Novel Role of IMPA2 in AIFM2-Mediated Apoptosis of Cervical Cancer by Targeting p53

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Abstract

Background

Cervical cancer continues to be concerned and the prognosis of locally advanced cervical cancer remains poor, which underscores pivotal needs to find novel therapeutic targets. Previously, we firstly identified Myo-inositol monophosphatase 2 (IMPA2) as a potential oncogene and verified its tumor-promoting role in vitro and in vivo. In this study, we further aimed to elucidate the underlying mechanisms of IMPA2 in regulation of tumor apoptosis.

Methods

Cell apoptosis was assessed by apoptosis-related proteins detecting, flow cytometry, immunofluorescence and immunohistochemical staining. Fluorescence microscope was used to analyze fluorescence signal. The string database was used to find molecules regulated by IMPA2. The expression of apoptosis inducing factor mitochondria associated 2 (AIFM2) was determined by qRT-PCR, western blot, immunohistochemical staining and immunofluorescence analysis. Function changes of mitochondria were evaluated by measurements of mitochondrial membrane potential and intracellular Ca\(^{2+}\) levels. CCK-8 assay was used to detected cell viability.

Results

Apoptosis of cervical cancer cells was markedly promoted when silencing IMPA2. AIFM2 was significantly up-regulated both in mRNA and protein levels, and inhibition of AIFM2 could reserve IMPA2 knockdown-induced apoptosis in a mitochondrial dependent manner. But the analysis of database and our experimental results showed that AIFM2 had little effect on cervical cancer progression and survival. Further mechanistic study revealed that IMPA2 and AIFM2 silencing apparently activated p53 and treatment of p53 inhibitor (Pithrinn α) could rescue IMPA2 knockdown-induced cell apoptosis.

Conclusion

Our findings displayed a novel function of IMPA2 in regulating cell apoptosis mediated by a disturbance of AIFM2 and p53 expression, potentially making it a novel therapeutic target for cervical cancer treatment.

Background

Cervical cancer is a common health problem that seriously threatened women’s health worldwide, with approximately 12,820 new cases and 4,210 deaths per year in the US(1, 2). Radiotherapy and chemotherapy are still the two main treatments for cervical cancer. However, the prognosis of locally
advanced cervical cancer remains poor, and the treatment still results in substantial morbidity owing to resistance of chemotherapy drugs\(^3\),\(^4\). Therefore, alternative therapeutic strategies are needed. Recently, clinical potential of activators of apoptotic pathways has been widely concerned in treatment of cervical cancer\(^5\). Thus, better knowledge of the molecular mechanisms that modulate the apoptotic pathways is urgently required.

Myo-inositol monophosphatase 2 (IMPA2) is a key lithium-sensitive enzyme involved in phosphoinositide (PI) signaling\(^6\). Most studies about IMPA2 focused on neuropsychiatric diseases and the pharmacological action of Lithium\(^7\),\(^8\). It was reported that altered \(IMPA2\) gene expression was closely related calcium homeostasis in bipolar disorder\(^9\). Recently, Lin HY found that dysregulation of \(IMPA2\) could promote metastatic progression of clear cell renal cell carcinoma\(^10\). In our previous study, we have demonstrated that \(IMPA2\) could play a tumor-promoting role in cervical cancer for the first time\(^11\), and the proteomic analysis results prompted that \(IMPA2\) may regulate apoptosis process of cervical cancer, but the underlying mechanisms are still unknown.

Apoptosis inducing factor mitochondria associated 2 (AIFM2) is an up-regulated gene in our previous proteomics analysis when silencing \(IMPA2\) gene expression in cervical cancer cells. It was reported that \(AIFM2\) could play a role in mitochondrial stress signaling and enhanced apoptosis of human lung cancer cells\(^12\). Tan JH et al also proved that ATF6 could aggravate acinar cell apoptosis and injury by regulating p53/\(AIFM2\) transcription in Severe Acute Pancreatitis\(^13\). Although evidence have shown that \(AIFM2\) may contribute to cell apoptosis, but little researches have focused on its role in cervical cancer.

Our previous study firstly showed that \(IMPA2\) may promote the progression of cervical cancer\(^11\). In this study, we further revealed that silenced-\(IMPA2\) could aggravate apoptosis regulated by activating the p53-signaling pathway through upregulating the expression of \(AIFM2\), suggesting a potential therapeutic target to cervical cancer treatment.

**Methods**

**Cells culture and cell transfection**

Cervical cancer cell line SiHa (#BNCC337881) was purchased from the Cell Bank of BeNa culture collection (Beijing, China). Cervical cancer cell line HeLa (#GCC-UT0002CS) was purchased from the Cell Bank of Genechem (Shanghai, China). The \(IMPA2\) silencing Siha and Hela cells were stably constructed as previously\(^11\). The cells were cultured in Dulbecco's modified eagle medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% antibiotics at 37°C in an atmosphere containing 5% CO2.

For AIFM2 knockdown, AIFM2 siRNAs were chemical synthesized by BioeGene (Shanghai, China) and the sequences were as following:

si-1: CCAAAUCAGUGGCUUCUAUTT (5’-3’);
si-2: GCACCGGCAUCAAGAUCAATT (5′-3′);
si-3: GCUGCCUCUCAAUGAGUAUTT (5′-3′).

The cells were transfected with LipoHigh (Sangon Biotech, China) according to the manufacturer’s instructions. After culture for 6h, replace the fresh medium and continue to culture for 24h for RNA extraction and 48h for protein extraction and other experiments.

Cell flow cytometry

After transfection for 6 h, cells were incubated for 48 h at 37 °C, 5% CO2 incubator. The collected cells were then rinsed twice with PBS and then prepared for subsequent testing. After IMPA2 silencing Siha and Hela cells were treated with 0μM and 20μM PFT-α (Sigma, USA) for 24h, apoptosis was analyzed by an Annexin-V-APC/PI staining kit (Biolegend, USA) according to the manufacturer’s instructions and finally analyzed in a Guava easyCyte HT flow cytometer (Millipore, USA).

Immunohistochemistry (IHC) staining analysis

The immunohistochemical staining procedure was performed using standard techniques. Tissue samples for Bak and AIFM2 detecting were xenografts from mice that injected silenced-IMPA2 Siha cells and control Siha cells. The staining positivity was determined by the following formula: IRS = intensity score × quantity score. The percentage of positive cells was divided into five score ranks: <10% (0), 10–25% (1), 25–50% (2), 50–75% (3), and >75% (4). The intensity of staining was divided into four score ranks: no staining (0), light brown (1), brown (2), and dark brown (3). Two different pathologists evaluated all the specimens in a blinded manner. The antibodies used were as following: anti-Bak (1:100, CST, USA); anti-AIFM2 (1:500, Affinity, USA).

Immunofluorescent analysis

Immunofluorescent staining was performed using the antibody of Caspase-3 (1:500, Abcam, UK) and AIFM2(1:100, Affinity, USA) following the protocol reported previously. The secondary antibody (1:1000, Abcam, UK) was followed and nucleus were stained by DAPI (Beyotime, China). The signals of immunofluorescence were examined using a fluorescence microscope.

Measurement of the intracellular Ca$^{2+}$ concentration

The mitochondrial Ca$^{2+}$ concentration was detected using a Rhod-2,AM probe (Maokang, China). The detection was carried out according to the manufacture’s instruction and signals of immunofluorescence were examined using a fluorescence microscope and Image J was used for fluorescence quantification.

Measurement of mitochondrial membrane potential

The extent of mitochondrial membrane potential (MMP) loss was measured using the potentiometric cation 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1)
(MedChemExpress, USA). Transfected Hela or Siha cells were incubated with JC-1 staining liquid for 20 minutes at 37°C and examined under fluorescence microscope.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted using the Trizol reagent (Sangon Biotech, Shanghai, China). RNA (1 ug) was reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany) according to the supplier's instructions. Quantitative real-time PCR analysis was performed with Stratagene Mx3000P qPCR system (Agilent Technologies, USA) using Thunderbird qPCR Mix (TOYOBO, Japan). cDNA samples were tested in triplicate and glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a reference gene. The expression of genes were quantified by measuring Ct values and normalized using the 2^ΔΔCt method relative to GAPDH. The primer pairs used for qRT-PCR were designed using the primer3 program. Primers used were shown in Table 1.

**Western bolt**

The cell extracts were prepared using RIPA buffer (KeyGEN Biotech, China) containing protease inhibitors (KeyGen Biotech). Equal amounts of protein samples were subjected to SDS-PAGE and transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were then blotted with primary antibodies overnight at 4 ℃, followed by incubation with the secondary antibody (1:5000, Beyotime, China). The proteins were then developed using enhanced chemiluminescence (Bio-rad, Berkeley, CA, USA). The band quantification was conducted using ImageJ (National Institutes of Health, Bethesda, MA, USA). The antibodies used were as following: anti-AIFM2 (1:1000, Biorbyt, UK); anti-Bcl2 (1:2000. Abcam, UK); anti-Bax (1:5000, Abcam, UK); anti-Caspase3 (1:5000, Abcam, UK); anti-Bak (1:1000, CST, USA); anti-Cytc (1:1000, Abcam, UK); p53 (1:2000, Abcam, UK); anti-p-mTOR (1:1000, CST, USA); mTOR (1:1000, CST, USA); anti-p-PI3K (1:1000, Affinity, USA); anti-PI3K (1:1000, Affinity, USA); anti-p-AKT (1:1000, CST, USA); anti-AKT (1:1000, CST, USA); anti-p-JAK2 (1:1000, Abcam, UK); anti-JAK2 (1:1000, Abcam, UK); anti-p-STAT3 (1:5000, Abcam, UK); anti-STAT3 (1:5000, Abcam, UK); anti-GAPDH (1:5000; BBI, China).

**Statistical analysis**

Data were shown as mean ± standard deviation (SD) based on at least three independent experiments. The results were analyzed using SPSS 22.0 (Chicago, IL, USA) and GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). The two-tailed Student's t-test was used to evaluate the difference between two groups of data. *P* < 0.05, **P* < 0.01, ***P* < 0.001. *P* value <0.05 was considered statistically significant.

**Results**

**IMPA2 knockdown induced apoptosis in cervical cancer cells**
To further investigate whether IMPA2 affects tumor progression by regulating apoptotic process of cervical cancer cells, we firstly detected expressions of key apoptosis-related proteins. As shown in Fig.1A and Fig.1B, IMPA2 knockdown significantly increased expression levels of pro-apoptotic proteins (Bax, Bak, and Caspase3), and decreased expression of apoptotic inhibitory molecule, Bcl-2. Also, apoptotic cells were measured using Annexin V-APC/PI staining assay. Compared to the negative control group, percentages of apoptotic cells in silenced-IMPA2 Siha and Hela cells were much higher ($P<0.01$) (Fig.1C-E). Besides, depletion of IMPA2 exhibited increased green fluorescence in Hela (Fig.1F) and Siha (Fig.1G) cells, which suggested higher expression of Caspase3. Furthermore, silenced IMPA2 could promote expression of Bak was also been verified in in xenografts derived from our previous study by Immunohistochemical staining (Fig.1H, I). Taken together, these data revealed that inhibition of IMPA2 could induce apoptosis in Siha and HeLa cells.

**IMPA2 knockdown upregulated AIFM2 gene expression to induce apoptosis of Hela and Siha cells**

To investigate the underlying mechanisms of the modulation of IMPA2 on apoptotic process of cervical cancer cells, we screened 8 molecules related to apoptosis from the previous proteomic results and a database for finding interacting molecules (Fig.2A) (http://string-db.org). After silencing IMPA2, we found only AIFM2 gene mRNA expression was up-regulated both in Hela and Siha cells (Fig.2B). Similar results were demonstrated in protein expression detected by western blot (Fig.2C). In addition, immunofluorescence results also showed that IMPA2 knockdown groups have an increase on AIFM2 expression both in Hela and Siha cells (Fig.2D, E). Meanwhile, the IHC staining also demonstrated clearly higher AIFM2 protein expression in in xenografts from mice after injecting siIMPA2-Siha cells ($P<0.05$) (Fig.2F, G). These results indicated that inhibition of IMPA2 could upregulate AIFM2 gene expression. Furthermore, IMPA2 knockdown-induced changes of apoptosis related proteins were reversed by co-transfecting AIFM2 siRNA, with an increase of Bcl-2 expression and repression of Bax and Caspase3 expression (Fig.2H-J). Similarly, apoptotic cells were also decreased when simultaneously knocking-down AIFM2 and IMPA2 genes in cervical cancer cells (Fig.2K-M). All these data suggested that IMPA2 inhibition could promote apoptosis of Hela and Siha cells by activating AIFM2 expression.

**Downregulation of AIFM2 repressed mitochondria-dependent apoptosis**

There were some articles have proved the crucial role of AIFM2 in apoptosis, but few researches focus its function on cancer cells, including cervical cancer. In above section, we have found AIFM2 could be upregulated in silenced-IMPA2 Hela and Siha cells. To further explore whether AIFM2 affects the apoptosis in cervical cancer cells, we designed three siRNAs to knockdown AIFM2 gene expression. From Fig.3A and Fig.3B, we could see AIFM2 was significantly downregulated both in mRNA level and protein level ($P<0.01$). Among the 3 siRNAs, si-1 had the most significant inhibitory effect to Hela and si-2 suppressed most to Siha cells. Based on this we chose si-1 and si-2 for our subsequent experiments. As shown in Fig.3C and Fig.3D, AIFM2 knockdown cells have a lower expression of Bax and Caspase3, consistent with an increase of Bax. Likewise, percentages of apoptotic cells in cancer cells transfected with AIFM2 siRNA also decreased compared with the control group (Fig.3E-G). On the other hand, in
AIFM2 knockdown Hela and Siha cells, the intensity of green fluorescence was weaker and the red fluorescence was stronger after JC-1 dye staining, which was opposite in the control cells. The result indicated that there was a redistribution of mitochondrial membrane potential (Fig.3H, I). In addition, AIFM2 knockdown cells also have a lower concentration of mitochondrial Ca\(^2+\) (P<0.001). These results demonstrated that AIFM2 could regulate apoptosis of cervical cancer cells in a mitochondria-dependent way.

**AIFM2 inhibition had little effect on cervical cancer progression and survival**

Analysis of a public CESC (Cervical squamous cell carcinoma) dataset from The Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/) showed that there was no significant difference of AIFM2 expression between cancer tissues and normal tissues (Fig.4A). And survival rates of high AIFM2 expression patients and low AIFM2 expression patients also had no obvious difference (P=0.55) (Fig.4B). Similar with results from database, silencing AIFM2 in Siha and Hela cells had little effect on the cervical cancer cell growth (Fig.4C, D). Also, AIFM2 expression on cervical cancer tissues and normal tissues showed no significant difference detected by IHC staining (Fig.4E, F). In sum, AIFM2 expression may have little effect on cancer progression and survival, which may be explained by the complex functions of AIFM2 including induction of apoptosis and suppression of ferroptosis.

**IMPA2 knockdown triggered AIFM2-mediated apoptosis via p53 signaling pathway in Hela and Siha cells**

To understand further mechanisms involved in IMPA2-AIFM2 regulated cell apoptosis, we identified some classical pathways of apoptosis regulation in cancer cells. Proteins of PI3K/AKT/mTOR, JAK/STAT3, and p53 were detected by western blot in IMPA2 silencing cells and the control cells (Fig.5A). The results showed that mTOR protein was activated but PI3K and AKT only activated in Hela or Siha cells, suggesting PI3K/AKT/mTOR may partially involve in IMPA2-regulated cell apoptosis. In addition, IMPA2 silencing significantly increased JAK/STAT3 phosphorylation and p53 expression in Siha and Hela cells, indicating IMPA2 may affect apoptosis via JAK/STAT3 pathway or p53 pathway. Also, those proteins of apoptotic pathways also detected in AIFM2 knockdown cell groups and the control groups. From Fig.5B, we could see the phosphorylation of JAK/STAT3 were repressed and p53 expression was also decreased after inhibiting AIFM2 gene. Furthermore, IMPA2 silencing-induced p53 activated was suppressed by inhibition of AIFM2, while the phosphorylation of JAK/STAT3 have not been totally rescued by siAIFM2 (Fig.5C). Besides, the relationship of AIFM2 and p53 also verified from database (Fig.5D) (http://string-db.org). Thus, p53 may be a key molecule for IMPA2-AIFM2 regulated tumor apoptosis.

**PFT-α treatment successfully rescued p53-induced cell apoptosis in IMPA2 silencing Hela and Siha cells**

To further identified the roles of p53 and JAK/STAT3 in cervical cancer apoptosis induced by IMPA2 and AIFM2, we examined the effects of PFT-α and AG490 on apoptosis proteins. As shown in Fig.6A and Fig.6B, PFT-α treatment made silenced-Hela and Siha cells express lower level of Bax and Caspase3, and higher level of Bcl-2 than the 0μM PFT-α treatment group (P<0.05). Similarly, apoptotic cells significantly decreased in IMPA2 knockdown cells after 20μM PFT-α treatment both in Hela and Siha cells using

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Annexin-V-APC /PI staining (Fig.6E-G). While after AG490 treatment, there were no statistical changes of apoptosis proteins compared with the control group (Fig.6C, D). Overall, IMPA2 silencing, with activation of AIFM2, promoted apoptosis of cervical cancer cells via p53 signaling pathway.

### Discussion

Due to an increase in cervical screening and popularization of HPV vaccine, deaths from cervical cancer have reduced in recent years. However, prognosis of cervical cancer remains poor, especially in developing countries(14). Resistance of chemotherapy drugs contribute to treatment failure or tumor recurrence and molecular target therapy displays outstanding curative effect. Thus, the development of new molecules for cervical cancer screening and treatment is urgently needed. In this study, IMPA2 was a novel gene we previously found from transcriptomics results that has been proved to be a potential tumor-promoting gene(11). And we further confirmed that IMPA2 may affect cancer cell death through inducing cell apoptosis. AIFM2, a NAD(P)H-dependent oxidoreductase involved in cellular oxidative stress response(15), has also been found to involved in IMPA2 knockdown-mediated cell apoptosis by activating p53. These evidences suggested IMPA2 as a potential target for cervical cancer therapy.

IMPA2 irregularity always proved to contribute to the pathophysiology of bipolar disorder(6, 16, 17). Few researches are focused on its other functions. In this study, we found IMPA2 could affect survival of cervical cancer cells by disturbing apoptotic process. And one article reported that altered IMPA2 gene expression was related to calcium homeostasis in bipolar disorder(9), giving indirect evidence of relationship between IMPA2 and cell apoptosis. But a comprehensive understanding of the mechanism of a potential cancer-promoting gene is necessary before its clinical use. Another article reported that IMPA2 downregulation enhances mTORC1 activity and restrains autophagy initiation in metastatic clear cell Renalca(18). In Fig. 2A, we also observed accumulation of autophagy lysosomes after IMPA2 knockdown. Besides, our previous proteomic prole of shCtrl and shIMPA2 cervical cancer cells also prompted differentially expressed proteins are enriched in autophagy biological process(11). These information suggest IMPA2 may also involving in cell autophagy, and the functional relationship between apoptosis and autophagy is admittedly complex existing mutual control and mutual conversion in different cellular settings(19, 20). Exploring the relationship between IMPA2 and autophagy is necessary and would be our next research direction.

Although we have confirmed the pro-apoptosis function of AIFM2 in cervical cancer, conflicting data exist on the pro-apoptotic function of the protein in different disease(21–23). We have detected the cell viability of AIFM2 silencing cells and the control cells, but there was no significant difference (Fig. 4), indicating that apart from apoptosis, there are other processes regulated by AIFM2 involving in cervical cancer cell death. A latest article has reported that FSP1, a protein encoded by AIFM2, is a glutathione-independent ferroptosis suppressor and acts parallel to GPX4 to inhibit ferroptosis(24, 25). Ferroptosis is a newly discovered form of regulated cell death that is the nexus between metabolism, redox biology, and human health(26). As AIFM2 not only encoding FSP1 to block ferroptosis, but also induce mitochondria-dependent cell apoptosis, we have not observed apparent changes of cell viability after inhibiting AIFM2
expression. Moreover, to explore whether ferroptosis involved in IMPA2-regulated cell death, we detected glutathione peroxidase 4 (GPX4) expression. The results showed no significant changes between IMPA2 knockdown cells and the control cells (sFig.1), hinting that IMPA2-induced cancer progression may be independent of ferroptosis.

The tumor-suppressor protein p53 is mutated in approximately half of all cancers, whereas the p53 signaling network is perturbed in almost all cancers(27). Based on this, p53 gives rise to people’s attention and becomes an outstanding target for cancer therapy. Numerous studies focused on p53 for its clinical treatment, some agents even have entered into phase II clinical study(28–30). Among them, APR-246 (PRIMA-1MET) is the first drug that restores transcriptional activity of unfolded wild-type or mutant p53 and had its in-human study performed in refractory hematologic malignancies and prostate cancer, which showed a favorable pharmacokinetic profile(31). But on December 28, 2020, NASDAQ-listed company Aprea Therapeutics announced that its p53 mutation inhibitor Eprenetapopt combined with azacitidine for the first-line treatment of myelodysplastic syndrome (MDS) has not reached the primary clinical endpoint in Phase III clinical trials, revealing immature acknowledge of p53 in tumorigenesis. In this study, we displayed a novel gene involving in p53-regulated cell apoptosis, indicating that IMPA2 may be a key regulatory factor for clinical treatment of p53 inhibitor.

**Conclusion**

In conclusion, we identified that IMPA2 downregulation promoted cervical cancer cell apoptosis and the mechanistic investigation revealed that IMPA2 knockdown triggered AIFM2 expression and activated p53, thereby induced cancer cell apoptosis. Taken together, our study provided a potential target for cervical cancer therapy.

**Abbreviations**

IMPA2
Myo-inositol monophosphatase 2; AIFM2: Apoptosis inducing factor mitochondria associated 2; qRT-PCR: Quantitative real-time PCR; IHC: Immunohistochemistry; CCK-8: Cell counting kit 8; HPV: Human papillomavirus; MMP: Mitochondrial membrane potential; siRNA: Small interfering RNA

**Declarations**

*Ethics approval and consent to participate*

Not applicable

*Consent for publication*

Not applicable
Availability of data and materials

The datasets generated and/or analysed during the current study are available in the Figshare repository Liu (2021): Origial data.rar. figshare. Dataset. https://doi.org/10.6084/m9.figshare.14069522.v1.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Min Wang: Conceptualization and methodology; Lei liu: Data curation, writing original paper and draft preparation; Xianping Li: Visualization and investigation; Bingqi Wang: Supervision; Sheng Yin: Writing reviewing and editing.

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### Table 1
Primer sequences used for performing qRT-PCR

| Gene name | Forward (5'-3') | Reverse (5'-3') |
|-----------|----------------|-----------------|
| AIFM2     | CAAGATCAACAGCTCCGCCTACC | CGTCGGCACAGTCACCAATGG |
| BLOC1S2   | ACTGGCGACCCGGAGTGATG | GTCAGCTTCAGCAGGCTCCTTTG |
| MYDGF     | CGGCGTCTGCCATTCCCTTCTC | CCATTGCTCATTGGTCGCCCTCCTTTG |
| MLH1      | TCTCAGGTATCGGAGCCAGC | ATCTTCCTCTGTCCAGCCACTCTC |
| HERPUD1   | ATCAGGGGCTTTTGTCCACCAC | ACAACCACTTGAGGAGCAGCTTTC |
| CAV1      | GCAGAAGGAGGACACACACAG | CCAAGAGGAGGAGACACAGCAAGC |
| HSF       | CGACAGTGCTCAGCACATTCC | ACAGCAGGGGCAGTAGAGG |
| MTM-1     | ATCCAGTTGCCAGTATGCGTCAC | TCGGCTGTGTGCTGATCCTG |

### Figures
Figure 3

AIFM2 knockdown repressed mitochondrial-dependent apoptosis of Hela and Siha cells. To analyze the function of AIFM2 on cell apoptosis, we designed 3 siRNAs to inhibit AIFM2 expression. The inhibitory effects of AIFM2 in Hela and Siha cells were validated by western bolt (A) and qRT-PCR (B). As si-1 and si-2 have the most prominent effects to Hela and Siha cells respectively, we chose these two siRNAs for our following research. After inhibiting AIFM2 expression, apoptosis-related proteins were detected and
analyzed by western blot (C)(D) and apoptotic cells were measured and quantified using flow cytometry(E-G). (H)(I) The mitochondrial membrane potential of AIFM2 silencing cervical cancer cells and the control cells were detected by JC-1 assay. The loss of mitochondrial membrane potential was represented by the fluorescence change from red to green. (J) The intracellular Ca2+ concentrations were detected using Rhod-2,AM probe. The fluorescence intensity is proportional to the intracellular Ca2+ concentration. (K) A histogram represented statistical analysis of intracellular Ca2+ concentration. Data are represented as mean ± SDs of 3 replicates. *P<0.05; **P<0.01; ***P<0.001.

A

Expression of AIFM2 in CESC based on Sample types

B

Effect of AIFM2 expression level on CESC patient survival

C

Relative viability

D

Relative viability

E

Normal

Cancer

F

IHC Score

Normal tissue

Cancer tissue
AIFM2 expression had little effect on cervical cancer progression and survival. (A) Expression levels of AIFM2 in cervical cancer tissues and normal tissues were analyzed based on CESC data set (https://tcga-data.nci.nih.gov/). (B) Effect of AIFM2 expression level on CESC patient survival was analyzed (https://tcga-data.nci.nih.gov/). Cell viability of Hela (C) and Siha cells (D) transfected with siAIFM2 or siNC were measured by CCK-8 assay. (E)(F) AIFM2 expression was measured and quantified in cervical tissues and normal tissues by IHC staining. Data are represented as mean ± SDs of 3 replicates.
IMPA2 inhibition regulated AIFM2-mediated cell apoptosis through p53 activation. To explored the mechanisms responsible for IMPA2 knockdown-induced cell apoptosis, we screened the classical apoptotic pathway through detecting the protein expressions after inhibiting IMPA2. The classical pathway proteins were also measured in IMPA2(A) and AIFM2(B) knockdown cells and the control cells. Based on above results, p53 and JAK2/STAT3 had the significant difference both in siIMPA2 and siAIFM2 Hela and Siha cells. So siIMPA2 and siAIFM2 were further co-transfected in cervical cancer cells and proteins of p53 and JAK2/STAT3 are detected (C). The results showed that only p53 expression has been successfully rescued. (D) A database analysis of the relationship between AIFM2 and p53 was exhibited (http://string-db.org).
Figure 6

PFT-α treatment rescued IMPA2 knockdown-induced apoptosis in Hela and Siha cells. After gradient concentrations of p53 inhibitor (PFT-α) treatment, apoptosis-related proteins are measured in IMPA2 silencing Hela(A) and Siha(B) cells to validate whether IMPA2 knockdown-induced cell apoptosis could be revised by PFT-α. Apoptosis-related proteins were also detected in IMPA2 inhibition and the control Hela (C) and Siha (D) cells after treatment of AG490, an inhibitor of JAK2/STAT3. (E-G) Apoptotic cells
were also analyzed and quantified by Annexin-V-APC staining after 0 and 20 μM PFT-α treatment in IMPA2 inhibition Hela and Siha cells.

**Supplementary Files**

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- sFig.1.tif