Overexpression of FUBP1 is associated with human cervical carcinoma development and prognosis

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Abstract

**Background:** Far upstream element-binding protein 1 (FUBP1) has been shown to involve in the tumorigenesis and tumor progression of various cancers. However, the expression and function of FUBP1 in cervical carcinoma remains unknown.

**Methods:** Transcriptional expression of FUBP1 was initially evaluated using the Oncomine database, followed by evaluation of FUBP1 protein levels using immunohistochemistry in 119 cervical carcinoma patient tissues. *In vitro* experiments were performed to assess the tumorigenic role of FUBP1. Besides, Gene Set Enrichment Analysis, EnrichmentMap analysis, and protein-protein interaction (PPI) networks were used to evaluate the potential mechanisms of FUBP1 in promoting cervical cancer progression.

**Results:** In this research, we found both FUBP1 mRNA transcription and protein expression levels increased significantly in cervical carcinoma tissues compared with adjacent normal cervical tissues. Furthermore, elevated FUBP1 expression was positively correlated with age, T classification, N classification, tumor recurrence, Ki67 expression, and poor prognosis in cervical carcinoma patients. Besides, elevated FUBP1 expression acted as an independent unfavorable predictor for overall survival and disease-free survival in cervical carcinoma. Overexpression of FUBP1 significantly promoted cervical carcinoma cell proliferation and inhibits cell apoptosis *in vitro*, while knockdown of FUBP1 showed the opposite effect. Mechanistically, bioinformatics analysis revealed that FUBP1 promoted the biological function of cervical carcinoma cells via enhancing DNA repair signal pathways.

**Conclusions:** Our results demonstrate for the first time that FUBP1 is a novel prognostic factor and therapeutic target for cervical carcinoma.

**Background**

As the fourth common malignancy and the fourth leading cause of death in women, cervical carcinoma is one of the most prominent gynecologic malignancies among women worldwide, especially in developing countries[1, 2]. It is reported that about 530,000 female patients are newly diagnosed, and approximately 275,000 patients die of cervical carcinoma every year[3]. With the widespread application of thin-preparation cytologic test and human papillomavirus prophylactic vaccine, the incidence rate of cervical carcinoma in developed countries is decreasing[4]. Although surgery, radiotherapy, and chemotherapy have been applied to treat cervical carcinoma with promising efficacy, the patients with the advanced clinical stage still suffered from an unfavour prognosis[5]. Unlike breast cancer and colorectal cancer, the significant molecular therapeutic targets have not been identified clearly in cervical carcinoma[6, 7]. Therefore, it is necessary to clarify the underlying pathogenesis and provide molecular targeted therapy in cervical carcinoma.

Far upstream element-binding protein 1 (FUBP1), a single-stranded DNA-binding protein, is highly expressed in various tumor cell lines and tissues, including liver cancer, breast cancer, gastric cancer, non-small lung cancer, renal cell carcinoma, nasopharyngeal carcinoma, and Hodgkin's lymphoma[8–12].
Additionally, the mutation in the FUBP1 gene is associated with an unfavorable prognosis of astrocytoma, oligodendrogliomas, oligoastrocytomas, and glioblastoma[13, 14]. FUBP1 forms a complex with the far upstream element (FUSE) site to regulate c-Myc expressions, displaying a broad spectrum of activities, such as promoting proliferation and inhibiting apoptosis of cancer cells[15, 16]. Other studies have also revealed that the abnormal expression of FUBP1 may not be related to the regulation of c-Myc in prostate and bladder cancers[17]. Our recent studies have shown that elevated FUBP1 inhibits the degradation of HIF1 α by binding with VHL promoter, thus accelerating glycolysis and leading to neuroblastoma cell proliferation[18]. However, the expression and role of FUBP1 in cervical carcinoma has never been reported.

In this study, we investigate the expression of FUBP1, a critical novel oncogene in cervical carcinoma and its underlying clinical significance and molecular mechanism. These findings may provide a new target for the diagnosis and treatment of cervical carcinoma.

**Materials And Methods**

**Human samples**

The cervical carcinoma tissue microarrays (HUtS154Su01-M-060, 119 cases) were purchased from Shanghai Outdo Biotech (Shanghai, China). All procedures were performed under consensus agreements and in accordance with the Chinese Ethical Review Committee. The clinical and biological characteristics of the patients were described in Table 1.

**Cell lines and culture**

The human cervical carcinoma cell lines (AV3, C33A, Ca Ski, HeLa) were obtained from the American Type Culture Collection (Manassas, USA). The normal cervical squamous epithelial cell lines (H8) were provided by Professor Zhonghan Yang, from Sun Yat-sen University. Cell lines were authenticated by Cellcook Biotech Co., Ltd, (Guangzhou, China). All these cells were cultured in RPMI1640 (Gibco, New York, USA, Cat. No. 61870044) and supplemented with 10% FBS (Gibco, Uruguay, 10270-106) at 37°C in a humidified incubator containing 5% CO2.

**RNA isolation and qRT-PCR**

Total RNA was extracted using TRIzol (Thermo Scientific, 15596026) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed using 500 nanograms of total RNA, and the qRT-PCR analysis system was performed using iQ SYBR Green Supermix and the iCycler Real-time PCR Detection System (Bio-Rad, USA). β-actin was used for normalization. Primer sets for SYBR Green analysis of human FUBP1 and β-actin are as follows: FUBP1 forward: TCTTTCTAGCCCTAACCCA; FUBP1 reverse: CTTGTCCAAGAGCCATCTCCAT; β-actin forward: GCACCTTCCAGCCTTCCTT; β-actin reverse: GTCGGCGTACAGGTCTTTGC.

**Immunohistochemistry staining**
Immunohistochemistry was performed according to a standard protocol as described previously. The slides were incubated with anti-FUBP1 antibodies (Merck Millipore, ABE1330) at 4°C overnight. On the second day, the slides were treated with HRP-conjugated secondary antibody and the antigen-antibody complex was visualized by incubation with the DAB kit. Finally, all sections were counterstained with hematoxylin and photographed through a slide scanner (Axio Scan. Z1, ZEISS, Oberkochen, Germany). The degree of immunostaining was determined by the staining index (SI). The SI was calculated as the product of the grade of tumor cell proportions and the staining intensity score. The tumor cell proportions were graded as follows: 0, no positive tumor cells; 1, <10% positive tumor cells; 2, 10-35% positive tumor cells; 3, 35-75% positive tumor cells; and 4, >75% positive tumor cells. Staining intensity was scored as follows: 1, no staining; 2, weak staining (light yellow); 3, moderate staining (yellow-brown); and 4, strong staining (brown). Accordingly, the protein expression as evaluated by SI has a possible score of 0, 1, 2, 3, 4, 6, 8, 9, 12, or 16. Samples with SI $\geq 6$ were determined as high expression, and those with SI < 6 were determined as low expression.

**Cell Counting Kit-8 (CCK8)**

Cell proliferation was measured via cell viability with a Cell Counting Kit-8 (Dojindo, Japan). Cervical carcinoma cells were seeded into 96-well plates and cultured for 24, 48 and 72 h. Then, 10 μl CCK8 reagent was added to 96-well plates and incubated for 2 h. The absorbance (OD450 nm) was measured using a microplate reader (TECAN, Switzerland) and calculated.

**Colony formation assay**

Cervical carcinoma cells were plated in 6-well dishes (500 cell/dish) and then incubated for 2 weeks for colony formation. After 14 days, cell colonies were then fixed in 4% polyformaldehyde and stained with 0.1% crystal violet. All colonies were counted separately for each sample, and the relative colony numbers were calculated.

**EdU staining**

EdU staining was performed by the protocol of EdU staining kit (KeyGEN, Nanjing, China), then the pictures were taken with ZEISS Axio Imager Z1 (ZEISS, Jena, Germany).

**Plasmids, retroviral infection, and transfection**

All lentiviral vectors contained the puromycin resistance gene. Vectors encoding FUBP1 plasmid and FUBP1 shRNA were purchased from Hanbio Biotechnology Co., Ltd, (Shanghai, China). After transfections 48 hours, confirmation of overexpression or interference was carried out using qRT-PCR.

**Annexin V/propidium iodide flow cytometric analysis**

Cervical carcinoma cell staining with Annexin V and PI was carried out using an Annexin V-FITC/PI Apoptosis Detection kit (Merck, Germany). A total of $1 \times 10^6$ cells were incubated at 37°C for 30 minutes.
before centrifugation to collect the cell pellet, then resuspended in a Ca\textsuperscript{2+}-enriched binding buffer and analysed using a Beckman Coulter flow cytometry. Data were calculated using Cell Quest software.

**TCGA Data Analysis**

The RNASeq data and clinical data for cervical carcinoma were obtained from The Cancer Genome Atlas (TCGA) databases (https://genome-cancer.ucsc.edu). For the association of FUBP1 expression with survival, patient vital status (dead and alive) was used as a surrogate end-point and patients dichotomized by FUBP1 expression. Kaplan-Meier survival curves were constructed, and the log-rank test was carried out using univariate analysis. Gene Set Enrichment Analysis (GSEA) was manipulated to predict the biological processes based on FUBP1 high and low expressed phenotype. A leading edge analysis was performed by GSEA 4.0.3 to elucidate key genes related to selected genes sets. EnrichmentMap plugin in Cytoscape 3.7.1 software was utilized with the following parameters: p-value cutoff = 0.005; similarity coefficient cutoff = 0.5. The protein-protein interaction (PPI) networks were constructed using The Search Tool for the Retrieval of Interacting Genes (STRING), which is a publicly available software for assessing the interaction between proteins and proteins (https://string-db.org/).

**Statistical analysis**

The variability of the data is presented as the SD (mean\pm SD) and was assessed with Student’s \(t\) test between two groups. For multiple groups, significant differences were determined using one-way ANOVA. The relationships between FUBP1 expression and clinicopathological characteristics were determined using the \(\chi^2\) test. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Statistical significance was defined at \(P<0.05\).

**Results**

**Elevated levels of the FUBP1 mRNA and protein in cervical carcinoma tissues.**

To explore the expression of FUBP1 in cervical carcinoma, we firstly analyzed the mRNA expression of FUBP1 in the normal Cervix Uteri tissues and cervical carcinoma tissues through the Oncomine database (Fig. 1A). As displayed in the Fig. 1B, the mRNA expression of FUBP1 in cervical carcinoma tissues was 2.23 times higher than that in the normal Cervix Uteri tissues. To further confirm whether the protein levels of FUBP1 change, we detected the expression of FUBP1 in a cervical carcinoma Tissue Microarray by IHC staining. Impressively, compared to adjacent tissues, FUBP1 expression was significantly increased in cervical carcinoma tissues (Fig. 1C-D). Furthermore, statistical analyses of the relationship between FUBP1 expression and cervical carcinoma clinicopathological characteristics were performed using the chi-square test. The result showed that the expression of FUBP1 was associated with age (\(P<0.001\)), T classification (\(P=0.007\)), N classification (\(P=0.003\)), tumor recurrence (\(P=0.004\)), and Ki67 expression (\(P=0.026\)), but not with clinical stage, HPV, EGFR expression, and PDL1 expression (Table. 1).
To analyze the influence of FUBP1 on the prognosis of cervical carcinoma patients, the relationship between FUBP1 and the prognosis of cervical carcinoma patients was analyzed by Kaplan–Meier Survival analysis. The results revealed that FUBP1 protein expression was negatively associated with cervical carcinoma overall survival ($P < 0.001$, Fig. 1E) and disease-free survival ($P = 0.003$, Fig. 1F). Moreover, FUBP1 mRNA expression was negatively associated with disease-free survival in TCGA cervical carcinoma dataset ($P = 0.005$, Supplementary Fig. 1). Taken together, these data indicate that the upregulation of FUBP1 contributes to cervical carcinoma progression and might be a prognostic factor for cervical carcinoma.

**Detection of FUBP1 transcription level in cervical carcinoma cell lines.**

The transcription of FUBP1 in the four cervical carcinoma cell lines (AV3, C33A, Ca Ski, HeLa) was shown in the Fig. 2A, and the H8 cells were used as the normal control. FUBP1 mRNA transcription level was significantly higher in the cervical carcinoma cell lines than in the immortalized H8 human cervical squamous epithelial cell line (Fig. 2A). The results also showed that Ca Ski and HeLa cells' transcription levels were higher, while AV3 and C33A cells were relatively lower (Fig. 2A). Building on these observations, FUBP1-overexpressing lentivirus were transfected into AV3 and C33A cells, while FUBP1-knockdown lentivirus were transfected into Ca Ski and HeLa cells. qRT-PCR was utilized to confirm transfection efficiency (Fig. 2B-C). Furthermore, CCK-8 results showed that overexpression of FUBP1 strongly promoted the cell viability of AV3 and C33A cells, whereas silenced FUBP1 expression markedly attenuated the cell viability of Ca Ski and HeLa cells (Fig. 2D-E). These data indicate that upregulation of FUBP1 plays a vital role in the biological function of cervical cancer cells.

**FUBP1 promotes cervical carcinoma cell proliferation.**

To determine the effect of altered FUBP1 expression on the proliferation of cervical carcinoma cells, FUBP1 overexpression and knockdown cervical carcinoma cells were used for colony formation and EdU staining assays. Notably, the number of colonies from FUBP1-transduced AV3 and C33A cells were more than the vector control cells, and conversely downregulated in FUBP1-silenced Ca Ski and HeLa cells (Fig. 3A-B; Supplementary Fig. 2). Furthermore, EdU staining assays showed that the proliferation ability was substantially enhanced in FUBP1 overexpressed cells while suppressed in FUBP1 knockdown cells (Fig. 3C-F). Taken together, these results demonstrate that FUBP1 could promote cervical carcinoma cell proliferation.

**FUBP1 inhibits apoptosis of cervical carcinoma cell.**

We further detected apoptosis of cervical carcinoma cells with overexpression or silencing of FUBP1. Cervical carcinoma cells were incubated with Annexin V and propidium iodide, and then detected by flow cytometer. Our results showed that the apoptosis rate was significantly increased after FUBP1 knockdown, while FUBP1 overexpression reduced the rate of apoptosis (Fig. 4). These results indicate that FUBP1 inhibits cervical carcinoma cell apoptosis.
**FUBP1 involves in the DNA repair signal pathways of cervical carcinoma.**

To gain insight into the biological functions of FUBP1 in cervical carcinoma, Gene Set Enrichment Analysis (GSEA) was manipulated to predict the biological processes based on FUBP1 expression. According to the top 10 ranking of NES (normalized enrichment score) and the nominal \( P \) value < 0.05, high FUBP1 expression were mainly enriched in DNA repair associated signal pathways (Fig. 5A), such as “Regulation of DNA repair”, “Non_recombinational repair”, “Double strand break repair” and “Regulation of double strand break repair” pathways (Fig. 5B). To research the associations between these enriched terms, EnrichmentMap plugin in Cytoscape 3.7.1 software was utilized. Likewise, as shown in the corresponding association network, FUBP1 high-expressed phenotype exhibited strong associations between several DNA repairs associated signal pathways (Fig. 5C). Furthermore, leading edge analysis was performed to extract the core genes in the EnrichmentMap network. Heatmap showed that several vital genes are markedly related to the above pathways (Fig. 5D). Besides, the protein-protein interaction (PPI) networks constructed by the STRING database showed that FUBP1 might interact with the above key genes related to DNA repair signal pathways (Fig. 5E). Collectively, these results suggest that FUBP1 promotes the DNA repair signal pathways of cervical carcinoma.

**Discussion**

This study was the first time revealed that the expression of FUBP1 was higher in cervical carcinoma cell lines and cervical carcinoma tissues than that in immortalized cervical squamous epithelial cell line and adjacent tissues (Fig. 1–2). Moreover, cervical carcinoma patients with high level of FUBP1 had lower overall survival and disease-free survival time than patients with low FUBP1 expression (Fig. 1E-F). Furthermore, overexpression of FUBP1 significantly promoted cervical carcinoma cell proliferation and inhibited cell apoptosis, while knockdown of FUBP1 showed the opposite effect (Fig. 3–4). Our investigation put forward that FUBP1 played an important role in developing cervical cancer and the possibility of a new treatment target for cervical cancer.

Through the analysis of the Oncomine database, we found that the mRNA expression of FUBP1 in cervical carcinoma tissues was higher than that in the normal Cervix Uteri tissues (Fig. 1A-B). Moreover, FUBP1 mRNA expression was negatively associated with disease-free survival in TCGA cervical carcinoma dataset (Supplementary Fig. 1). Furthermore, the FUBP1 protein level results analyzed by Tissue Microarray IHC staining were consistent with that of the FUBP1 mRNA level (Fig. 1C-F). Our results suggest that FUBP1 may serve as a useful prognostic biomarker for cervical carcinoma.

Further study on the mechanism of abnormal increase of FUBP1 mRNA expression provided more information for the targeted treatment of cervical carcinoma. Previous studies have found that FUBP1 increases in various tumors, but the underlying mechanisms were various[17]. It was reported that miR-16 inhibited the transcription of FUBP1 by targeting TGCTGCTA sequence. Low expression of miR-16 promoted gastric cancer and breast cancer cell proliferation by relieving the transcription inhibition of FUBP1[19]. Interestingly, FUBP1 was one of the hydrolytic substrates of caspase3[16]. The abnormally
activated PI3K/Akt/mTOR signal pathway reduced the degradation of FUBP1 by inhibiting the activity of caspase3, which led to the more stable expression of FUBP1 in hepatoma cells and thus promoted cell proliferation[20]. In addition, it was found that the ubiquitin ligase Smurf2 mediated ubiquitin degradation of FUBP1 in lung cancer cells. Furthermore, previous studies have found that FUBP1 gene mutation exists in oligodendroglioma, similar to the exon mutation of the TP53 gene[21, 22]. However, there was no report on the mechanism of the abnormal increase of FUBP1 in cervical carcinoma. The mechanism of the abnormal rise of FUBP1 mRNA expression in cervical carcinoma needed to be further clarified.

Although many studies have explored the roles of FUBP1 in the pathogenesis and pathophysiology of a variety of cancer cells, the functions of FUBP1 in human cervical cancer progression remained unclear. The statistical analyses of clinicopathological characteristics of cervical carcinoma suggest that the high expression of FUBP1 were associated with T classification, N classification, tumor recurrence, poor prognosis, and Ki67 expression, all of which indicated that FUBP1 played an important role in the proliferation of cervical carcinoma cells (Table. 1). Furthermore, we used four cervical carcinoma cell lines with different basic FUBP1 expression and manipulated FUBP1 expression by overexpression or knockdown. In consistence with our clinicopathological characteristics statistical analyses, overexpression of FUBP1 significantly promoted cervical carcinoma cell proliferation in vitro, while knockdown of FUBP1 showed the opposite effect (Fig. 3–4). However, the relationship between FUBP1 and tumor metastasis was still controversial[23]. It has been reported that FUBP1 promotes the metastasis of non-small cell lung cancer by increasing Stathmin through transcriptional regulation[24]. Some studies have also found that FUBP1 does not affect the mobility of Hep3B cells[25]. Our result showed that the expression of FUBP1 was not related to the clinical stage, suggesting that FUBP1 may not affected the metastasis of cervical carcinoma (Table. 1). Transwell migration assay and wound healing assay are needed to verify this conclusion in vitro further. Human papillomavirus (HPV) infection is the leading risk factor for cervical carcinoma, occurring in 80% of women[26]. Our study did not find that FUBP1 expression was related to cervical cancer HPV infection, while FUBP1 persisted Hepatitis B Virus (HBV) and Hepatitis C virus (HCV) replication[25, 27, 28].

Through Gene Set Enrichment Analysis, EnrichmentMap analysis, and protein-protein interaction (PPI) networks, high FUBP1 expression were mainly enriched in DNA repair associated signal pathways (Fig. 5). Especially, FUBP1 could interact with the key genes related to DNA repair signal pathways, such as PRKDC[29]. This gene encodes the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), which functions with the Ku70/Ku80 heterodimer protein in DNA double strand break repair and recombination[30]. DNA repair signal pathways are closely related to cell proliferation and apoptosis, suggesting that FUBP1 promotes cervical carcinoma progression through this mechanism. According to the top 10 ranking of NES (normalized enrichment score) and the nominal P value < 0.05, high FUBP1 expression was negatively correlated with the oxidative phosphorylation. Importantly, oxidative phosphorylation stability was beneficial to maintained the DNA damage repair function of tumor cells (Supplementary Fig. 3). FUBP1 is generally considered to be a transcription factor that promotes c-Myc expression. Moreover, our recent studies have shown that elevated FUBP1 accelerated glycolysis by
binding with VHL promoter[18]. Whether FUBP1 act as a transcription factor to promote the expression of PRKDC deserves further investigation.

**Conclusions**

In summary, this study provided, for the first time, clinical and experimental evidence that FUBP1 is highly expressed in cervical carcinoma and is associated with poor prognosis. FUBP1 promotes proliferation and inhibits apoptosis of cervical carcinoma cells, potentially in relation to the maintenance of DNA repair signal pathways. FUBP1 may be a vital prognostic indicator and molecular target for cervical carcinoma.

**Declarations**

**Authors’ contributions**

MCQ, HHH, and HM were responsible for designing and supervising the entire study and revised the manuscript. YHF, HZJ, and WZK performed the experiments and analyzed the data. LXP, and DCG contributed the data analysis and discussion. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare no conflict of interest.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Consent for publication**

The authors agree to publish this manuscript.
Ethics approval and consent to participate

This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center.

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Tables
### Table 1: Correlation between expression of FUBP1 and clinicopathological features in 119 cases of cervical carcinoma

| Characteristics          | No. of patients | Expression of FUBP1 | P value |
|--------------------------|-----------------|---------------------|---------|
|                          |                 | Low      | High     |         |
| Patients                 |                 |          |          |         |
| Malignant tumor          | 119             | 56       | 63       | 0.039   |
| Adjacent tumor           | 36              | 24       | 12       |         |
| Age                      |                 |          |          |         |
| ≤46                      | 54              | 35       | 19       | <0.001  |
| >46                      | 65              | 21       | 44       |         |
| Clinical stage           |                 |          |          |         |
| I+II                     | 27              | 14       | 13       | 0.570   |
| III+IV                   | 92              | 42       | 50       |         |
| T classification         |                 |          |          |         |
| T1-T2                    | 96              | 51       | 45       | 0.007   |
| T3-T4                    | 23              | 5        | 18       |         |
| N classification         |                 |          |          |         |
| N0                       | 97              | 52       | 45       | 0.003   |
| N1                       | 22              | 4        | 18       |         |
| Tumor recurrence         |                 |          |          |         |
| No                       | 80              | 45       | 35       | 0.004   |
| Yes                      | 39              | 11       | 28       |         |
| HPV                      |                 |          |          |         |
| Negative                 | 27              | 9        | 18       | 0.104   |
| Positive                 | 92              | 47       | 45       |         |
| Ki67 expression          |                 |          |          |         |
| Negative                 | 68              | 38       | 30       | 0.026   |
| Positive                 | 51              | 18       | 33       |         |
| EGFR expression          |                 |          |          |         |
| Low                      | 15              | 9        | 6        | 0.283   |
| High                     | 104             | 47       | 57       |         |
| PDL1 expression          |                 |          |          |         |
| Low                      | 91              | 43       | 48       | 0.939   |
| High                     | 28              | 13       | 15       |         |