RNA-Binding Protein Dnd1 Promotes Breast Cancer Apoptosis by Stabilizing the Bim mRNA in a miR-221 Binding Site

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RNA-binding proteins (RBPs) and miRNAs are capable of controlling processes in normal development and cancer. Both of them could determine RNA transcripts fate from synthesis to decay. One such RBP, Dead end (Dnd1), is essential for regulating germ-cell viability and suppresses the germ-cell tumors development, yet how it exerts its functions in breast cancer has remained unresolved. The level of Dnd1 was detected in 21 cancerous tissues paired with neighboring normal tissues by qRT-PCR. We further annotated TCGA (The Cancer Genome Atlas) mRNA expression profiles and found that the expression of Dnd1 and Bim is positively correlated (p = 0.04). Patients with higher Dnd1 expression level had longer overall survival (p = 0.0014) by KM Plotter tool. Dnd1 knockdown in MCF-7 cells decreased Bim expression levels and inhibited apoptosis. While knockdown of Dnd1 promoted the decay of Bim mRNA 3′ UTR, the stability of Bim-5′ UTR was not affected. In addition, mutation of miR-221-binding site in Bim-3′UTR canceled the effect of Dnd1 on Bim mRNA. Knockdown of Dnd1 in MCF-7 cells confirmed that Dnd1 antagonized miR-221-inhibitory effects on Bim expression. Overall, our findings indicate that Dnd1 facilitates apoptosis by increasing the expression of Bim via its competitive combining with miR-221 in Bim-3′UTR. The new function of Dnd1 may contribute to a vital role in breast cancer development.

1. Introduction

RNA-binding proteins (RBPs) play vital roles in regulating RNA biology through the interplay with RNAs within dynamic ribonucleoproteins [1]. Numerous diseases have been linked to abnormal expression of RBPs, including metabolic disorders [2], germ-cell development [3], muscular atrophies [4], and cancer [5].

Dnd1, an evolutionary conserved RBP, is implicated in mediating germ-cell activity and inhibits the germ-cell tumors formation [6] and regulates the male germ-cell development by serving as an essential partner of NANOS2 [3]. On the other hand, Dnd1 expression is repressed in primary acute myeloid leukemia patients and inhibition of Dnd1 mRNA expression significantly attenuated NB4 differentiation [7]. Moreover, Dnd1 expression change mediated by miR-24 could suppress the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B) and also led to enhanced proliferation and reduced apoptosis in tongue squamous cell carcinoma cells [8]. These findings imply that Dnd1 not only regulates germ-cell development but also has wide-ranging roles in tumor development, like in skin oncogenesis [9]. However, limited information is available on the role of Dnd1 in breast cancer.

3′ UTR has various effects on mRNA translation, subcellular localization, and stability through the multiple regulatory elements within them [10]. And 3′UTR could exert tumor-suppressive or tumor-promotive effects via regulating gene expression [11, 12]. In addition, the recent exploration of 3′ UTR functions suggests the potential involvement of 3′ UTR as a new marker in the preclinical oncology [13]. Therefore, focusing on 3′ UTR-mediated effects may provide novel opportunities for 3′UTR-based therapy. Most importantly, Dnd1 has been proved to counteract the function of several miRNAs in human cells by binding to mRNA 3′UTRs and thus inhibiting the association of miRNAs with their
target mRNAs [14]. miRNAs are small noncoding single-stranded RNA molecules that regulate gene expression at a posttranscriptional level via binding with the 3′ UTR [15]. However, the counteracting roles of Dnd1 with miRNAs in breast cancer development remain unclear.

Based on the above-described link of Dnd1, 3′ UTR, and miRNA in tumor development, we investigate their roles in apoptosis of breast cancer. The expression level of Dnd1 was examined in 21 breast cancerous tissues paired with adjacent tissues through qRT-PCR analysis. We further annotated the TCGA microarrays samples by taking into account gene expression levels of breast cancer and analyzed the expression correlation between Dnd1 and other genes. We revealed that low Dnd1 level was a potential marker for breast cancer and that the expression level of Dnd1 was correlated with Bim, which is hall-marker of apoptosis. miR-221 has been proved to promote laryngeal cancer proliferation by suppressing Apaf-1 [16]. Most importantly, miR-221 has been proved to inhibit the apoptosis via targeting Bim or confer a poor prognosis in breast cancer patients [17, 18]. As the specific counteracting between Dnd1 and Bim, it is reasonable to assume that Dnd1 binds to Bim-3′ UTR through counteracting miR-221 function in breast cancer.

Here, we first survey the levels of Dnd1 and Bim in clinical breast cancer tissues and microarray, aiming to investigate whether the expression levels of Dnd1 and Bim are correlated with breast cancer development and whether the expression level of Dnd1 is positively correlated with Bim level. In addition, we focus on the effect of Dnd1 on breast cancer apoptosis in vitro. Finally, the mechanism of the interaction between Dnd1 and Bim is identified, in order to determine whether Dnd1 competitively bind to the Bim-3′ UTR with miR-221, thus promoting breast cancer apoptosis. To the best of our knowledge, the function of Dnd1 validated here has not yet been reported anywhere. Together, we show that the RBP Dnd1 prohibits miRNA-dependent inhibition of Bim expression in breast cancer cells and underscores the tumor-inhibitory roles of the Dnd1 in breast cancer apoptosis, so it may be a possible therapeutic target of breast cancer.

2. Materials and Methods

2.1. Cell Culture and Patient Samples. Human breast cancer cell line MCF-7 and normal mammary gland epithelial cell line HBL-100 were purchased from the Cobioer Bioscience Co., Ltd., and 67NR, 168FARN, 4TO7, 66Cl4, and 4T1 were purchased from the cell bank in Chinese Academy of Sciences of China. All of the above cell lines were maintained in a humidified atmosphere. Twenty-one pairs of breast tumors with neighboring mammary normal epithelial tissues were obtained from 21 patients who underwent surgery at the Central Hospital of Lishui City from February 2015 to December 2015 and all the 21 cases had no metastasis. Approval from the Institute Research Ethics Committee was obtained for the use of these clinical materials for research purposes. Paired mRNA profiling data was downloaded from the TCGA data portal (http://cancergenome.nih.gov/) and a total of 351 TCGA breast cancer samples were utilized as a research set. The dataset from the Tumor Breast-EXPO-351-MASS.0. ut133p2 (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi), which includes 351 breast cancer samples, was acquired as a discovery set. The microarray data set was reserved in the Gene Expression Omnibus (GEO) (accession number GSE2109) according to “minimum information about a microarray experiment” (MIAME) guidelines. The R2 platform was used to analyze the microarray (http://r2.amc.nl/).

2.2. Plasmid and Reporter Constructs. Reverse transcription PCR (RT-PCR) was used to obtain the human Bim and Dnd1 complementary DNA (cDNA) in MCF-7 cells, followed by amplification of the 3′ UTR and 5′ UTR of Bim mRNA using a cDNA template and the following the primers: Bim-3′ UTR forward: 5′-GAATCCACTCTGCAGCAGCGACCGCC-TGGT-3′ and reverse 5′-GGATCTTGGCTCTTTTTCCTGCTTTTCCTC-3′. The PCR products were subcloned into EcoRI- and BamHI-digested pd2EYFP-N1 reporter vector (Clontech, CA, USA): Bim-5′ UTR forward: 5′-GAATCCCTGGTCGTGAGCTTGGAGGCT-3′ and reverse 5′-ACCGGTCACTTTATACAGCCGAGGTAC3′ The PCR products were subcloned into EcoRI- and AgeI-digested pd2EYFP-N1 reporter vector. And miR-221-mutated binding site in YFP-Bim was inserted by employing overlap extension Polymerase Chain Reaction (PCR). Bim promoter sequence was introduced into the pGL3 vector (Promega) for Bim promoter transcriptional activity assay. The coding sequence of Dnd1 was cloned into the plasmid pcDNA3.1(+), referred to as Dnd1-CDS. Primer sequences were as follows: forward: 5′-AAGCTTATGCAGTCGCTAAGCGGGATTGTGAGC-3′; reverse: 5′-GAATCCCTAAGCCTTAAACCCTATGGTACC-TGCC-3′.

2.3. Transfection. For the transfection of siRNAs (Santa Cruz), synthetic siRNAs were transfected into cells that reached 40–60% confluence at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 6-well plates following the recommend protocols. And a universal negative control siRNA (siRNA NC) was used. For the transfection of plasmids, cells that reached 80% confluence were transfected with 2.5 μg plasmid using Lipofectamine 2000 in 6-well plates following the recommend protocols. 35 nM siRNA or miRNA mimics or anti-miRNAs and 1.6 μg plasmid were used for cotransfection with Lipofectamine 2000.

2.4. Real-Time Quantitative PCR (qRT-PCR). Total RNAs were extracted from cells using the Trizol (Invitrogen, USA) reagent according to the manufacturer’s instructions. The first-strand cDNA was synthesized using M-MLV (Promega, USA) following standard protocols. Dnd1, Bim, and GAPDH mRNA expression levels were detected using the specific primers and SYBR Green Master Mix (Biomics Biotechnol- ogy Inc., China). The miRNA first-strand cDNA was generated with miRNA reverse transcription kit (Amb, Canada). miRNA qRT-PCR kit and miR-221 primer were purchased
from Abm. U6 snRNA was used as an endogenous quantity control for miRNA quantification. And qRT-PCR was performed on an ABI Prism 7500 Sequence Detector (Applied Biosystems, Life Technologies). Melting curve analysis was used routinely to check the specificity of amplification.

2.5. Western Blot. Detailed procedure was described elsewhere [19]. The antibodies against Dnd1 (Catalog number ab104792) and β-actin (Catalog number ab8226) were purchased from Abcam, the antibodies against Bim (sc-11425), PARP (sc-23461-R), and caspase-3 (sc-65496) were purchased from Santa Cruz. Protein expression levels were quantified by density analysis using Quantity One Software and normalized to β-actin.

2.6. RIP (RNA Immunoprecipitation) Assays. MCF-7 cells were lysed with 25 mM Tris-HCl buffer (pH 7.5) and 100 U/mL RNase inhibitor (Sigma), and then whole-cell extracts were incubated with protein-A Sepharose beads precleared with anti-Dnd1 antibody or anti-Ago2 antibody or control rabbit IgG for 2h at 4°C. After washing with NT2 buffer, the beads were incubated with 30U of RNase-free DNase I in NT2 buffer for 20 min at 37°C, regulation of Bim mRNA turnover by Dnd1 (Invitrogen) in NT2 buffer for 30 min at 37°C and further incubated in NT2 buffer containing 0.2% SDS and 0.5 mg/mL proteinase K for 20 min at 55°C. RNA was extracted with Trizol and mRNA levels were measured by qRT-PCR.

2.7. Clinical Data. The KM Plotter tool (http://kmplot.com/ analysis/) [20], a meta-analysis-based biomarker assessment tool, was used to compare the survival of breast cancer patients whose Dnd1 and Bim mRNA levels were in the top 1/3 (high) versus the bottom 1/3 (low) groups in publicly available breast cancer gene expression data (Affymetrix (Santa Clara, CA, USA) ProbeID) and selected on the basis of the following parameters: overall survival (OS, 1117 patients), upper versus lower tertile of Dnd1 expression, and including both ER positive (+) and negative (−) breast cancer.

2.8. Statistical Analysis. All data are presented as mean ± SD. For three independent experiments, the differences between the groups were analyzed with Student’s t-test, and *p < 0.05 or less was considered significant.

3. Results

3.1. Dnd1 Expression Level Is Reduced in Breast Tumors and Correlates with Bim Expression. mRNA microarrays were annotated by looking into the mRNA-based subtypes of breast cancer according to the TCGA. The mRNA expression was analyzed in the mRNA microarrays, and we found that Dnd1 expression level was downregulated in the recurrence subtypes compared with the no-recurrence subtypes (Figure 1(a)). Furthermore, the expression levels of Dnd1 or Bim are positively correlated (p = 0.04) (Figure 1(b)). The KM Plotter tool was employed to assess whether Dnd1 mRNA level correlated with the survival of breast cancer patients. The Dnd1 low-expression patients had significantly shorter overall survival than those of Dnd1 high-expression group (p = 0.014) (Figure 1(c)). Further qRT-PCR and western blot results exhibited lower Dnd1 expression levels in MCF-7 cells than in normal mammary gland epithelial cells HBL-100 (Figures 1(d) and 1(e)). And to examine whether Dnd1 levels were associated with tumor aggressive character, Dnd1 mRNA level was determined in five cell lines (67NBR, 168FARN, 4TO7, 66cl4, and 4T1) with unique tumorigenic feature and increased metastatic capability in order [21]. As shown in Figure 1(f), Dnd1 exhibited the least level in 4T1 cells which is the most tumorigenic cells than in other cell lines. Finally, the Dnd1 level was further analyzed in cDNA samples from 21 pairs of breast tumors and their neighboring mammary normal epithelial tissues. An decreased mRNA level of Dnd1 was observed in breast cancer tissues with twofold lower than adjacent normal breast tissues (Figure 1(g)). These data suggest that Dnd1 could be a tumor suppressor in breast cancer and correlated with Bim.

3.2. Dnd1 Stabilizes Bim mRNA and Increases Apoptosis in MCF-7 Cells. As Dnd1 was a conserved RBP which could bind to the AU-rich sites in mRNA 3′ UTRs [14], we hypothesized that Dnd1 could also bind to Bim-3 UTR, thus enhancing the stability of Bim mRNA and promoting the apoptosis of breast cancer cells. Firstly, we examined whether the stability of Bim mRNA was affected by Dnd1 knockdown. Dnd1-Cds and its siRNAs were transfected into MCF-7 cells for 48 h and remarkably upregulated or downregulated the mRNA and protein levels of Dnd1 (Figures 2(a)–2(d)). Meanwhile, the expression levels of Bim were increased in Dnd1-Cds-transfected cells and decreased in Dnd1 siRNA-transfected cells (Figures 2(e)–2(g)). In addition, Dnd1 was overexpressed or knockdown and then blocked de novo synthesis with actinomycin D; the decay rate of Bim mRNA in Dnd1 siRNA-transfected cells was faster than that of NC-transfected cells (t1/2 = 3.2 ± 0.3 h and t1/2 = 4.1 ± 0.3 versus t1/2 = 6.0 ± 0.4 h), whereas it is slower in Dnd1-CDS-treated group (Figure 2(h)). Notably, the luciferase reporter assay showed that Bim promoter transcriptional activity was not affected by Dnd1 ectopic expression (Figure 2(i)), demonstrating that Dnd1 could not alter the transcriptional activity of Bim. In addition, MCF-7 cells were used to examine whether Dnd1 knockdown could actually decrease the susceptibility to apoptotic cell death via decreasing the expression of Bim. Transfection of the Dnd1 siRNA into 5-fluorouracil- (5-FU-) treated MCF-7 cells prohibited the cleavage of poly (ADP-ribose) polymerase (PARP) and activation of caspase 3 (Figure 2(j)). Finally, to determine whether Dnd1 induced cell death by apoptosis, PARP and caspase-3 expression were further measured in cells transfected with Dnd1-Cds. As shown in Figure 2(k), Dnd1 caused cleavages of caspase-3 and PARP in a time-dependent manner. Overall, our results indicate that Dnd1 may modulate the proapoptotic properties of Bim and thus promotes the apoptosis in breast cancer cells.

3.3. Dnd1 Binds to Bim-3′ UTR. To further validate the association of Dnd1 with Bim mRNA, an YFP reporter construct
of a chimeric RNA was constructed with bridging the YFP protein and Bim-5′ UTR or 3′ UTR (YFP-Bim-5′UTR; YFP-Bim-3′UTR; Figure 3(a)). MCF-7 cells were cotransfected with YFP-Bim-3′UTR and YFP-Bim-5′UTR plus Dnd1-CDS or Dnd1 siRNA. The degradation rate of YFP mRNA was detected with actinomycin D treatment via qRT-PCR. As shown in Figure 3(b), the mRNA half-life period of chimeric YFP-Bim-3′UTR was increased by Dnd1 overexpression but shortened by Dnd1 knockdown, whereas the decay rate of YFP-Bim-5′UTR did not change (Figure 3(c)). In addition, to determine whether Dnd1 bound to Bim-3′ UTR, Dnd1 expression was induced in MCF-7 cells, and then Dnd1-binding complex with Dnd1 antibody was pulled down, followed by examining the bound mRNAs by qRT-PCR. As shown in Figures 3(d) and 3(e), RIP analyses indicated that the association between Dnd1 and the chimeric RNA containing Bim-3′ UTR (Figure 3(d)) or Bim (Figure 3(e)) was more specially than control. Most importantly, qRT-PCR assays were performed...
Figure 2: Continued.
to detect the tissue distribution of Dnd1 and Bim transcripts in normalized pooled cDNA samples from 21 pairs of breast tumors with neighboring mammary normal epithelial tissues. An decreased expression level of Bim mRNA was observed in breast tumor tissues over normal tissues (Figure 3(f)), and its expression was correlated with Dnd1 expression positively (Figure 3(g)). Overall, these results indicate that Dnd1 stabilizes Bim mRNA via interacting with Bim-3′UTR.

3.4. Dnd1 and miR-221 Competitively Regulate Degradation of Bim mRNA. As Bim had been proved to target mRNA of miR-221, the competitive effect of miR-221 and Dnd1 on the expression of Bim was further confirmed. The effects of Dnd1 siRNAs in MCF-7 cells overexpressing miR-221 were examined. As shown in Figure 4(a), MCF-7 cells transfected with miR-221 mimics could significantly increase the level of miR-221, and upregulation of miR-221 level significantly decreased Bim mRNA and protein levels (Figures 4(b) and 4(c)), which is consistent with the previous study that miR-221 can target Bim in breast cancer [17]. And Dnd1 downregulation in miR-221 mimics-transfected cells accelerated the miR-221-mediated degradation of Bim mRNA and thus decreased Bim protein level (Figures 4(d) and 4(e)). We also confirmed that Dnd1 mRNA level was not affected by upregulating or down-regulating miR-221 level (Figure 4(f)). To accurately examine the interplay between miR-221 and Dnd1 on Bim-3′UTR, we constructed reporter plasmid that expressed chimeric RNAs including the sequences for Bim-3′UTR harboring mutated binding site for miR-221 and YFP (YFP-Bim-miR-221mut; Figure 4(g)). As shown in Figure 4(h), in cells transfected with NC, the quantity of YFP mRNA generated by the YFP-Bim-5′UTR vector was significantly less than the amount produced by the control YFP-Bim-5′UTR vector, which could be due to the miR-221 regulation and the fact that Dnd1 knockdown additionally accelerated the reduction of the mRNA level of YFP depending on Bim-3′UTR. On the contrary, the levels of YFP mRNA were reversed and the reduction was erased in cells expressing YFP-Bim-221mut, and knockdown Dnd1 did not affect the YFP mRNA level. Additionally, upregulation of miR-221 facilitated the association between Ago2 and Bim mRNA, which was further promoted by knockdown of Dnd1 (Figure 4(i)). Our results indicate that the binding sites of miR-221 and Dnd1 overlap, thus competitively regulating the degradation of Bim mRNA.
4. Discussion

Although the expression of Dnd1 is necessary for germ-cell development, little is known about its function in other physiological conditions, like breast cancer development. Here, the Dnd1 mRNA level was investigated in breast cancer tissues or cells and normal tissues or cells via qRT-PCR analysis. The results showed that Dnd1 expression level was lower in breast cancer cells or tissues and aberrant expression of the Dnd1 correlated with prognosis of patients with breast cancer. In addition, Dnd1 expression level was positively associated with the expression of Bim. And our further studies showed that knockdown of Dnd1 can lead to the decreased stability and expression of Bim but not affect the promoter activity of Bim. Finally, we provided evidences that Dnd1 protected the expression of Bim from repression of miR-221 by competitively binding to the of Bim, thus promoting the apoptosis of breast cancer. Our results pinpoint the mechanism by which Dnd1 exerts its function in breast cancer apoptosis. Binding of Dnd1 to Bim-3' UTR prohibits the interaction between miR-221 and Bim.

The mRNA stabilization is regulated by RBPs and miRNAs posttranscriptionally. RBPs modulate mRNA translation and stability by interacting mostly with the 3' UTR [22]. miRNAs frequently bind to "seed matches" in the 3' UTRs of target mRNAs resulting in translational inhibition or degradation of
Figure 4: Continued.
protein-coding transcripts [23]. Amount of evidence shows that RBPs posttranscriptionally regulate mRNA levels via the joint influence of miRNAs [24]. Such that miRNA processing is regulated by ADAR1 in a catalytically independent manner, which is required for differentiation and neural induction [25]. miR-21 is sequestered by HuR to prevent translation repression of proinflammatory tumor suppressor gene-programmed cell death 4 in breast cancer cells [26]. Lin28A could enhance chemosensitivity of colon cancer cells to 5-FU by promoting apoptosis in a let-7 independent manner [27]. And MCPIP1 can selectively destabilize transcripts associated with an antiapoptotic gene expression program in breast cancer cells that can elicit complete tumor regression [28]. In contrast, let-7-mediated RISC could also be recruited to the 3' UTR of Myc by HuR and thus downregulates c-Myc expression [29]. Therefore, when the binding sites for RBPs overlap with or close to miRNA-binding sites, RBPs could either cooperate or compete with miRNAs via their physical interactions. Here, luciferase reporter assays showed that the binding sites for Dnd1 in Bim overlap with the miR-221 binding sites; therefore Dnd1 could compete with miR-221 in Bim-3'UTR.

Dnd1 plays a wide role in regulating gene expression and miRNA processing. It must be noted that, in addition to the competitive interactions between miR-221 and Dnd1, other potential mechanisms may be involved in the regulation for Bim expression. Nevertheless, the competitive binding between miR-221 and Dnd1 could give novel views into the modulation of Bim level, thus promoting the apoptosis in breast cancer cells. Based on the positive correlation between Dnd1 and Bim in breast cancer tissues, increased Dnd1 expression levels could act in concert with Bim or other tumor suppressors to promote breast cancer cell apoptosis, which indicates that Dnd1 possesses a tumor-suppressive role in breast cancer development. However, based on the fact that the functions of RBPs are different in various tumors, further animal studies should be performed to examine our conclusions, and it would be interesting to see whether this study could be extended to other tumors; thus then the results may have clinical applications.

In the present study, both miR-221 and Dnd1 posttranscriptionally regulate their target, Bim mRNA. Knockdown Dnd1 in breast cancer cells promotes Bim mRNA decay by competitive inhibiting the combination of miR-221 with Bim-3'UTR. Hence, knockdown of Dnd1 decreases the apoptosis of breast cancer cells and defining Dnd1 as one of the key players that regulate breast cancer development provides the possibility of controlling the apoptosis of breast cancer cells by modulating the expression of the Dnd1 protein.

Competing Interests
The authors declare that they have no conflict of interests.

Authors’ Contributions
Feng Cheng and Ying Pan contributed equally to the work.
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