Knockdown of RNF6 Inhibits Cervical Cancer HeLa Cells Growth by Suppressing the MAPK/ERK Signaling

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Abstract

Background

Given its crucial role in human malignancies, how Ring finger protein 6 (RNF6) functions in cervical cancer has yet to be elucidated. In our research, we explored the biological significance of RNF6 in cervical cancer HeLa cells and its possible regulatory mechanism.

Methods

The expression levels of RNF6 mRNA and protein in cervical cancer tissues and cells were both analyzed, the former by Gene Expression Profiling Interactive Analysis (GEPIA), and the latter by quantitative real-time PCR (qRT-PCR) and immunohistochemistry assays. In vitro cell proliferation was tested through MTT assay and flow cytometer was used to detect cell apoptosis. The activation of ERK (extracellular signal regulated kinase) was explored by Western Blot.

Results

In the present research, we found that the expression of RNF6 was high in both primary tissues and cervical cancer cells. RNF6 could promote cervical cancer HeLa cells growth. Once knockdown of RNF6 in cervical cancer cells, cell proliferation could be suppressed and cell apoptosis was promoted. Moreover, its elevation had an adverse effect on the prognosis of cervical cancer. Further studies showed that ERK activation is one of the potential mechanisms.

Conclusion

These findings provided evidence that the up-regulated RNF6 could activate the MAPK/ERK pathway to regulate the cell growth in cervical cancer, which suggested that RNF6 could be a promising target for diagnosis and treatment for cervical cancer.

1 Background

Cervical cancer is the most common gynecological malignant tumor, seriously endangering the health and lives of women in developing countries. Classical treatment for cervical cancer includes surgery, chemotherapy or radiotherapy or both. However, the patients with advanced cervical cancer still have a poor prognosis, which requires early diagnosis and prediction to greatly improve the treatment outcome. Therefore, further studies are needed to find novel methods for cervical cancer.

RNF6 is an E3 ligase ^[1] and its controversial role in the field of tumorigenesis has been debated hotly. The Mutated RNF6 was firstly recognized to regulate human esophageal squamous cell carcinoma as a

tumor suppressor and it is on chromosome 13q12 [2]. More recently, its role in carcinogenesis is emerging as the study of cancer progresses further and further.

RNF6 exerted different effects in various cancers, which could be used as a tumor-specific target. Huang et al. Research confirmed that RNF6 was an oncogene for gastric cancer. The research proposed the promising role of RNF6-SHP-1-STAT3 in gastric cancer. Many studies had recently proved high expression of RNF6 to have a poor prognosis for colorectal cancer Independent factors. RNF6 was high-regulated in colorectal tumors and activated JAK/STAT3 pathway or Wnt/ β -catenin pathway to promote colorectal tumorigenesis [3]. Patients with cisplatin-resistant lung adenocarcinoma had increased RNF6 expression in tumor cells [4]. RNF6 could atypically polyubiquitinate at Lys-6 and Lys-277 and then RNF6 could benefit the transcriptional activity of Androgen receptor (AR) due to its overexpression in prostate cancer. By regulating AR function, RNF6 promoted the prostate cancer tumor cell growth, and mutations in RNF6 gene and specific inhibition could change AR transcriptional activity in xenograft models and delay the progression of prostate cancer [5]. Transcription factor PBX1 can directly target on RNF6 and the tumor cells with increased levels of RNF6 expression induced leukemia cell growth [6]. However, research on RNF6 remains largely unknown, and there is only limited information available about its biological functions. The role of RNF6 in cervical malignancies has not yet been discovered.

In this study, we aimed to detect the expression of RNF6 in cervical cancer and its probable molecular mechanisms. We found that the tumor cells proliferated significantly in cervical cancer as a result of increased expression of RNF6. Moreover, RNF6 might function via the activation of protein kinases/extracellular signal-regulated kinase (MAPK/ERK) pathway.

2 Methods

2.1 Cells and tissues

Cervical cancer HeLa cell line was provided by the Key Laboratory of Environment and Disease, Medical College of Xi'an Jiaotong University. Cell line was cultured in 10% FBS and 1% penicillin/streptomycin in a standard CO₂ (5%) cell incubator at 37 °C.

A total of 40 cases of pathologically confirmed specimens were surgically sectioned and biopsied from the Department of Pathology, the Second Affiliated Hospital of Xi'an Jiaotong University. These cases included 20 paraffin-embedded cervical squamous cell carcinoma tumor tissue blocks and adjacent non-tumor tissue blocks. All of the patients were not given any other treatments before surgery, such as chemotherapies or radiotherapy.

All samples were obtained with informed consent under a protocol approved by the Ethics Committee of Medical School of Xi'an Jiaotong University, China. The study is compliant with all relevant ethical regulations regarding research involving human participants.

Ethical approval was obtained from the ethics committee of Medical School of Xi'an Jiaotong University, China. All samples were obtained in accordance with the relevant ethical regulations.

2.2 Gene construction and transfection.

We designed and validated two effective small interfering RNA (siRNA) sequences to knock down endogenous RNF6 expression (Gima, Shanghai, China). These sequences were: Human RNF6-1 (siRNF6-1):

Sense 5'-CCCGAACAAUGGAGAGUUUTT-3';

Antisense 5'-AAACUCUCCAUUGUUCGGGT-3'; Human RNF6-2 (siRNF6-2):

Sense 5'-GGAUCCGUCCUGGAGAAAATT-3'; Antisense 5'-UUUUCUCCAGGACGGAUCCTT-3'; Negative scramble control sequences (si-NC) were: Sense 5'-UUCUCCGAACGUGUCACGUTT-3'; Antisense 5'-ACGUGACACGUUCGGAGAATT-3'; We designed and synthesized the RNF6 overexpression plasmid to enforce the RNF6 expression in cervical cancer HeLa cells. According to the instructions, we used Lipofectamine 3000 reagent (Invitrogen, USA) in Opti-MEM (Gibco, USA). The overexpression plasmid map was as follows.

2.3 Quantitative real-time PCR (qRT-PCR)

Cells were harvested 24 hours after transfection. Firstly, we extracted the total RNA using Trizol reagent, Chloroform and isopropanol according to the protocols (Invitrogen, USA). Secondly, the transcription cDNA was reversed by PrimeScript™ RT reagent Kit (GeneStar). Then we performed qRT-PCR to detect the mRNA levels of RNF6 by using a SYBR Supermix kit (Bio-Rad Laboratories, Richmond, CA, USA). The thermocycling conditions were as follows: 40 cycles of 10 min at 95 °C, 1 min at 60 °C and 30 sec at 72 °C. A β -actin primer was used as an internal reference. The primers used were as follows: RNF6, forward 5'-AGAAGATGGCAGCAAGAGCG-3' and reverse 5' TCAAGTCAGGCTGAGATGCTAGT-3'; β -actin forward, 5'-ATCTGGCACACCTTCTA-3' and reverse, 5'-GGATAGCACAGCCTGGATAC-3';

2.4 MTT assay

Cell proliferation was detected and analyzed by standard 3-(4,5-cimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) assay. After transfection for 24 h, 48 h, and 72 h, cells were harvested and seeded at a density of 3500 cells per well in 100 μ l culture medium in 96-well culture plates. MTT and dimethyl sulfoxide (DMSO) were employed into plates and cell proliferation was assessed using a microplate reader. The optical density of the wells was measured at 492 nm. Each experiment was repeated at least three times.

2.5 Immunohistochemical (IHC) assay

IHC staining was performed to measure the expression of RNF6 protein in cervical cancer tissue samples. In brief, tissues were obtained in wax blocks after being soaked with 4% paraformaldehyde, ethanol, xylene, etc. DAB was used for counterstained. Then all tissue sections were dehydrated, and mounted.

Finally, sections were viewed under a brightfield microscope. RNF6 expression was initially defined as IHC scores. The IHC scores was percentage of nuclear positive cells $(1-4) \times$ staining intensity $(0-3)$.

2.6 Western blot analysis

After transfection for 24 hours, cells were harvested and were lysed in RIPA buffer (Beyotime, China), and Equivalent amount of the cell protein samples were measured using the BCA protein assay kit (Biyuntian biotech, Shanghai, China). Protein was subjected to electrophoresis using an 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PDVF membrane (Millipore, Billerica, MA, USA). The blot was incubated successively with the primary antibodies overnight at 4 °C. The primary antibodies used in this study were as follows: anti-RNF6 (1:1000 dilution), anti-p-ERK1/2 (1:1000 dilution) and anti-GAPDH (1:3000 dilution) (Cell Signaling Technology, Inc., Beverly, MA, USA). And then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000 dilution) for 1 h, and signals were detected using enhanced chemiluminescence reagents (Pierce, USA). Quantity One Software (Bio-Rad, USA) was used to analyze the intensity of blots. GAPDH was used as the loading control.

2.7 Cell apoptosis assay

Cell apoptosis assay was performed by flow cytometry with propidium iodide (PI) staining. After transfection for 24hours, cells were harvested and seeded at a density of 1.0×10^5 cells per well in 100 μ l culture medium in 12-well culture plates. Cells were washed twice with PBS resuspended in 400 uL Annexin V-FITC binding buffer. Then 5 uL of Annexin V-FITC and 10 ul propidium iodide (PI) were added to stain cells according to the Apoptosis Detection Kit (Qihai biotech, Shanghai, China) instructions. Then, cells apoptosis was detected on a flow cytometer (BD Biosciences, USA). We repeated this experiment at least three times.

2.8 Database analysis

The GEPIA (Gene Expression Profiling Interactive Analysis) database is developed by Peking University. Based on the information in the Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression (GTEx) projects, the differential expression of genes in tumors and normal tissues can be analyzed by single gene, multiple genes, and tumor types. GEPIA can be directly linked to other large databases or literature libraries, such as GeneCard, Pubmad, COSMIC, HPA, etc.

2.9 Statistical analysis.

All data were analyzed for statistical significance using SPSS 22.0 software (SPSS, USA). Data were subjected to a one-way ANOVA. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 RNF6 was overexpressed in cervical cancer

To evaluate the expression level of RNF6 in cervical cancer, GEPIA was first used for analysis, and the expression of RNF6 in tumor tissues of cervical cancer was significantly increased as shown in Fig. 1A. Then, we collected 20 pairs of adjacent normal tissues and tumor tissues from cervical cancer patients to detect the protein levels of RNF6, which were also up regulated in tumor tissues (Fig. 1B). Moreover, we investigated the prognostic significance of RNF6 expression in cervical cancer patients using GEPIA with the log-rank test (Fig. 1C). We found that the high expression of RNF6 indicted that cervical cancer patients had a poor prognostic.

Figure 1 RNF6 was overexpressed in cervical cancer.

3.2 RNF6 promoted cervical cancer cell growth

We knocked down the expression of RNF6 and detected the cell activity in order to determine the biological functions of RNF6. First of all, we examined the RNF6 expression in HeLa cells by Western blot (Fig. 2A). Consistent with the tumor tissues, the results showed high RNF6 expression in the cervical cell line. To further explore the biological functions of RNF6, we validated the two effective siRNA sequences to knock down endogenous RNF6 expression (Fig. 2B). We found that the proliferation of HeLa cells was significantly suppressed using MTT assay after RNF6 knockdown. In parallel, RNF6 was obviously elevated and RNF6 overexpression significantly enhanced the cell proliferation (Fig. 2C). Combined with results that the expression of RNF6 was increased either in tumor tissues and HeLa cells, we deduced the cell growth in cervical cancer could be promoted by RNF6 overexpression.

Figure 2 RNF6 regulated cervical cancer cell growth.

3.3 RNF6 inhibited cell apoptosis in cervical cancer

Apoptosis has been implicated in tumorigenesis and development. After transfection for 24 hours, we performed flow cytometry to detect cell apoptosis to find out whether cell apoptosis was also triggered by silenced RNF6. As compared to the control group, the total apoptosis ratio was significantly increased after RNF6 knockdown. (Fig. 3A). On the contrary, cell apoptosis was significantly inhibited when the expression of RNF6 was enforced. And the total apoptosis ratio was evidently decreased compared with blank plasmid transfected cells (Fig. 3B).

Figure 3 RNF6 inhibited cell apoptosis in cervical cancer.

3.4 RNF6 activated MAPK/ERK pathway in cervical cancer

ERK1/2 has been reported to be overexpressed in cervical cancer^[7, 8]. In addition, most studies currently believe that abnormal activation of ERK signaling pathway can inhibit cell apoptosis in multiple aspects and promote tumor cell growth^[9]. We hypothesized that RNF6 might also mediate tumor cell biological behaviors through the ERK pathway in cervical cancer.

First, we found that RNF6 and p-ERK1/2 (phosphorylated ERK1/2) were co-expressed. Then, western blot was performed and as shown in Fig. 4. It was observed that the silencing of RNF6 resulted in decreased

p-ERK1/2 after 24 hours transfection (Fig. 4A). Conversely, the expression levels of p-ERK1/2 were increased when RNF6 was upregulated, whereas the total ERK1/2 expression was unaffected (Fig. 4B).

Figure 4 RNF6 activated MAPK/ERK pathway in cervical cancer

4 Discussions

The presence of high-risk human papillomavirus (HR-HPV) types, such as 16 and 18, was a well-established cause in the development of cervical carcinomas^[10]. Of course, only a small percentage of HPV-infected patients suffered from cervical cancer, and other cofactors may served to the development of cervical cancers. Therefore, looking for factors other than HPV infection has become a hot spot in the research of cervical cancer. Studies have found that UPP was closely related to the occurrence and development of tumors in some female-related diseases. Studies related to cervical cancer originated from HPV virus, and p53 was degraded through the ubiquitin-proteasome pathway^[11, 12], leading to cells cycle changes and loss of apoptotic function. As an upstream component of the UPS, RNF6, a E3 ligase, has attracted more and more attention in the tumor field. Growing evidence has emerged indicating that overexpression of RNF6 was observed in cervical cancer and could develop distant metastasis to a great extent, which ultimately lead to a poor prognosis in CC patients.

However, the role in cancer pathogenesis played by RNF6 remain mysterious. The function and clinical role of RNF6 expression in cervical cancer is has not yet been fully elucidated. In our research, RNF6 acted as an oncogene in cervical cancer. To characterize the expression pattern of RNF6, we performed immunohistochemistry in 20 cervical cancer tissues and western blot in HeLa cells. Consistent with the data from GEPIA, RNF6 protein expression levels is substantially increased compared with normal tissues, which indicated that RNF6 expression participated in the tumorigenesis of cervical cancer. Moreover, the data from GEPIA showed that the clinical prognosis of patients with RNF6-positive staining is significantly poor. According to multivariate analysis, RNF6 overexpression was considered to act as an independent prognostic indicator. In contrast, RNF6 was first found mutated on chromosome 13q12 and performed as a tumor suppressor in human esophageal squamous cell carcinoma^[2]. Along with these data, our study confirmed that RNF6 may exert variable or even opposing roles in different tumor contexts and could be expected to be a novel candidate for cancer immunotherapy.

Proliferation of cancer cells is one of signs of malignant tumor and dedicates a poor prognosis of cancer patients. In our study, we designed two effective small interfering RNA (siRNA) sequences to knock down endogenous RNF6 expression and RNF6 overexpression plasmid to enforce the RNF6 expression in cervical cancer HeLa cells to detect the effects of RNF6 in order to obtain a greater understanding of the potential association between the cell-biological characters and the expression levels of RNF6. In vitro studies suggested that RNF6 is capable of inhibiting cell apoptosis to promote cell proliferation, consistent with the results obtained in tumor tissues. The above results indicated that RNF6 exerted its oncogenic role through promoting proliferation and suppressing apoptosis of CC cells.

MAPK/ERK pathway functions a lot in regulating various physiological and pathophysiological processes in the initiation and progression of different types of cancers^[13, 14], and ERK is a key member, which can transmit various extracellular stimulation signals into the cell^[15]. In addition, most studies currently believe that abnormal activation of ERK signaling pathway can inhibit cell apoptosis in multiple aspects and promote tumor cell growth^[9]. Recent studies have demonstrated that RNF135 and RNF138 amplification activated ERK signaling pathway in glioblastoma, which promoted cellular proliferation, migration and invasion^[16, 17]. Ruan et al. found that ERK was over expression in cervical cancer. And during the development from CIN I to CIN III, the MAPK/ERK pathway was significantly activated^[7]. This conclusion was consistent with the research by Li Gang et al., which suggested that the MAPK/ERK signaling pathway played an important role in the evolution of cervical cancer and could promote the cancerous tendency of cervical cells^[8]. Therefore, we explored whether the high expression of RNF6 could promote cell proliferation together with its possible molecular mechanisms.

The activation of the ERK signaling pathway requires the activation of ERK by phosphorylation, which can mediate the transmission of signals from the cytoplasm to the nucleus, by up- or down-regulating nuclear transcription factors, such as c-myc, c-Fos, c-Jun, cyclin-D1 and Bcl-2^[18]. c-Fos and c-Jun, as oncogenes encoding nuclear proteins, are highly expressed in cervical cancer^[19]. And it has been demonstrated that ERK may affect Hela cell proliferation by regulating the expression of c-Fos and c-Jun proteins^[20]. In present study, we performed western blot analysis to detect the expression of p-ERK1/2 and total ERK1/2 when enforced RNF6 expression. While the former was notably elevated, the latter were not altered. Given these evidences, we speculated that we could knocked down RNF6 accompanied by inhibition of MAPK/ERK signaling pathway as well as its downstream genes to treat cervical cancer. The intrinsic mechanisms involved remain unknown and still require further study.

However, our research had some limitations that future research should address. Due to inadequate funding, in vitro and animal experiments have not yet been implemented to determine the specific molecular mechanisms of RNF6-ERK signaling. When we get enough support, we would continue to perfect this deficiency in future experiments.

In summary, we confirmed that RNF6 expression was significantly upregulated in cervical cancer samples and HeLa cell lines than in normal tissues, which also facilitated a poor prognosis for cervical cancer patients. At the same time, RNF6 knockdown might inhibit cell proliferation and promote cell apoptosis by suppression of MAPK/ERK signaling pathway in cervical cancer. Our research has provided a direction in cervical cancer and might important information for better understanding of the mechanics of RNF6-related cervical carcinogenesis. Further experimental studies for RNF6 could develop better therapeutic strategies for cervical cancer.

5 Conclusion

This study demonstrated that illustrated that RNF6 acted as a tumor suppressor to promote cell proliferation by activation MAPK/ERK signaling pathway in cervical cancer cells. Consequently, targeting RNF6-ERK pathway may be effective and promising treatment against cervical cancer.

Abbreviations

RNF6
Ring Finger Protein 6
CC
Cervical cancer
MAPK
mitogen-activated protein kinases
ERK
extracellular signal regulated kinase
TCGA
the Cancer Genome Atlas
GEPIA
Gene Expression Profiling Interactive Analysis
siRNA
small interfering RNA
qRT-PCR
quantitative real-time PCR

Declarations

Ethics approval and consent to participate

This study was conducted at The Second Affiliated Hospital, School of Medicine, Xi'an Jiaotong University, China. The research on tumor tissues was approved by the Ethics Committee of Medical School of Xi'an Jiaotong University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Competing Interests

The authors have declared that no competing interest exists.

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Author contribution

KZ and MZM collected associated clinical data and performed the experiments. HB and YYX contributed to statistical analyses of the data. ZD planned and designed the research. KZ and HB wrote the manuscript and conceptualized the framework for this research. All authors read and approved the final manuscript. KZ and HB contributed equally to this paper.

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Abbreviations

RNF6: Ring Finger Protein 6

CC: Cervical cancer

MAPK: mitogen-activated protein kinases

ERK: extracellular signal regulated kinase

TCGA: the Cancer Genome Atlas

GEPIA: Gene Expression Profiling Interactive Analysis

siRNA: small interfering RNA

qRT-PCR: quantitative real-time PCR

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Supplementary Figures

Supplementary Figures were not provided with this version of the manuscript.

Figures

Figure 1

The overexpression plasmid map

Figure 2

RNF6 was overexpressed in cervical cancer. Notes: (A) GEPIA database was used to detect the expression level of RNF6. T, tumor tissue; N, normal tissue. * $p < 0.05$ (B) IHC analysis of the expression of RNF6 in cervical cancer tissues and adjacent normal tissues. (a) normal cervical tissues, no brown or yellow particles detected; (b) cervical cancer tissues, a large number of brown yellow particles are visible. (C) GEPIA database was used to evaluate the association between RNF6 mRNA expression and prognostic significance in cervical cancer. (a) RNF6 high-expression and its positive correlation to poorer overall survival in cervical cancer patients. (b) RNF6 high-expression and its positive correlation to poorer disease free survival in cervical cancer patients.

Figure 3

RNF6 regulated cervical cancer cell growth. Notes: (A) The protein levels of RNF6 were detected by Western blot. * $p < 0.05$ (B) The transfection efficiency was detected by qRT-PCR. * $p < 0.05$ (a) RNF6 siRNA-1, RNF6 siRNA-2, or si-NC was transfected into HeLa cells. (b) RNF6 overexpression plasmid or blank plasmid was transfected into HeLa cells. (C) MTT was used to detect the cell viability at certain time points. * $p < 0.05$

Figure 4

RNF6 inhibited cell apoptosis in cervical cancer. Notes: (A) Cell apoptosis was promoted after RNF6 downregulation in HeLa cells. Quadrants: upper left (dead cells); lower left (live cells); lower right (early apoptotic cells); and upper right (late apoptotic cells). (B) Cell apoptosis was suppressed after RNF6 upregulation. * $p < 0.05$

Figure 5

RNF6 activated MAPK/ERK pathway in cervical cancer Notes: (A) After transfection for 24 hours, cells were harvested and detected the expression of RNF6, p-ERK1/2 and ERK1/2 by western blot. GAPDH was used as an internal control. RNF6 knockdown resulted in decreased p-ERK1/2. (B) Enforced expression of RNF6 significantly upregulated levels of p-ERK1/2 expression in HeLa cells, while there had no effect on the total ERK1/2 expression. * $p < 0.05$ Full-length blots are presented in Supplementary Figures.