Ubiquitin ligase *RNF5* serves an important role in the development of human glioma

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Abstract. The ubiquitin ligase ring finger protein 5 (*RNF5*) has previously been associated with the development of breast cancer. Patients with breast cancer and high *RNF5* expression have been demonstrated to have a shorter survival time compared with patients with low *RNF5* expression. However, the role of RNF5 in human glioma has not been determined. The present study analyzed the role of RNF5 in gliomas using bioinformatics analysis. The results revealed that RNF5 was differentially expressed in non-cancerous brain tissues and different grades of glioma. Furthermore, a high *RNF5* expression in patients with glioma was associated with an improved prognosis compared with patients with low expression. Gene Set Enrichment Analysis revealed that *RNF5* was particularly associated with 'Wnt signaling pathway', 'apoptosis', 'focal adhesion' and 'cytokine-cytokine receptor interaction' in patients with glioma. Additionally, 4 potential ubiquitination substrates for *RNF5* were predicted, including sorting nexin 10, proprotein convertase subtilisin/kexin type 1, leucine rich glioma inactivated 1 and solute carrier family 39 member 12. These findings provided the basis for further investigation on the role of *RNF5* in tumors.

Introduction

Gliomas are the most common primary brain tumors with an incidence rate of ~5/100,000). Despite comprehensive treatment strategies, including surgery, radiotherapy and chemotherapy, the prognosis of patients remains unsatisfactory, with a median survival time of 12-18 months (1-4). This poor outcome is largely associated with the difficulty of curing gliomas and the high relapse rates (5,6). Therefore, the identification of novel therapeutic targets has become a particular focus of research.

Ring finger protein 5 (*RNF5*) belongs to the ring finger family of ubiquitin ligases (7,8), which are anchored to the endoplasmic reticulum (ER) membrane and are important components of the ER-associated degradation (ERAD) mechanism. RNF5 serves a role in monitoring the folding of CF transmembrane conductance regulator (CFTR), CFTRDF508 and nascent CFTRΔF508 in the ER membrane (9,10). Furthermore, a previous study reported that *RNF5* regulates cell movement by targeting paxillin ubiquitination and altering its localization (11). *RNF5* participates in the inflammatory response in viral infections by ubiquitinating transmembrane protein 173 and inhibiting the activation of virus-induced interferon regulatory factor 3, expression of interferon β1 and the cellular antiviral response (12). In breast cancer cells, *RNF5* ubiquitination degrades the L-glutamine carrier proteins solute carrier family 1 member 5 and solute carrier family 38 member 2, which reduces glutamine uptake and levels of the tricarboxylic acid cycle components, decreases mechanistic target of rapamycin signaling and cell proliferation, and increases autophagy and apoptosis (13). *RNF5* is highly expressed in breast cancer and related cell lines, and inhibition of its expression decreases cell proliferation (13). The present study attempted to characterize the role of *RNF5* in human glioma and to determine its association with tumor grade and survival time in patients with glioma.

The present study revealed that *RNF5* was differentially expressed in patients with different grades of glioma and was closely associated with the prognosis of patients with anaplastic glioma (AG) and glioblastoma multiforma (GBM). Moreover, Gene Set Enrichment Analysis (GSEA) identified the Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathways that were significantly associated with RNF5. Additionally, a correlation analysis was used to predict the potential ubiquitination substrates for *RNF5* in human glioma.

Materials and methods

Patient samples. mRNA microarray expression for patients were obtained from the Chinese Glioma Genome Atlas

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(CGGA; cgga.org.cn) and the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo). The CGGA contains 301 glioma samples (including 84 astrocytoma, 89 oligodendroglioma and 128 glioblastoma samples). The grouping of low-grade glioma (LGG), AG and GBM was performed as previously described (14). The GSE16011 dataset (www.ncbi.nlm.nih. gov/geo/query/acc.cgi?acc=GSE16011) contains 276 glioma samples (including samples from 8 patients with epilepsy and 24 astrocytoma, 85 oligodendroglioma and 159 glioblastoma samples) and 8 control samples, totaling 284 specimens (15). The GSE4290 dataset (www.ncbi.nlm.nih.gov/geo/query/acc. cgi?acc=GSE4290) contains 23 samples from patients with epilepsy as non-cancerous samples and 157 tumor samples, including 26 astrocytoma, 50 oligodendrogliomas and 81 glioblastoma samples (16). Gene mutation data were obtained from The Cancer Genome Atlas (TCGA; cancergenome.nih.gov).

RNF5 expression and its association with patient prognosis. The expression levels of *RNF5* in the CGGA database and GSE16011 and GSE4290 datasets were analyzed using GraphPad Prism software version 6.01 (GraphPad Software, Inc.). In addition, the association between the expression level of *RNF5* and the prognosis of patients was obtained using the CGGA database. In order to analyze patient prognosis, patients were equally divided into two groups according to *RNF5* expression levels.

Cell culture. The glioblastoma cell line U251 was obtained from The Type Culture Collection of The Chinese Academy of Sciences. U251 cells were cultured using DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C and 5% CO_2.

Plasmid and transfection. The *RNF5* cDNA sequence was inserted between the *Hin*dIII and *Xba*I restriction sites in the p3XFLAG-CMV-14 vector (Shanghai GenePharma Co., Ltd.). The p3XFLAG-CMV-14 plasmid was used as an empty vector. A total of 3 μg plasmid was transfected into U251 cells using 9 μl Polyjet transfection reagent (SignaGen Laboratories) according to the manufacturer's protocol. The culture medium was changed after 12 h and cells were transfected for 72 h.

Reverse transcription-quantitative PCR (RT-qPCR). RNF5 expression was assessed using RT-qPCR. Total RNA was extracted from U251 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse-transcribed into cDNA using the Quant One-Step RT-PCR kit (Tiangen Biotech Co., Ltd.). qPCR was performed using FastStart Universal SYBR Green Mix (Roche Diagnostics) and an ABI 7300 real-time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers for RNF5 and β-actin were designed as follows: RNF5, forward 5'-GTACCC ATACGATGTTCCAGATTACGC-3', reverse 5'-CTGAGC AGCCAGAAAAAGAAAAAGATG-3'; and β-actin forward, 5'-CATGTACGTTGCTATCCAGGC-3', and reverse, 5'-CGC TCGGTGAGGATCTTCATG-3'. Thermocycling conditions included pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and extension

at 72°C for 1 min for 35 cycles. Expression level of *RFN5* was calculated using the $2^{-\Delta\Delta Cq}$ method (17).

Cell colony formation assay. U251 cells $(1x10^5)$ overexpressing *RNF5* were seeded into 6 cm dishes and cultured for 14 days at 37°C. Cells were subsequently fixed in 4% paraformaldehyde for 30 min at room temperature and stained with 0.05% crystal violet for 30 min at room temperature. A light Canon 70D camera (Canon, Inc.) was used to capture images (magnification, x1).

GSEA to evaluate RNF5-enriched KEGG pathways. In order to elucidate the signaling pathways associated with the possible actions of *RNF5* in human glioma, enrichment analysis was performed using GSEA software version 6.2 (software.broadinstitute.org/gsea/login.jsp) in the CGGA database. *RNF5*-enriched KEGG (www.genome.jp/kegg) signaling pathways were identified through this analysis.

Identification of differentially expressed genes (DEGs). To predict the potential substrate(s) of *RNF5*, CGGA, GSE16011 and GSE4290 data were sorted according to *RNF5* expression level from low to high. Data were subsequently divided into four groups: A, B, C and D according to the number of patients following RNF5 determination. To avoid data with no significant differences from groups B and C, comparative analysis was performed between groups A and D to identify the DEGs from the three databases. To narrow the scope, the DEGs that overlapped among the three databases were identified using limma package of R software 3.4.4 (www.r-project.org) and selected for subsequent analysis. A gene with |logFC|>1 was defined as DEG.

Correlation analysis between RNF5 and overlapping genes. To demonstrate the correlation between *RNF5* and the five overlapping genes, analysis using R and GraphPad Prism software was performed. Correlation analysis was performed using Pearson's correlation coefficient test.

Statistical analysis. GraphPad Prism software was used for statistical analysis. Results are presented as the means ± standard error of the mean. Statistical significance was analyzed using Student's t-test (two groups) and one-way analysis of variance (multiple groups) followed by Dunnett's post hoc test. Kaplan-Meier survival analyses for overall survival were performed and compared with the log-rank test. P<0.05 was considered to indicate a statistically significant difference. For GSEA, a normalized enrichment score >1, nominal P<0.05 and false discovery rate q-value <0.25 were considered to indicate a statistically significant difference.

Results

Expression of RNF5 and its association with prognosis. To characterize the expression of *RNF5* and its association with patient prognosis, the CGGA database and the GSE16011 and GSE4290 datasets were used. *RNF5* was differentially expressed in LGG, AG and GBM. *RNF5* expression was significantly higher in LGG and AG compared with GBM
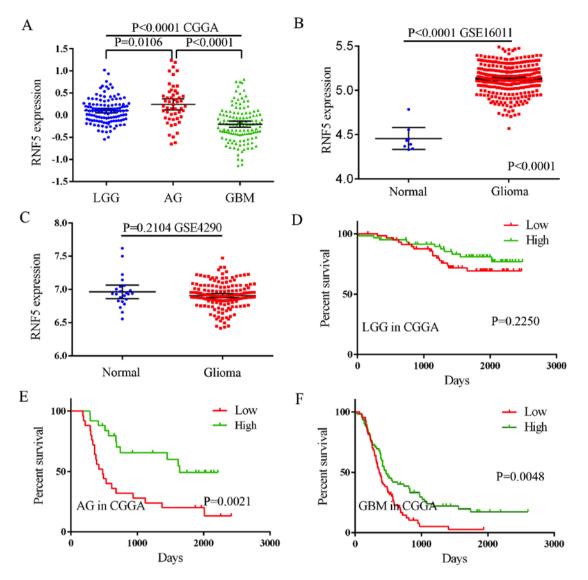
Figure 1. Expression levels of *RNF5* in non-cancerous brain tissue and glioma samples from patients with different disease grades and association with patient prognosis. (A) RNF5 was differentially expressed in different grades of glioma and exhibited the highest and lowest expression in AG and GBM, respectively. (B) *RNF5* expression significantly differed between non-cancerous brain and glioma tissues in the GSE16011 dataset. (C) Expression of *RNF5* did not significantly differ between non-cancerous brain tissue and glioma tissues in the GSE4290 dataset. Kaplan-Meier survival curve analysis of the prognostic significance of *RNF5* expression in patients with (D) LGG, (E) AG and (F) GBM. *RNF5*, ring finger protein 5; AG, anaplastic glioma; GBM, glioblastoma multiforme; LGG, low-grade glioma; CGGA, Chinese Glioma Genome Atlas.

(Fig. 1A). The GSE16011 and GSE4290 datasets were selected to investigate the difference in *RNF5* expression between non-cancerous brain tissue and glioma; however, a consistent conclusion was not reached owing to the small number of non-cancerous brain tissue samples in the datasets (Fig. 1B and C). However, an association between *RNF5* expression levels and patient prognosis was determined using the CGGA database. In LGG, the expression level of *RNF5* and patient prognosis were not significantly associated, while in AG and GBM, patients with high *RNF5* expression had an improved prognosis compared with patients with low expression (Fig. 1D-F).

To further investigate the association between high *RNF5* expression and prognosis in patients with glioma, tumor protein 53 (*TP53*) mutations were analyzed as indicated by the literature (18-21). An analysis of TCGA database revealed that *TP53* has a high mutation rate in GBM, while only

two *RNF5* mutations were identified (Fig. 2A). Additionally, *in vitro* analysis revealed that cells overexpressing *RNF5* exhibited increased colony formation compared with control cells (Fig. 2B-D). Subsequently, the role of *RNF5* in glioma and its possible target proteins were further analyzed using a bioinformatics approach.

GSEA of KEGG signaling pathways associated with RNF5 in human glioma. To further analyze the role of *RNF5* in human glioma, GSEA of the CGGA database was performed. The expression levels of *RNF5* in the samples were sorted from low to high, and the samples were divided into four groups: A, B, C and D. Group A contained samples with low expression of *RNF5*, while Group D contained samples with a high expression level of *RNF5*. The newly grouped data were subsequently analyzed by GSEA. The GSEA revealed that *RNF5* was significantly associated with
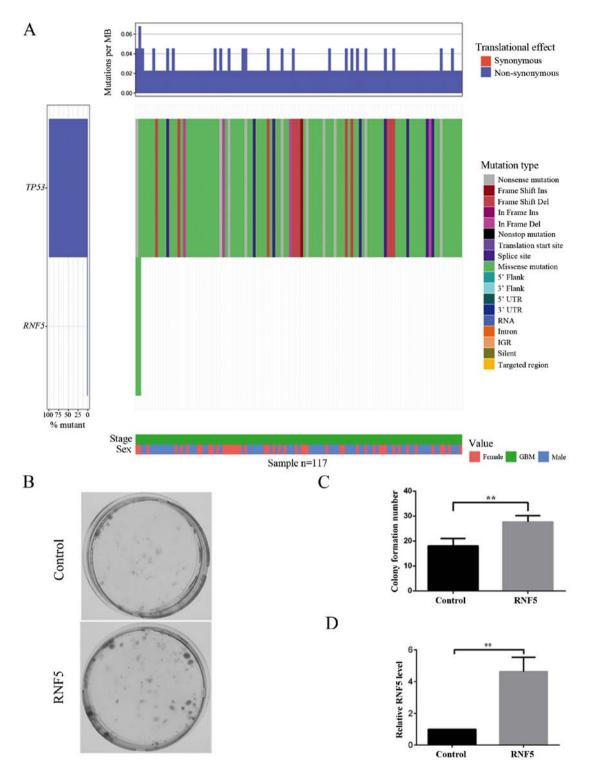
Figure 2. *RNF5* and *TP53* mutations identified in low-grade glioma and GBM and colony formation assay. (A) A total of 117 mutations in *TP53* and two mutations in *RNF5* in GBM were identified in The Cancer Genome Atlas. (B) Cell colony forming ability was increased in U251 cells overexpressing *RNF5* compared with control cells. (C) Cell colony formation analysis. (D) Reverse transcription-quantitative PCR analysis of the expression levels of RNF5 in U251 cells overexpressing RNF5 compared with control cells. [**]P<0.01. *RNF5*, ring finger protein 5; *TP53*, tumor protein 53; 3'UTR, 3'untranslated region; GBM, glioblastoma multiforme.

the following KEGG signaling pathways: 'Wnt signaling pathway', 'apoptosis', 'cell adhesion molecules CAMs', 'cytokine-cytokine receptor interaction', 'focal adhesion' and 'ECM-receptor interaction' (Fig. 3). Therefore, *RNF5* may affect the development of glioma through these KEGG signaling pathways.

Prediction of RNF5 ubiquitination substrates using a bioinformatics approach. In order to identify the potential ubiquitination substrates of RNF5 in human glioma, CGGA, GSE16011 and GSE4290 data were divided into groups of low and high RNF5 expression. Differential genetic analysis was then performed on these groups to identify the DEGs.
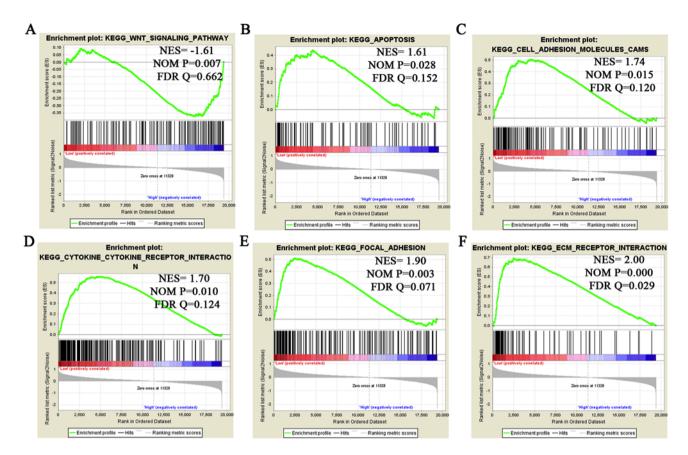
Figure 3. GSEA to identify significant *RNF5*-enriched KEGG signaling pathways. GSEA revealed that *RNF5* is enriched in the following pathways: (A) The 'Wnt signaling pathway', (B) 'apoptosis', (C) 'cell adhesion molecules CAMs', (D) 'cytokine-cytokine receptor interaction', (E) 'focal adhesion' and (F) 'ECM-receptor interaction'. GSEA, Gene Set Enrichment Analysis; *RNF5*, ring finger protein 5; KEGG, Kyoto Encyclopedia of Genes and Genomes; ECM, extracellular matrix; NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.
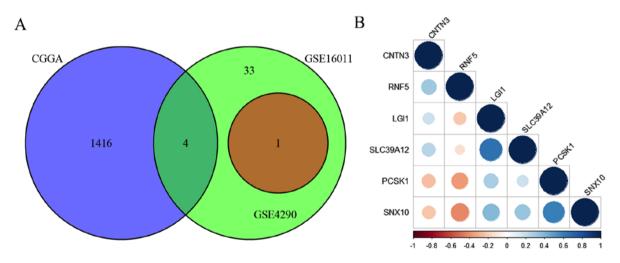

Figure 4. *RNF5* ubiquitin substrate prediction. (A) Venn diagram showing 4 overlapping genes between the CGGA database and the GSE16011 dataset and 1 overlapping gene between the GSE4290 and GSE16011 datasets. (B) Associations between *RNF5* and these 5 genes. Red and blue dots indicate a negative and positive association, respectively. The greater the size and color intensity of the dot, the stronger the association. *RNF5*, ring finger protein 5; CGGA, Chinese Glioma Genome Atlas; *CNTN3*, contactin 3; *LGI1*, leucine rich glioma inactivated 1; *SLC39A12*, solute carrier family 39 member 12; *PCSK1*, proprotein convertase subtilisin/kexin type 1; *SNX10*, sorting nexin 10.

To further clarify the possible ubiquitination substrates of *RNF5*, the overlapping genes between the CGGA database and GSE16011 and GSE4290 datasets were identified (Fig. 4A). A total of 4 overlapping genes were identified between the CGGA database and the GSE16011 dataset, 1 overlapping gene was identified between the GSE16011 and GSE4290 datasets, and no overlapping genes were identified between the CGGA database and the GSE4290 dataset. The 5 overlapping genes included contactin 3 (*CNTN3*), leucine rich glioma inactivated 1 (*LGI1*), proprotein convertase subtilisin/kexin type 1 (*PCSK1*), sorting nexin 10 (*SNX10*) and solute carrier family 39 member 12 (*SLC39A12*; Fig. 4B).
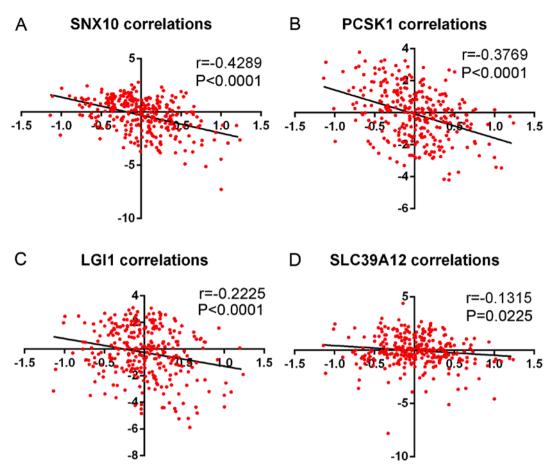
Figure 5. Correlation between *RNF5* and the 4 negatively correlated genes. Correlation between *RNF5* and (A) *SNX10*, (B) *PCSK1*, (C) *LGI1* and (D) *SLC39A12*. *RNF5*, ring finger protein 5; *SNX10*, sorting nexin 10; *PCSK1*, proprotein convertase subtilisin/kexin type 1; *LGI1*, leucine rich glioma inactivated 1; *SLC39A12*, solute carrier family 39 member 12.

RNF5 expression was positively associated with *CNTN3*, while a negative association was demonstrated for the remaining four genes (SNX10, PCSK1, LGI1 and SLC39A12) (Fig. 4B). In addition, the results from Fig. 5 demonstrated that RNF5 was negatively correlated with SNX10, PCSK1, LGI1 and SLC39A12.

Discussion

Previous studies have demonstrated that ubiquitin ligase is closely associated with tumor development and metastasis (13,22-25). The present study used human glioma data to reveal that *RNF5* was differentially expressed in patients with different levels of glioma and was correlated with prognosis in patients with AG and GBM. Specifically, an improved prognosis was observed in patients with AG and GBM with a high expression of *RNF5* compared with a low expression. Subsequently, *RNF5* was overexpressed in U251 cells *in vitro*, and it was revealed that colony forming ability was enhanced in cells overexpressing *RNF5* compared with controls. The authors speculate that silencing *RNF5* reduces the colony forming ability. Through GSEA enrichment analysis, KEGG signaling pathways that were significantly associated with *RNF5* were identified. To further explore possible ubiquitination substrates for *RNF5* in human glioma, correlation analysis were performed. A total of 4 genes were negatively associated

with *RNF5* expression, and may serve as potential *RNF5* ubiquitination substrates.

Previous studies revealed that *RNF5* was highly expressed in breast cancer specimens and cell lines. Additionally, tumor cell proliferation was inhibited after silencing *RNF5*, and patients with breast cancer with high *RNF5* expression have a poor prognosis compared with patients with low expression (13,21). Cell proliferation was inhibited after silencing *RNF5* expression in MCF-7 cells. However, cell proliferation was not affected in MDA-MB-231, MDA-MB-435 and BT-474 cells following *RNF5* silencing due to differences in *TP53* status, as *TP53* is only functional in MCF-7 cells (21,26,27). Furthermore, *TP53* expression was increased following the silencing of *RNF5* in MCF-7 cells, suggesting that RNF5 may be involved in the inhibition of *TP53* by Rho GTPase, Src networks or ERAD (21). The present study demonstrated that higher *RNF5* expression was associated with an improved prognosis in patients with glioma, which may be consistent with *TP53* mutations in glioma, particularly in GBM (28,29). Previous studies revealed a higher rate of *TP53* mutations in patients with glioma compared with healthy subjects (30-33). Therefore, it is possible that in patients with glioma, mutations in *TP53* may reverse the inhibition of proliferation induced by *RNF5* silencing. The apparently opposite prognostic effect of *RNF5* expression levels in patients with glioma necessitates further investigation of the mechanism of action of *RNF5*. The

present study investigated cell colony formation of U251 cells overexpressing *RNF5*. However, experiments on apoptosis, invasion and migration should be performed in future studies. Furthermore, the lack of validation on clinical samples is a limitation of the present study.

In summary, the present study revealed that *RNF5* is differentially expressed in patients with glioma with different disease grades and is associated with patient prognosis. Moreover, GSEA revealed KEGG pathways that are significantly associated with *RNF5*. Through correlation analysis, possible ubiquitin substrates for *RNF5* in patients with glioma were predicted. These results provided a meaningful insight into the treatment of glioma.

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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.

Authors' contributions

YG, CX, MJ, QS and YS conceived and designed this study. YG, CX, MJ, QA, BZ, XC, LW and YW performed the experiments. YG, CX and LW conducted statistical analysis. YG, CX, QA, QS and YS wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study protocol was approved by the Ethics Committee of Xuzhou Children's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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