Identification of the potential roles of ring finger protein 8 in TP53-mutant breast cancer

FENG ZHAO1,2*, PEIBIN WANG1,2*, YAN GUO3*, QI LU1,2, XU KONG1,2, DONGWEI SU4, HENGYU LI5, GUOPING LIU4 and CHAOQIAN LIU4

1Anhui Province Engineering Laboratory of Occupational Health and Safety, Anhui University of Science and Technology, Huainan, Anhui 232000; 2Department of General Surgery, Huainan First People's Hospital Affiliated to Bengbu Medical College, Huainan, Anhui 232000; Departments of 3Endocrinology and 4General Surgery, Changhai Hospital, Second Military Medical University, Shanghai 200433, P.R. China

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Abstract. Breast cancer is one of the malignant tumors with the highest mortality rate. With the development of precise treatment technology for cancer, numerous molecular targets have been identified and applied in the treatment of diseases. The present study investigated the potential role of ring finger protein 8 (RNF8) in TP53-mutant breast cancer and explored its possible mechanisms of action through a combination of bioinformatics techniques and cell biology. The results revealed that significantly different genes were expressed in RNF8-knockout mice sequencing data compared with the control group in the presence of TP53 mutations. Downregulated genes were significantly enriched in several pathways of cell proliferation and apoptosis regulation, development and transcription regulation, while upregulated genes were mainly enriched in immune response-associated signaling pathways. Therefore, the consensus genes of the major signaling pathways were further analyzed, revealing that among patients with TP53 wild-type breast cancer, the prognosis of patients with low expression levels of fibroblast growth factor receptor 1, LIM homeobox 2 and EPH receptor B2 was improved compared with that of patients with high expression levels. Finally, cell biology experiments demonstrated that in TP53-mutant breast cancer cells (HCC1937), inhibition of RNF8 significantly inhibited the proliferation of TP53-mutant HCC1937 cells and promoted their apoptosis. The present findings may enrich the understanding of the role of RNF8 and indicated that RNF8 may be used as a potential molecular target in TP53-mutant breast cancer, which may lead to the development of clinical treatment strategies.

Introduction

Breast cancer is one of the most common types of cancer in the world. Breast cancer cases in the world have continued to increase from 2005, with 12% of women expected to develop this disease in their lifetime (1). Ongoing research on breast cancer is trying to improve its treatment to assist in dealing with the issue (2).

Ring finger protein 8 (RNF8) is a gene in the human body that encodes the E3 ubiquitin-protein ligase RNF8 enzyme (3). It is involved in various activities of the body associated with immune system functions and DNA repair (4,5). Previous studies have indicated that RNF8 is a damage-responsive protein that integrates both protein phosphorylation and ubiquitination, serving an essential role in disease treatment (6,7). Additionally, depletion of RNF8 in the body can lead to cell cycle arrest and inhibition of cell proliferation (8).

The vital role of RNF8 in diseases is evident in the treatment of various types of cancer (9). It was reported that RNF8 can act as a co-activator to promote cell proliferation of estrogen receptor (ER)α-positive breast cancer via post-transcriptional signaling pathways (8). Furthermore, RNF8 was reported to promote epithelial-mesenchymal transition of breast cancer cells and facilitate the metastasis of breast cancer cells in vivo (10). By contrast, another study demonstrated that mice with RNF8 deficiency have an increased risk of mammary tumorigenesis, since RNF8 can regulate Notch signaling by ubiquitylating the active NOTCH1 protein, leading to its degradation (7), while abnormal activation of Notch signaling would promote the risk of breast cancer.
Additionally, Gao et al. (11) revealed that RNF8 can negatively regulate the activation of NF-κB, of which excess activation can lead to cancer. Therefore, there are controversial roles of RNF8 in tumorigenesis, and the function of RNF8 in breast cancer remains unclear.

In the present study, a series of bioinformatic approaches were used to analyze breast cancer sequencing data to explore the underlying mechanism of RNF8 in breast cancer. Subsequently, further experiments were performed to verify the bioinformatic results. The present study suggested that breast cancer progression may be closely associated with RNF8 expression.

Materials and methods

Bioinformatics analysis. RNA sequencing data from TP53-mutant mammary tumor cells was downloaded from the GSE76075 dataset containing mRNA sequencing data for two independent mouse TP53-mutant breast cancer cell lines (RNF8−/− and RNF8-wild-type) (12) from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76075). The gene names and gene count files were obtained, and the GFOID values of each gene were calculated using the open source GFOID software v1.1.4 (13). A total of 500 differentially expressed genes with the largest (upward) and minimum (downward) GFOID values were selected, and Gene Ontology (GO) enrichment analysis was performed on these genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/). Furthermore, the GO enrichment results of each group were clustered using the GOSemSim v3.11 R package (http://www.bioconductor.org/packages/release/bioc/html/GOSemSim.html) (14), and the clustering results between the groups were visualized using the ggtree v3.11 R package (http://www.bioconductor.org/packages/release/bioc/html/ggtree.html) (15). Subsequently, survival analysis of key genes was performed using Kaplan-Meier (KM) Plotter (http://kmplot.com/analysis/) with the log-rank test.

Cell culture. The HCC1937 cell line was purchased from the China Infrastructure of Cell Line Resources (Institute of Basic Medical Sciences; Chinese Academy of Sciences). The HCC1937 cell line was selected due to its TP53-negative status, which can eliminate the uncertainty derived from TP53 mutations. Using the HCC1937 cell line helps to maintain consistency between human and mouse cell models. The cells were cultured in the RPMI 1640 (Thermo Fisher Scientific, Inc.) at 37˚C in the presence of 5% CO2 and 5% FBS (Gibco; Thermo Fisher Scientific, Inc.) medium containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.)

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| siRNAs   | Sequence          |
|----------|-------------------|
| siRNA1   | 5'-UGCGAGAUAUGAAUUGAAU-3' |
| siRNA2   | 5'-GGACAAUUUGGCAACAAAGA-3' |
| siRNA3   | 5'-UAAGGAGAUAUGGGAGAAT-3' |
| Control   | 5'-GGUACGGCUUACAGUCCGAGUAATT-3' |

siRNA, small interfering RNA.

Cell transfection. According to the sequence of the RNF8 gene, three small interfering (si)RNAs (Table I) with different target sequences of RNF8 were synthesized by Shanghai Shenggong Biology Engineering Technology Service, Ltd., to decrease the expression levels of the RNF8 gene in the TP53-mutant HCC1937 cells. Untreated cells were used as negative control 1. An non-specific scrambled siRNA (50 nM) was used as negative control 2 and specific siRNAs (siRNA1, siRNA2 and siRNA3; 50 nM), as well as a siRNA mix (consisting of all three specific siRNAs) against RNF8 were directly transfected into HCC1937 cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Transfected cells were cultured at 37˚C in the presence of 5% CO2 for 48 h, after which subsequent experiments were performed.

Cell proliferation assay. Cell Counting Kit-8 (CCK-8; Takara Bio, Inc.) was used to evaluate cell proliferation. Briefly,
5x10^5 cells/well were seeded into 96-well plates and cultured in medium containing 10% FBS for 24, 48, 72 or 96 h at 37°C in the presence of 5% CO₂. After 2 h of culture with the CCK-8 reagent, the cells were analyzed at a wavelength of 450 nm using a microplate reader.

**Flow cytometric assay.** The pretreated cells were harvested by trypsinization and fixed with 70% ethanol at 4°C for 2 h. Subsequently, the cells were stained with 10 μl Annexin V-FITC and 5 μl propidium iodide (both Shanghai Yeasen Biotechnology Co., Ltd.) for 1 h at room temperature. Finally, cells were analyzed using a FACS instrument (FACSCelesta; BD Biosciences) and corresponding data were analyzed using the FlowJo v10.6.2 software (FlowJo LLC).

**Statistical analysis.** All experiments were performed in triplicate. GraphPad Prism 8.0 (GraphPad Software Inc.) was used for statistical analysis. The data are presented as the mean ± SD. The difference between two groups was analyzed by unpaired Student's t-test, while the comparisons among >2 groups were performed by one-way ANOVA followed by Bonferroni’s multiple comparisons test as the post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Transcriptome analysis of RNF8-/- TP53-mutant cells.** To evaluate the signaling pathways affected by RNF8-knockdown in TP53-mutant breast cancer cells, the GSE76075 dataset was downloaded from the GEO database, containing mRNA sequencing data for two independent mouse TP53-mutant breast cancer cell lines (RNF8-/- and RNF8-wild-type). The reads per kilobase of exon per million mapped sequence reads (RPKM) values were used to quantify the expression intensity of the genes, and the GFOLD values were used to evaluate the degree of differential gene expression, and a heat map was used to present the differential expression between the two cell lines. The results revealed that the GFOLD values of most of the genes between the two samples had different expression patterns (Fig. 1A). In addition, GFOLD values >1 or <1 were used as the threshold for differential expression. The results revealed that there were 2,932 upregulated genes (~12%) and 2,255 downregulated genes (~10%) in RNF8-/- cells compared with wild-type cells (Fig. 1B and C).

**Enrichment and clustering.** To further analyze the molecular mechanism of RNF8 in TP53-mutant breast cancer, a GO enrichment analysis of the top 500 downregulated and upregulated genes among the differentially expressed genes was performed using DAVID. The GO analysis yielded a large number of terms, making it difficult to quickly extract effective information; therefore, statistically significant GO terms were clustered by semantic similarity analysis. The results revealed that in RNF8-knockout TP53-mutant breast cancer cells, downregulated genes were significantly enriched in several pathways involved in cell proliferation and apoptosis regulation, development and transcription regulation, while upregulated genes were mainly enriched in immune response-associated pathways (Fig. 2).

**Survival analysis based on key genes.** A previous study has demonstrated that RNF8 is involved in the reproductive development of the body (17). Therefore, the present study investigated whether RNF8 regulated genes in development-associated pathways in TP53-knockout breast cancer cells. A statistical analysis of all the genes enriched in the GO ‘development’ cluster was performed, and genes that appeared in >3 GO terms were selected. Finally, a total of 10 genes were obtained, namely Myosin Heavy Chain 10, Paired Box 6, LHX2, GATA Binding Protein 3, Fibroblast Growth Factor Receptor 1 (FGFR1), EPHB2, Sphingosine-1-Phosphate Receptor 1, Empty Spiracles Homeobox 2, BRSK1 and GDNF family receptor α-2. Subsequently, the association between these 10 genes and the prognosis in patients with breast cancer was analyzed using KM Plotter (Table III). The results revealed that in patients with TP53-wild-type breast cancer, the prognosis in patients with low expression levels of LHX2 and EPHB2 was significantly improved compared with that in patients with high expression (LHX2, P=0.00045; EPHB2, P=0.024; Fig. 3A and B), while the prognosis in patients with low expression levels of FGFR1 was significantly decreased compared with that in patients with high expression (FGFR1, P=0.026; Fig. 3D). In patients with TP53-mutant breast cancer, there was no significant difference in survival status, except for the prognosis in patients with high BRSK1 expression, which was significantly improved compared with that in patients with low expression (P=0.0014; Fig. 3C).

**RNF8-knockdown inhibits cell proliferation and promotes apoptosis in human TP53-mutant breast cancer cells.** To further elucidate the role of RNF8 in TP53-mutant breast cancer,

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Table II. Primer sequences of the target genes.

| Gene name | Forward primer | Reverse primer |
|-----------|----------------|----------------|
| RNF8      | 5'-AAGCGACGGGCAGCAGAA-3' | 5'-AGCACCTTCACCTTCCTCAG-3' |
| GAPDH     | 5'-GCACCGTCCAAGGCTGAGAC-3' | 5'-TGGTGAGAACGGCCAGTGGAG-3' |
| EPHB2     | 5'-TGAGTGCCCTCAGATGCTCA-3' | 5'-AGGCGAGGTTACATCACATGTAATG-3' |
| LHX2      | 5'-GGAAGCATCTACTGCAAGGAAG-3' | 5'-GAGGTTGATAAAACCAAGTCCCC-3' |
| BRSK1     | 5'-CGGGAACTTCACTCTCCTTGGAC-3' | 5'-ACAGCACACTGTGACTCAGGGT-3' |

RNF8, ring finger protein 8; LHX2, LIM homeobox 2; EPHB2, EPH receptor B2; BRSK1, BR serine/threonine kinase 1.
RNF8 mRNA and protein expression was knocked down in the TP53-mutant human breast cancer HCC1937 cell line using three different siRNAs and a siRNA mix consisting of a; three siRNAs (Fig. 4A) In order to achieve the best interfering
effect, the siRNA mix was used in subsequent experiments. The results demonstrated that RNF8-knockdown in HCC1937 cells significantly decreased cell proliferation compared with that in control cells (Fig. 4B). Since the aforementioned bioinformatics analysis revealed that downregulated genes after RNF8-knockout were mainly enriched in apoptosis-associated signaling pathways, the effect of RNF8-knockdown in siRNA-treated HCC1937 cells was compared with that in control siRNA-treated HCC1937 cells using flow cytometry. The results demonstrated that apoptosis in HCC1937 cells with RNF8-knockdown was significantly higher compared with that in control cells (Fig. 4C). Overall, the present results indicated that downregulation of RNF8 expression inhibited proliferation and enhanced apoptosis in TP53-mutant breast cancer cells.

Table III. Association between 10 differentially expressed genes and the prognosis in patients with breast cancer.

| Gene      | Count | Frequency, % | GFold | Rank | Kaplan-Meier analysis P-value (TP53-mutated) | Kaplan-Meier analysis P-value (TP53-wild-type) |
|-----------|-------|--------------|-------|------|--------------------------------------------|---------------------------------------------|
| MYH10     | 5     | 45.45        | -6.07418 | 196  | 0.57000                                    | 0.77000                                    |
| PAX6      | 4     | 36.36        | -7.30754 | 98   | 0.17000                                    | 0.84000                                    |
| LHX2      | 4     | 36.36        | -7.12856 | 116  | 0.60000                                    | 0.00045                                    |
| GATA3     | 4     | 36.36        | -4.67309 | 372  | 0.37000                                    | 0.62000                                    |
| FGFR1     | 4     | 36.36        | -4.43547 | 418  | 0.81000                                    | 0.05800                                    |
| EPHB2     | 3     | 27.27        | -7.27086 | 101  | 0.49000                                    | 0.02400                                    |
| S1PR1     | 3     | 27.27        | -7.51289 | 82   | 0.52000                                    | 0.74000                                    |
| EMX2      | 3     | 27.27        | -5.30267 | 286  | 0.86000                                    | 0.51000                                    |
| BRSK1     | 3     | 27.27        | -6.58582 | 153  | 0.00140                                    | 0.23000                                    |
| GDNF      | 3     | 27.27        | -5.50851 | 254  | 0.61000                                    | 0.66000                                    |

MYH10, Myosin Heavy Chain 10; PAX6, Paired Box 6; LHX2, LIM Homeobox 2; GATA3, GATA Binding Protein 3; FGFR1, Fibroblast Growth Factor Receptor 1; EPHB2, EPH Receptor B2; S1PR1, Sphingosine-1-Phosphate Receptor 1; EMX2, Empty Spiracles Homeobox 2; BRSK1, BR serine/threonine kinase 1; GDNF, GDNF family receptor α-2.

Figure 2. Gene Ontology term clusters of upregulated and downregulated genes in ring finger protein 8-knockdown/TP53-mutant breast cancer cells. Enrichment P-values are represented by the red scale.

qPCR and western blot analysis of the expression levels of LHX2, EPHB2 and BRSK1 in TP53-mutant human breast cancer cells with low RNF8 expression. Since low FGFR1 expression was associated with a poor prognosis, which indicated that it may have a different function compared with LHX2 and EPHB2 in breast cancer, FGFR1 was excluded from subsequent evaluations. The association between RNF8 and the three aforementioned genes associated with prognosis, LHX2, EPHB2 and BRSK1, was based on sequencing data from mouse breast cancer cells. To verify whether knockdown of RNF8 in human breast cancer cells also caused a decrease in the expression levels of LHX2, EPHB2 and BRSK1, qPCR was performed, revealing that the expression levels of EPHB2, BRSK1 and LHX2 were significantly downregulated in human...
Figure 3. Kaplan-Meier curve analysis of overall survival based on Kaplan-Meier plotter mRNA expression levels of (A) LHX2, (B) EPHB2, (C) BRSK1 and (D) FGFR1 in patients with breast cancer. HR, hazard ratio; LHX2, LIM homeobox 2; EPHB2, EPH receptor B2; BRSK1, BR serine/threonine kinase 1; FGFR1, fibroblast growth factor receptor 1.
breast cancer HCC1937 cells after treatment with the siRNA mix (Fig. 5A). A similar trend was also observed in protein expression in RNF8-silenced cells (Fig. 5B).

Discussion

Previous studies have indicated that TP53 mutations have important functions in multiple subtypes of breast cancer, such as triple-negative and HER2+ breast cancer (18-20). Similarly, some studies have reported that RNF8 is associated with the oncogenesis of breast cancer (10,11). For example, Kuang et al (10) demonstrated that RNF8 promotes epithelial-mesenchymal transition in breast cancer cells (including TP53 wild-type and mutant cells); however, the aforementioned study did not investigate the mechanism of RNF8 promoting epithelial-mesenchymal transition in TP53-mutant breast cancer. Other studies have tried to investigate the specific mechanism of RNF8 in breast cancer (21,22). For example, it has been reported that RNF8 cooperates with RNF168 to mediate Forkhead Box M1 ubiquitination and degradation in breast cancer (21); however, the aforementioned study used the MCF7 cell line, which is a TP53-wild-type breast cancer cell line. In the present study, different datasets were used to investigate the role of RNF8 in TP53-mutant breast cancer and to explore the specific mechanism of RNF8 promoting TP53-mutant breast cancer through transcriptome sequencing, and verified the co-expression of RNF8, LHX2 and EPHB2 in the HCC1937 cell line.

RNF8 is an E3 ubiquitin ligase that acts primarily on the DNA damage repair process and can rapidly accumulate at the site of DNA damage through Fork Head Associated domain-mediated phosphorylation of Mediator Of DNA Damage Checkpoint 1 (22-24). Numerous proteins associated with DNA repair, such as P53 binding protein and BRCA1, can accumulate and function through its signal amplification (25). Therefore, RNF8 is a key factor that serves an important role in telomere protection, maintaining genomic integrity and regulating the cell cycle (26).
A previous study has revealed that the function of RNF8 is highly dependent on the P53 protein, suggesting that RNF8 serves a biological role in synergy with P53 (27). However, numerous studies have revealed a number of mutations in the P53 gene in tumor cells (28,29); therefore, it is increasingly important to investigate the functional role of RNF8 under the condition of P53 mutation (27). The present study identified the differentially expressed genes in mouse transcriptome data from the GEO database by comparing the low and normal expression levels of RNF8 in the TP53-mutant samples, revealing that downregulated genes were mainly enriched in several pathways involved in cell proliferation and apoptosis regulation, development and transcription regulation. This is consistent with previous studies (28,29), indicating that even in the presence of P53 mutations, the main function of RNF8 is still focused on the regulation of the cell cycle and transcription factors, thus confirming that the function of RNF8 is not completely P53-dependent and that TP53 mutations have a relatively small effect on the physiological function of RNF8.

The role of RNF8 in tumor development and progression exhibits a double-sided action, resulting in either tumor inhibition or promotion. In a mouse model, the downregulation or loss of RNF8 results in a significant increase of tumor incidence, including the incidence of leukemia in tumorigenesis of lymphoma (50%), thymoma (38%), mammary carcinoma (13%), skin tumor (13%) and sarcoma (13%) (30). A previous study has revealed that the expression levels of P53 are significantly increased in RNF8-knockout mice (12). At the same time, RNF8 has a strong inhibitory effect on breast cancer and can downregulate the Notch signaling pathway by deleting the C-terminal of the NOTCH1 intracellular domain (7), thereby limiting the spread of mammary luminal progenitors. Data from The Cancer Genome Atlas also support a negative association between RNF8 expression and the Notch signaling pathway (7).

However, a previous study has revealed that decreased RNF8 expression can significantly improve radiotherapy sensitivity in nasopharyngeal cancer, while inhibiting cell proliferation and increasing apoptosis (31). In breast cancer MCF-7 cells, overexpression of RNF8 can significantly increase epithelial-mesenchymal transition and it mainly promotes phosphorylation of GSK3β, further inhibiting its activity (10). In addition, an increase in phosphorylation of β-catenin has been observed in RNF8-overexpressing cells (10). Furthermore, in the treatment of breast cancer using tamoxifen to block ERα, tamoxifen exerts anticancer effects mainly by blocking the ERα signaling pathway (31), but a previous study has revealed that RNF8 can activate the ERα signaling pathway and thus promote tumor cell proliferation (8). The aforementioned studies did not focus on p53 mutations. Therefore, the present study investigated the effects of RNF8 in TP53-mutant breast cancer cells, revealing that downregulation of RNF8 expression in a cell model with TP53 mutation significantly inhibited the proliferation of tumor cells. In addition, the current study hypothesized that the tumorigenesis and development of breast cancer may be regulated by a sophisticated network, and the role of RNF8 in the tumorigenesis of TP53-mutant breast cancer may not be unique. Therefore, future studies should further explore RNF8/TP53 and immune response-associated pathways (32).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request, and the GSE76075 dataset is available from the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76075).

Authors' contributions

FZ performed the cell experiments, prepared the figures and wrote the manuscript. PW and YG performed the qPCR and western blot analysis, and data interpretation. QL and XK performed flow cytometry. DS and HL performed the bioinformatics analysis. GL and CL designed the study and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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