have not been studied in breast cancer, we investigated the expression profile of ZWINT in The Cancer Genome Atlas (TCGA) breast cancer database and used bioinformatic analyses to explore the potential biological function of ZWINT in breast cancer tissues and cells. In addition, we identified a microRNA (miRNA) that could directly target ZWINT, serving as a tumor suppressor in breast cancer. Our results showed that the miR-204/ZWINT axis plays a critical role in progression of breast cancer and may serve as a promising biomarker and novel therapeutic target in breast cancer.

Material and Methods

Data source and bioinformatic analyses

RSEM-normalized RNA-seq data and miRNA-seq data of donated normal breast tissues (retrieved in the Genotype-Tissue Expression [GTEx] database) and para-tumor tissues and breast cancer tissues (retrieved in TCGA database) were accessed from UCSC Xena (https://xenabrowser.net/) [12,13].

The weighted gene co-expression network analysis (WGCNA) was performed based on the transcriptome profiling data of 1097 breast cancer samples with ZWINT expression value as a trait. This work was performed using R package “wgcna” [14]. The module which depicted the highest correlation value with ZWINT expression value was considered as the ZWINT-related gene module, and gene significance of hub-genes involved in the module bigger than 0.6 were set as the selective criteria. Then, a website tool Metascape was used to visualize the result of Gene Ontology (GO) enrichment analysis on these genes [15].

CancerSEA (http://biocc.hrbmu.edu.cn/Cancer SEA/) [16], which provides a cancer single-cell functional states atlas, involving 14 functional states of 41 900 cancer single cells from 25 cancer types, was used to explore the potential roles of ZWINT in breast cancer.

Small interfering RNA (siRNA) and miRNA mimic transfections

The human normal mammary epithelial cell line MCF-10A and breast cancer cell lines MCF-7, MDA-MB-231, BT474, and MDA-MB-453 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Lipofectamine™ Rania Regent (Thermo Fisher Scientific, USA) was used to transfect cells according to the manufacturer’s protocols. Nonsense scrambled small interfering RNA (siRNA) was designed as a negative control. A miry-204 mimic, negative control mimics, and siRNA targeting ZWINT was designed by Gene hem (Shanghai, China). The sequences of siRNA were as followed:

forward: 5’-CECA GAG GAA ACG GAD ACA A-3’ and reverse: 5’-UNG UGU CCG UUU CLU CU G-3’.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols, and was quickly reversely transcribed to complementary DNA (cDNA) using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Japan; cat No. RR036A). The quality of extracted RNA and cDNA were measured using the NanoDrop 2000 (Thermo Fisher, USA). Experiments were repeated if the ratio of OD260 and OD280 value was not in the range from 1.8 to 2.2. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed in a 50 µL reaction system with SYBR Select Master Mix (Applied Biosystems, Cat No. 4472908). The reaction system and thermal cycle conditions were used as previously described [17]. The sequences of primers in our study were listed as followed: ZWINT (forward): 5’-AAC TCC GGG AAG CCT TTG AG-3’ and
ZWINT (reverse): 5’-TTC TGG ACT GCT CGT TT-3;
GAPDH (forward): 5’-AAT GGG CAG CCC TTA GGA AA-3’ and
GAPDH (reverse): 5’-GGC CCC AAT ACG ACC AAA TC-3’.
Each experiment was performed in triplicate.

Western blot

We strictly followed the protocols described in our previous study [17]. In brief, the total protein from cultured cells with different treatments was isolated by 1% phenylmethanesulfonyl fluoride (PMSF) and radioimmunoprecipitation assay (RIPA) lysis buffer. Boiling with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer for 5 minutes, then the samples were separated in SDS-PAGE gels. Then the protein was transferred on a polyvinylidene difluoride (PVDF) membrane (Millipore). Incubation overnight with a dilution of 1: 1000 of rabbit polyclone anti-mouse ZWINT, cyclin D1, cyclin E1, CDK4, and CDK18 antibodies. Then, after incubation with a secondary antibody (1: 2000) for 2 hours at room temperature, the ECL chemiluminescence detection kit (Bio-Rad) was used to visualize blots on polyvinylidene difluoride membrane, and the luminescent-image analyzer (ThermoFisher Scientific, USA) was used to evaluate band intensity.

Tissue microarray (TMA) and immunohistochemistry (IHC)

Tissue microarray (TMA) with 139 formalin-fixed paraffin embedded primary breast cancer tissue dots with corresponding clinical annotations and follow-up information was purchased from the Biochip Co., Ltd. (Shanghai, China).

Immunohistochemistry (IHC) staining was used to detect ZWINT protein expression of tumor and para-tumor tissues in the microarray. Tissue sections on the microarray were deparaffinized and rehydrated via alcohol solutions of different grades. Endogenous peroxidase activity was blocked in H2O2. Antigen retrieval was performed with citrate buffer and the heating treatment of microwave. IHC staining of each dot was evaluated and scored by 2 pathologists independently. The staining intensity was evaluated into 4 grades: 0 (almost negative), 1 (weak), 2 (moderate), and 3 (strong). The outcome of the percentage of staining region multiplied by intensity grade was calculated as a final immunoreactive score with a range from 0 to 300.

Proliferation assay and cell cycle distribution analysis

The proliferation assay of Cell Counting Kit-8 (CCK-8) and colony formation assays were performed as previously described [17]. Experiments were repeated in triplicate.

For the cell cycle distribution, flow cytometry analysis was performed to measure the cell cycle distribution of cell lines with different treatments as previously described [17].

Statistical analysis

Data were presented as the mean±standard deviation. SPSS software (SPSS Inc., Chicago, IL, USA), R software (version 3.5.0), and GraphPad Prism 8 software (San Diego, CA, USA) were used to perform analyses and plot graphs. Student’s t-test or one-way analysis of variance (ANOVA) were used to analyze differences between different groups or treatments. The Kaplan-Meier method was used to plot survival curve, and log-rank test was used to evaluate the difference. P<0.05 was considered statistically significant.

Results

Elevation of ZWINT is correlated with advanced stage and worse survival

RSEM-normalized RNA-seq data of ZWINT was collected and investigated in 179 GTEx donated normal tissues, 1092 TCGA breast cancer tissues along with 113 para-tumor tissues. Significant elevation of ZWINT was observed in breast cancer tissues compared to normal or para-tumor tissues P<0.0001; Figure 1A). IHC analysis on TMA indicated that protein expression of ZWINT was significantly upregulated in tumor tissues, especially in samples with more advanced tumor stages (Figure 1B). Moreover, Kaplan-Meier curve indicated that patients with higher expression of ZWINT exhibited worse overall survival than those with lower expression, with a hazard ratio (HR) of 5.897 in a cohort of 139 patients (Figure 1C).

Bioinformatic analyses indicated that ZWINT might exert its oncogenic role via cell cycle regulation in breast cancer

First, WGCNA was performed based on whole transcriptome profiling data and ZWINT expression levels of 1097 breast cancer samples (Figure 2A). A total of 30 non-grey modules were assigned with a power of β=7 as the optimal soft threshold to ensure a scale-free co-expression network (Supplementary Figure 1). Among these modules, the yellow module depicting a highest correlation (r=0.69, P=4e-151) was considered most correlated with ZWINT, and genes involved in yellow module were considered as ZWINT-correlated hub-genes (Figure 2B). Then, we used Metascape to perform enrichment analysis on these genes; a network of biological functions of these genes is shown in Figure 2C. We observed that ZWINT-correlated hub-genes were enriched in cell cycle-related processes such as cell division, cell cycle phase transition, DNA replication, etc. In order to explore the biological function of ZWINT in breast cancer cells, single-cell analysis was performed using CancerSEA. Among various cancer-related hallmarks across 4 different datasets, the heatmap suggested that ZWINT might regulate cell cycle progression primarily (Figure 2D). In detail, ZWINT...
expression is positively correlated with cell cycle in these 4 datasets of single-cell analysis, with correlation coefficients of 0.77, 0.56, 0.86, and 0.74, respectively (Figure 2E).

**ZIWNT promotes breast cancer proliferation by regulating cell cycle process**

As shown in Figure 3A, MCF-7 and MDA-MB-231 were chosen for further study due to their relatively highest expression levels. An effective siRNA targeting ZWINT was designed to knockdown its expression in the 2 cell lines, and the transfection efficiency was evaluated using western blot analysis (Figure 3B). The role of ZWINT in cell cycle was measured using flow cytometry analysis. Silencing of ZWINT significantly resulted in G1 phase arrest in both cell lines compared to si-scramble groups (Figure 3C). Moreover, si-ZWINT treatment group exhibited significantly fewer colony numbers than the matched si-scramble groups in both cell lines (Figure 3D). As shown in Figure 3E and 3F, CCK-8 assays revealed that silencing of ZWINT markedly weakened the proliferative abilities of MCF-7 and MDA-MB-231 cells. In addition, we performed western blot to detect some cell cycle regulators involved in G1 phase or G1/S...
Figure 2. Bioinformatic analyses showed that ZWINT highly correlated with cell cycle process in breast cancer. (A) WGCNA was performed with transcriptome data and ZWINT expression level. (B) Different modules were generated, and the yellow module was identified as ZWINT-correlated one. (C) Biological network of ZWINT-correlated hub-genes. (D) Heatmap of relationships between ZWINT expression and different cancer hallmarks in 4 single-cell studies. (E) In detail, ZWINT expression is positively correlated with cell cycle in these 4 datasets. ZWINT – ZW10 binding factor; WGCNA – weighted gene co-expression network analysis.
Figure 3. ZWINT promotes breast cancer proliferation via cell cycle regulation. (A) MCF-7 and MDA-MB-231 were chosen for further study. (B) Transfection efficiency was measured using western blot. (C) Silencing of ZWINT induced G1 phase arrest in the 2 breast cancer cell lines. (D) ZWINT knockdown group exhibited significantly fewer colony numbers than the matched si-scramble groups in both cell lines. (E, F) CCK-8 assays showed that silencing of ZWINT markedly weakened the proliferative abilities of MCF-7 and MDA-MB-231 cells. (G) Western bolt analysis showed that ZWINT could influence some critical cell cycle regulators in MCF-7 cells. ZWINT – ZW10 binding factor; CCK-8 – Cell Counting Kit-8.

transition after si-ZWINT treatment in both cell lines. We observed that silencing of ZWINT could downregulate some positive cell cycle regulators such as CCND1, CCNE1, and CDK4, while negative regulator such as CDKN1B was significantly up-regulated in MCF-7 cells (Figure 3G). These results indicated that ZWINT could promote proliferation by regulating cell cycle process, via influencing some critical cell cycle regulators involved in G1 phase or G1/S transition.

MiR-204 targets ZWINT directly

To screen out microRNAs (miRNAs) which could target ZWINT in breast cancer, predictive results from 3 databases including Targetscan, miRDB, and PicTar were analyzed and intersected. Among overlapping candidates, a miRNA named miR-204 was finally filtered out due to its highest predictive score (upper panel of Figure 4A). As shown in lower panel of Figure 4A, 4326-4333
Figure 4. ZWINT is a direct target of miR-204. (A) miR-204 was identified as a promising candidate with high predictive score. (B) miR-204 was significantly downregulated in tumor tissues compared to para-tumor tissues. (C) Luciferase reporter assay showed that miR-204 could bind to wild-type 3’-UTR of ZWINT instead of mutated site. (D, E) Enforced overexpression of miR-204 could significantly reduce ZWINT mRNA and protein expression in breast cancer cells. (F) A significant negative correlation was observed between ZWINT and miR-204 expression in TCGA breast cancer samples. ZWINT – ZW10 binding factor; TCGA – The Cancer Genome Atlas; mRNA – messenger RNA.
in the ZWINT 3′-UTR region acted as a predicted targeting site of miR-204 with high predicted score. Subsequently, we investigated the expression profile of miR-204 in TCGA breast cancer database. MiR-204 was observed to be significantly downregulated in breast cancer tissues compared to para-tumor normal tissues (P<0.0001; Figure 4B). Luciferase reporter was carried out and validated that miR-204 could directly bind to the predicted site. Results indicated that miR-204 could significantly inhibit luciferase activity in both MCF-7 and MDA-MB-231 cells with a reporter plasmid carrying the wild-type ZWINT 3′-UTR sequence. In contrast, no significant inhibition was observed in cells with the reporter plasmid carrying a mutated sequence aforementioned (Figure 4C). Furthermore, the inhibitory effect of miR-204 on ZWINT expression was measured in both MCF-7 and MDA-MB-231 cells. Enforced overexpression of miR-204 could significantly reduce ZWINT mRNA and protein expression in breast cancer cells when compared to negative control groups (Figure 4D, 4E). In addition, we extracted breast cancer samples with both ZWINT and miR-204 expression in TCGA, and we observed that ZWINT expression was significantly negatively correlated with miR-204 in breast cancer samples (r=−0.3083, P<0.0001; Figure 4F).

**Discussion**

Alteration of cell cycle process serves as an important cancer hallmark [18]. Dysregulation of cell cycle process is a result of a series changes in the activation or inactivation of cell cycle-related regulators, which usually induces cancer cell proliferation and tumor growth. In breast cancer, some cell cycle-related genes were identified as promising biomarkers for prognosis and potential therapeutic targets [19–22].

ZWINT was first identified as a centromere-related protein in a yeast 2-hybrid screening. Starr et al. reported that ZWINT could directly interact with ZW10, and thus to regulate the process of mitotic spindle checkpoint [7]. Silencing of ZWINT could disrupt the localization of a complex which consists of Rod, ZW10, and Zwilch to centromeres, and reduces the activity of checkpoint [23]. Recently, accumulating evidences indicated that ZWINT functioned as an oncogene in some cancer types. For example, elevation of ZWINT could predict poor prognosis and promotes cell proliferation and tumor growth in hepatocellular carcinoma [11]. Moreover, overexpression of ZWINT predicts poor prognosis in lung cancer in a bioinformatic study [9]. Fang et al. reported that ZWINT might function as a potential therapy target in lung cancer [10]. However, the clinical significance and biological role of ZWINT in breast cancer were still unclear.

To our knowledge, this study is the first to report ZWINT in breast cancer. Our bioinformatic analyses strongly indicated that upregulation of ZWINT might play a role in cell cycle regulation in breast cancer, using WGCNA plus enrichment analysis and a single-cell analysis database. Under the guidance of these predicted results, in vitro experiments were carried out to reveal the oncogenic role of ZWINT in breast cancer cells. Our data showed that silencing of ZWINT could induce breast cancer cells arrest in G1 phase thus to weaken the proliferative ability of breast cancer cells. Furthermore, some critical regulators involved in G1 phase and G1/S transition were evaluated after treatment of ZWINT silencing, and we observed that these genes were significantly changed when ZWINT was silenced.

Because the development and progression of breast cancer is a comprehensive event which involves complicated networks of gene expression regulation and environmental changes, in which miRNAs play important roles, we used a bioinformatic algorithm for predicting potential miRNAs targeting ZWINT. With a high predictive score, miR-204 was identified and predicted as a promising candidate. Then, the expression profile and biological role of miR-204 were investigated in breast cancer tissues, and the direct binding of miR-204 to a specific site in ZWINT 3′-UTR region was validated by a series of experiments in vitro. In addition, we investigated the relationship between ZWINT and miR-204 in TCGA breast cancer tissues. A significant negative correlation was observed between ZWINT and miR-204 in breast cancer tissues, which supports our finding that miR-204 could negatively regulate ZWINT expression from the side.

**Conclusions**

In summary, we presented the first evidence that ZWINT was significantly elevated in breast cancer tissues, and ZWINT could promote breast cancer cell proliferation via cell cycle regulation, especially by influencing some critical cell cycle regulators involved in G1 phase. In addition, miR-204 was identified as a tumor suppressor miRNA which directly targets ZWINT. The miR-204/ZWINT/cell cycle axis might provide useful clues toward novel strategies in treating breast cancer patients.

**Data availability**

All presented data in this study are available from the corresponding author upon reasonable request.

**Conflicts of interest**

None.
Supplementary Data

Supplementary Figure 1. A power of $\beta=7$ was chosen as the optimal soft threshold to ensure a scale-free co-expression network.

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