Down-regulation of RFWD3 inhibits cancer cells proliferation and migration in gastric carcinoma

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Abstract. The E3 ligase RING finger and WD repeat domain 3 (RFWD3) can stabilize p53 in response to DNA damage, participate in replication checkpoint, and have an important role in multiple myeloma, testicular germ cell tumor and lung carcinogenesis. Its expression and molecular mechanisms have never been explored in gastric cancer. In present study, the RFWD3 was found over-expressed in the both AGS and HGC-27 gastric cancer cells. Knockdown of RFWD3 suppressed cells proliferation activity of gastric cancer cells. Further study showed that the down-regulation of RFWD3 promotes cell apoptosis, suppresses cell migration and invasion and blocks G2/M cell cycle progression, which may be related with AKT, ERK/P38 and Slug pathways. In summary, the results of the present study showed that RFWD3 might be an oncogene candidate for gastric cell proliferation and may have an important role in gastric carcinogenesis.

Key words: RFWD3 — Gastric carcinoma — Cell proliferation — Cell migration

Introduction

Gastric cancer is the fifth most frequent malignancy and the third leading cause of cancer death worldwide, accounting for ~723,000 deaths annually (Ferlay et al. 2015). However, most patients are diagnosed at an advanced stage of the disease. Although advanced gastric cancer is, in general treated with surgery and/or chemotherapy, the overall 5-year survival rate is less than 24% (Lordick et al. 2017; Russo et al. 2019). Gastric cancer is a complex disease characterized by the accumulation of multiple genetic and epigenetic alterations to oncogenes, which disturb the expression of genes controlling critical regulatory processes (Rocken et al. 2017; Birkman et al. 2018). However, the exact molecular mechanisms underlying gastric cancer initiation and progression are still not well understood, which prevent the development of personalized and suitable cancer therapy strategies. Therefore, it is critical to discover reliable prognosis and therapeutic molecular biomarkers for gastric carcinoma and targeted treatment gastric cancer.

The ubiquitin proteasome system (UPS) is one of the important signal transduction pathways (Mu et al. 2007). Ataxia Telangiectasia-mutated (ATM) and ATM-Rad3-related (ATR) are two key kinases in UPS (Mu et al. 2007; Kitajima et al. 2018). Recent studies have found that the E3 ligase RING finger and WD repeat domain 3 (RFWD3 or RNF201/FLJ10520) was identified as a phosphorylation substrate protein of the ATM/ATR (Mu et al. 2007; Kitajima et al. 2018). RFWD3 can positively regulate p53 stability in response to DNA damage when the G1 cell cycle checkpoint is activated (Fu et al. 2008). RFWD3 functionally interacted and participated in replication checkpoint control, and also respond to replication protein A (RPA)-mediated DNA damage in cancer cells (Gong et al. 2011; Liu et al. 2011). Besides, study showed that RFWD3 is necessary for replication fork restart, normal repair kinetics during replication stress, and homologous recombination (HR) at stalled replication forks (Inano et al. 2017). Biallelic mutations have been found in RFWD3 in patients with Fanconi anemia, which contributes to this disease (Knies et al. 2017). A genome-wide association study demonstrated
that RFWD3 is a susceptible site for malignant neoplasms, including multiple myeloma and testicular germ cell tumor (Chung et al. 2013; Mitchel et al. 2016). Recently, Zhang et al. (2019) found that RFWD3 is more elevated in tumor samples than in paired normal lung tissues and is inversely associated with the clinical outcome of patients with non-small cell lung cancer, which suggest that RFWD3 may have an important role in lung carcinogenesis. However, the significance and elaborate molecular mechanisms of RFWD3 in gastric cancer remains to be elucidated, although a research showed 125I seed irradiation can partly inhibit growth of gastric cancer xenografts via up-regulating the expression level of RFWD3 (Ma et al. 2019). Here, we obtained the RFWD3 through a series of data standardization and statistical analysis from The Cancer Genome Atlas Database (TCGA), and further examined the function of RFWD3, in terms of cell proliferation, migration, and explored possible molecular mechanism in gastric carcinoma.

Materials and Methods

TCGA analysis

We collected the transcriptomic and clinical phenotype information of the gastric cancer-related tissues from TCGA database. The database was applied to predict the target genes of the potential differentially expressed RNA and the considerable amount of transcriptomic data provided the gene expression level of RFWD3.

Cell culture

Human gastric epithelial cell lines GES-1, human gastric cancer cell lines SGC-7901, MGC-803, AGS and HGC-27 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% streptomycin, and all cells were cultured in incubator containing 5% CO2 at 37°C.

Gastric cell transfections for gene silencing

To knockdown RFWD3 expression, AGS and HGC-27 cells were plated in 6-well plates at a density of 3×10^5 and cultured for 24 h. shRNA against RFWD3 or non-targeting control were transfected using lipofectamine™ 2000 reagent (shRFWD3). Culture medium was replaced with fresh medium after 6 h and cells were incubated for an additional 24 h. A pool of shRNA against RFWD3 was used. Sequence: (sense: 5'-TGA AGATGCGAGGTGTACGT-3', antisense: 5’-TAGAAGCAGAAGGTAGCAGG G-3’).

Cell proliferation assay

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 96-well plates (5×10^4 cells/well) and were allowed to attach overnight in complete growth media. After culturing for 24, 48, 72 and 96 h, cell proliferation was evaluated by thiazolyl blue tetrazolium bromide (MTT) assay.

Flow cytometric analysis of cell apoptosis

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 6-well plates (3×10^5 cells/well) and were allowed to attach overnight in complete growth media. Cells were collected by the pancreatin without EDTA, stained with 5 μl AnnexinV-APC and 5 μl 7-AAD for 5–15 min at room temperature avoiding light, and immediately analyzed on FACSCalibur flow cytometer.

Cell migration assay

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 6-well plates (3×10^5 cells/well) and grown to 90% confluence. We scratched the cells in the same way with using a sterile pipette tip and washed with PBS to remove the floating cells, then cells were allowed to attach for 24 h. Resulting images were captured with a microscopic camera system.

Cell invasion assay

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 24-well plates (2×10^5 cells/well) and were allowed to attach overnight in complete growth media. Coating Matrigel was plated in the upper chamber before the seeding of cells. The lower chamber was filled with medium containing 10% FBS as a chemoattractant. Cells were incubated for 24 h at 37°C and invade through the matrigel. The cells adhered to the lower chambers were fixed using 4% paraformaldehyde for 30 min and stained using 0.5% crystal violet for 20 min, photographed by inverted microscope.

Flow cytometric analysis of cell cycle

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 6-well plates (3×10^5 cells/well) and were allowed to attach overnight in complete growth media. All cells were harvested by digesting with trypsin and washed with PBS and then fixed with ice-cold 70% ethanol in PBS for 30 min at –20°C. Fixed cells were washed with PBS, treated with 10 μl RNase A (1 mg/ml) and resuspended in 10 μl of 400 μg/ml propidium iodide (PI) for staining. Cell cycle distribution was performed with FACSCalibur flow cytometer.
**RT-PCR analysis**

AGS and HGC-27 cells (shRNA NC and shRFWD3) were washed with PBS and collected for RT-PCR. The primers designed for RFWD3 were 5’-TGAAGAGTTGCAAGGTGTCTAGT-3’ as forward and 5’-TAGAAGCAGAAGGTAGCAGAGG-3’ as reverse. Then, reverse transcription was performed as following: 50°C for 1 min for the reverse transcription. Thermal cycles were: at 95°C for 10 min, then 40 cycles at 95°C for 30 s, at 60°C for 30 s. For RT-PCR analysis, the GAPDH was used as the internal control.

**Western blot analysis**

AGS and HGC-27 cells (shRNA NC and shRFWD3) were seeded into 6-well plates (3×10⁵ cells/well) and were allowed to attach overnight in complete growth media. The cells were lysed in protein lysate buffer for 10 min. Total proteins were centrifuged at 12,000 rpm for 10 min at 4°C and quantified. Protein samples were separated with 10% SDS-PAGE gel. After electrophoresis, the proteins in the gel were transferred to PVDF membrane, incubated with 5% skim milk for 1.5 h. After overnight incubation with the primary antibody, protein samples were incubated with HRP-conjugated secondary antibodies for 1 h. The results were detected by Quantity One software. The anti-RFWD3, anti-P-P38, anti-P38, anti-P-ERK, anti-ERK, anti-Slug, anti-P-AKT, anti-AKT, anti-E-Cadherin, anti-N-Cadherin, anti-β-actin, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP antibodies were obtained from Santa Cruz Biotechnology.

**Statistical analysis**

Data were expressed as means ± SEM of three independent experiments. Student’s t-test and two-way ANOVA were employed to analyze the statistical comparisons between sets of data. Significant differences were established at \( p < 0.05 \).

**Results**

RFWD3 expression was increased in gastric carcinoma

We conducted a systematic analysis by data standardization and statistical analysis from TCGA, and RFWD3 was unveiled (Fig. 1A). Specifically, 32 pairs of gastric carcinoma samples were used in the analysis. The results showed that RFWD3 expression was significantly upregulated in gastric carcinoma tissues compared to normal tissues. The differences were statistically significant as determined by Student’s t-test and two-way ANOVA.

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**Figure 1.** RFWD3 expression was increased in gastric carcinoma. **A.** Schematic of the study workflow. **B.** Distribution of biological differences of 32 pairs of gastric carcinoma samples. **C.** Distribution of biological differences of 26 pairs of gastric carcinoma samples. **D.** Distribution of differentially expressed genes in 26 pairs of gastric carcinoma samples. **E.** RFWD3 level in the tumor samples of 26 gastric carcinoma from TCGA. N, normal sample; C, cancer sample; FC, fold change.
samples with RNAseq and pathological information from TCGA were downloaded; we performed data filtering and data standardization. The results showed that there are differences between the majority of the normal samples and cancer samples, while samples 27–32 lacked of biological differences (Fig. 1B). Therefore, a total of 26 pairs of gastric carcinoma samples were identified, and further performed data standardization (Fig. 1C). Next, we analyzed the differential genes of 26 pairs of gastric carcinoma samples candidate genes (Fig. 1D), and RFWD3 was identified. As shown in Figure 1E, we further found that RFWD3 is elevated in the tumor samples of 15 gastric carcinomas. Based on above results, RFWD3 may be a candidate tumor oncogene in gastric cancer.

**Low expression of RFWD3 suppressed gastric cancer cells proliferation**

Furthermore, the expression level of RFWD3 in gastric cells was evaluated by RT PCR. It is noted that compared with normal gastric cells GES-1, the RFWD3 was over-expressed in gastric cancer cells (Fig. 2A). These data demonstrated that RFWD3 overexpression in gastric carcinoma and down-regulation of RFWD3 may be a potential strategy for gastric cancer treatment. In order to illustrate RFWD3 is a candidate tumor oncogene in gastric cancer, based on the fact that RFWD3 expression displayed relatively higher level in AGS and HGC-27 cells compared with other gastric cancer cells (Fig. 2A), AGS and HGC-27 cells were chosen for the following experiments. We verified the results by transfecting shRFWD3 in AGS and HGC-27 cells (Fig. 2B–D). If true, the low expression of RFWD3 would be expected to decrease the proliferation effect in gastric cancer. To test this, we evaluated the inhibitory activity in vitro against the growth of AGS and HGC-27 cells. Our results showed, that down-regulation of RFWD3 reduces cell proliferation in time-dependent manner, which suggested that RFWD3 may have important roles in the development of gastric cancer (Fig. 2E,F).

**Low expression of RFWD3 promoted gastric cancer cells apoptosis**

Admittedly, apoptosis is recognized as the classical form of programmed cell death, which plays a crucial role in animal processes, tissue homeostasis and pathogenesis (Adams et
Down-regulation of RFWD3 inhibits gastric carcinoma growth

To explore whether the inhibitory effect of low expression of RFWD3 on gastric cell growth is related to cell apoptosis, AnnexinV-APC/7-AAD double-staining assay was performed. As shown in Figure 3A and B, the results indicate that compared with shRNA NC (negative control) group, down-regulation of RFWD3 results in an increasing number of apoptotic cells, suggesting that low expression of RFWD3 exhibits a powerful pro-apoptotic effect.

Low expression of RFWD3 suppressed gastric cancer cells migration and invasion

Metastasis is a major cause of death in gastric carcinoma patients because cancer cells have a potent ability of migration (Han et al. 2019; Jiang et al. 2019). Therefore, we further investigated the effect of RFWD3 on cells migration capability using wound healing assay. In this study, AGS and HGC-27 cells were transfected shRFWD3 before creating the scratch in the confluent monolayers of cultured cells. After the treatment of 0 h and 24 h, we captured the images of the injured area. As shown in Figure 4A and B, the scratched area of shRNA NC group was mostly healed, while cell migration was significantly decreased in shRFWD3 group after 24 h incubation. In general, tumor cells have greater adaptability to the environment and fortissimo independent survival, and can infinitely invade. To measure the effect of RFWD3 low-expression on tumor cell invasion, cells growing in the log phase were collected and cultured on transwell apparatus. After 24 h incubation, down-regulation of RFWD3 significantly decreased invasiveness, compared with the shRNA NC group. These results demonstrated that RFWD3 low-expression reduces the migration and invasion of gastric cancer cells (Fig. 4C,D).

Low expression of RFWD3 blocked cell cycle progression

Generally, preventing the growth of cancer cells by altering the regulation of cell cycle at a specific checkpoint is related with the various tumor developments (Wang et al. 2018). Fu et al. (2019) found that RFWD3 could stabilize p53 in response to DNA damage when the cell cycle checkpoint was activated, which showed RFWD3 may be associated with cell cycle arrest. In the present study, the cell cycle event mediated by RFWD3 was investigated on the gastric cancer cell lines AGS and HGC-27 cells. As shown in Figure 5A and B, down-regulation of RFWD3 resulted in an accumulation of cells in G2/M phase, which indicate that RFWD3-induced G2/M phase of cell cycle arrest may be one of the mechanisms of gastric cancer cell growth inhibition.

RFWD3 may be involved in AKT, ERK/P38 and Slug pathway

Evidence has revealed that many oncogenes and tumor suppressor genes are directly involved in regulation of

Figure 3. Gastric cancer cell apoptosis of the AGS (A) and HGC-27 (B) cells transfected with shRNA NC or shRFWD3. ** p < 0.01, *** p < 0.001 vs. shRNA NC.
intrinsic or extrinsic pathway (Desterro et al. 2019). In exploring the molecular mechanism caused by RFWD3 low-expression in AGS and HGC-27 cells, we found that P-P38, P-ERK, P-AKT protein expression levels are decreased in shRFWD3 cells compared with shRNA NC-treated cells. It also indicated that Slug and N-Cadherin protein expression levels are decreased, while the expression level of E-Cadherin is increased (Fig. 6A,B). These data all showed that RFWD3 may be involved in AKT, ERK/P38 and Slug pathway-related cell apoptosis.

Figure 4. The migration and invasion activity of gastric cancer cells AGS (A and C) and HGC-27 (B and D), transfected with shRNA NC or shRFWD3. *** p < 0.001 vs. shRNA NC.

Figure 5. The cell cycle progression of gastric cancer cells AGS (A) and HGC-27 (B) transfected with shRNA NC or shRFWD3.
Discussion and Conclusion

Gastric cancer is a common malignancy of human digestive system which has high incidence and mortality worldwide (Ferlay et al. 2015). Gastric cancer occurs without exhibiting specific clinical manifestations. Therefore, it is important to identify tumor markers with high sensitivity and specificity for clinical screening for early gastric cancer. Moreover, chemotherapies against gastric cancer with cytotoxic drugs are not sufficiently efficient.

Personalized postoperative adjuvant chemotherapy using biomarkers is a promising treatment strategy for improving outcomes of patients with advanced gastric cancer (Esfandi et al. 2019). Increasing studies have proved that tumor suppressor genes or oncogenes are aberrantly expressed and involved in the initiation and development of gastric cancer (Yan et al. 2018; Zhang et al. 2018; Jia et al. 2019). Thus, exploring the biological function of tumor suppressor genes or oncogenes can be useful for finding novel biomarkers and understanding the mechanism of gastric cancer progression.

In the present study, TCGA database showed that the expression of \textit{RFWD3} is significantly higher in gastric cancer tissues compared with those in matched para-tumorous tissues. Furthermore, RT-PCR showed that the level of \textit{RFWD3} was significantly increased among the gastric cancer cells, specially, in AGS and HGC-27 cells. In order to demonstrate that high expression of \textit{RFWD3} is related to the gastric cancer cells proliferation, we down-regulated the expression of RFWD3 using shRNA NC or shRFWD3. ** \( p < 0.01 \), *** \( p < 0.001 \) vs. shRNA NC.

**Figure 6.** The AKT, ERK/P38 and Slug pathway-related proteins expression level of gastric cancer cells AGS (A) and HGC-27 (B) transfected with shRNA NC or shRFWD3. ** \( p < 0.01 \), *** \( p < 0.001 \) vs. shRNA NC.
expression of RFWD3 in both AGS and HGC-27 cells. The results of MTT showed that down-regulation of RFWD3 expression increases the inhibitory effect of cells, which suggested RFWD3 is significantly associated with the development of gastric cancer. Moreover, our research showed that down-regulation of RFWD3 expression promotes gastric cell apoptosis, suppresses gastric cell migration and invasion and arrests gastric cell cells in G2/M phase. In addition, we discovered that these alterations are related with AKT, ERK/P38 and Slug pathways. Researches show that AKT, ERK/P38 and Slug pathways are all involved in the regulation of various biological processes and promote tumorigenesis (Cho et al. 2019; Oliveira et al. 2019; Revathiidevi et al. 2019). Moreover, the AKT, ERK/P38 and Slug pathways are activated by diverse cellular stimuli regulating various physiological functions such as cell growth, cell survival, cell cycle progression, protein translation and metabolism (Cho et al. 2019; Oliveira et al. 2019; Revathiidevi et al. 2019). Our results showed that down-regulation of RFWD3 significantly decreases the P-P38, P-ERK, P-AKT, Slug and N-Cadherin protein expression levels in AGS and HGC-27 cells, while the expression level of E-Cadherin is increased, suggesting that the downregulated expression of RFWD3 in gastric cell lines activated AKT, ERK/P38 and Slug signaling pathway. These results showed that RFWD3-mediated AKT, ERK/P38 and Slug signaling pathway may be one of the possible mechanisms of cell growth inhibition.

In conclusion, our results indicated that RFWD3 plays an accelerative role in the development of gastric carcinoma. Down-regulation of RFWD3 inhibited gastric cancer cell proliferation and promoted cell apoptosis. Moreover, down-regulated expression of RFWD3 inhibited cell migration, invasion and arrested cell cycle through the regulation of AKT, ERK/P38 and Slug pathways in gastric cancer cells. RFWD3 may be a vital regulator of gastric tumorigenesis and an important therapeutic target for gastric carcinoma, which deserves further investigation.

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