Original Article
p53 mediated apoptosis in osteosarcoma MG-63 cells by inhibition of FANCD2 gene expression

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Abstract: Purpose: The aim of this study was to investigate the association between osteosarcoma (OS) and Fanconi anemia (FA) related pathways and the molecular mechanisms. Methods: siRNA for Fanconi anemia complementation group D2 (FANCD2) was constructed and transfected into the osteosarcoma cell line MG-63 cells. Expression of TP53INP1, p53, p21, caspase-9, and caspase-3 mRNA in MG-63 cells were examined by real-time fluorescence quantitative PCR, and the protein levels were also determined by western blot. Results: After silence of the FANCD2 gene in MG-63 cells, cell proliferation was inhibited, cell cycle was arrested and cell apoptosis was induced. The apoptosis was mediated by the p53 signaling pathway. After FANCD2 expression was inhibited, TP53INP1 gene expression was up-regulated, phosphorylation of p53 was promoted and the p21 protein was activated, leading to cell cycle arrested in G1, finally resulted in caspase-dependent cell apoptosis. Conclusions: Inhibition of FANCD2 gene expression can induce apoptosis of osteosarcoma cells, which indicated that FANCD2 played an important role in the development of osteosarcoma and it might be a potential target for treatment of osteosarcoma.

Keywords: Fanconi anemia complementation group D2, osteosarcoma, RNA interference, p53, cell apoptosis

Introduction

Osteosarcoma (OS) is the most common primary malignant tumor of the bone tissue, mainly invading the metaphysis of long bones. OS has high degree of malignancy and poor prognosis. New drugs and treatment strategies based on full understanding of the mechanism of OS are required [1]. Dysfunction of proto-oncogenes and tumor suppressor genes is one of the pathogenic factors for OS. Like most other malignancies, OS involves multiple onco-genes activations and tumor suppressor genes mutations, including proto-oncogene c-myc, ras, fos, etc., and tumor suppressor gene p16, p53, Rb, etc [2-5].

Fanconi anemia (FA) is a rare autosomal or X-linked recessive hereditary disorder. It is found that FA could result from missing or mutation of 15 kinds of FA-related genes. These related genes form a complex network named FA pathway, in which Fanconi anemia complementation group D2 (FANCD2) is a key member. After post-translational modifications like phosphorylation and ubiquitination, [6, 7],

FANCD2 protein plays important role in regulating expression of genes which are involved in tumorigenesis, apoptosis, and other important life processes [8-10]. A FA patient has considerably higher risk of cancer [11]. Studies have shown that 28% of FA patients would have non-hematopoietic tumors before the age of 40, indicating that there is a strong positive correlation between FA and malignant tumors [12]. FA patients often suffer from head and neck, skin or anogenital squamous cell carcinomas [13]. But few researches have investigated the association between osteosarcoma and the FA pathway.

OS is one of the complications of FA, and both FA and OS are likely to occur in adolescence, but the interactions between them remains unclear. It is thought that FANCD2 is an important protein related to OS, while the associations and mechanisms need further study.

In this study, siRNA of FANCD2 was constructed and transfected to the osteosarcoma MG-63 cells, gene expressions after silence of FANCD2 gene were detected by gene chip. Cell prolifera-
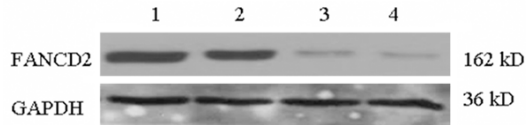
Figure 1. FANCD2 protein expression in MG-63 cells after RNAi. Lane 1: Control; Lane 2: siRNA-Control; Lane 3: siRNA-FANCD2 24 h; Lane 4: siRNA-FANCD2 48 h.

tion and apoptosis, and the apoptosis-related signaling pathway were investigated, in order to reveal the role of FANCD2 in OS development.

Materials and methods

Construction and transfection of the FANCD2 siRNA in MG-63 cells

siRNA-FANCD2 and a control siRNA plasmid were designed and synthesized by Santa Cruz Biotechnology, Inc. (Texas, USA). They were transfected into osteosarcoma MG-63 cells by liposome. Expression of FANCD2 proteins in MG-63 cells were detected by western blots 24 h and 48 h later in order to evaluate the efficacy of siRNA.

Cell proliferation

Cell Counting Kit8 (DOJINDO LABORATORIES, Kumamoto, JAPAN) was used to detect cell proliferations of MG-63 cells 4, 8, 12, 24, and 48 h after siRNA transfection.

Cell cycle and apoptosis detection

24 h and 48 h after siRNA transfection, PI staining combined with flow cytometry was used to detect the percentage of G_0/G_1, S-phase and G_2/M cells and cell cycle arrest in MG-63 cells. Annexin V-FITC/PI staining was used to detect apoptosis in MG-63 cells. Four groups, namely the control group (Control), siRNA-control group, siRNA-FANCD2 24 h group and siRNA-FANCD2 48 h group were detected.

Gene expression

24 h and 48 h after siRNA transfection, total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA) and the changes in gene expression in MG-63 cells after FANCD2 gene silence were analyzed by Affymetrix GeneChip microarray system (Affymetrix, CA, USA). Differential gene expression was detected by

real-time fluorescence quantitative PCR (Q-PCR).

The clustering graph and the differentially expressed genes were obtained, and a fold change of 2.0 was set as threshold for the screening of differentially expressed genes.

Apoptosis-related proteins

Tumor protein 53-induced nuclear protein 1 (TP53INP1), p53, p21 caspase-9 and caspase-3 mRNA expression in MG-63 cells were examined by Q-PCR method. And western blot assay was performed to detect the production of TP53INP1, phos-p53, p21 protein, activation of caspase-9 and caspase-3. As mentioned above, four groups, namely the control group (Control), siRNA-control group, siRNA-FANCD2 24 h group and siRNA-FANCD2 48 h group were detected.

Statistic analysis

The experimental data were expressed as mean±standard deviation (SD), and the two groups were compared using independent sample t-test after test for the homogeneity of variance. More than two groups were compared using ANOVA. $P<0.05$ indicates statistical significance.

Results

FANCD2 siRNA interfere FANCD2 expression in MG-63 cells

FANCD2 protein was detected both in the control group and the siRNA-control group, and no significant difference was observed between the two groups, indicating that the negative control did not prevent FANCD2 expression in MG-63 cells. The production of FANCD2 protein was significantly inhibited in the siRNA-FANCD2 group at 24 h (siRNA-FANCD2 24 h) and 48 h (siRNA-FANCD2 48 h), and the interfere effect was more pronounced at 48 h (**Figure 1**).

Cell proliferation

In the control group and the siRNA-control group, MG-63 cells showed rapid cell proliferation. There was no significant difference between the proliferation rates of the two groups. While the proliferation rates of the siRNA-FANCD2 group were significantly lower than the control group at 8, 12, 24 and 48 h (**Table 1**).

Table 1. Absorbance of MG-63 cells after siRNA-FANCD2 interfere by CCK-8 assay ($\bar{x}\pm s$, n = 6)

Groups	Changes of absorbance value (A_{450})				
	4 h	8 h	12 h	24 h	48 h
Control	0.268±0.012	0.297±0.006	0.289±0.007	0.397±0.016	0.463±0.02
siRNA-control	0.275±0.007	0.288±0.009	0.281±0.013	0.382±0.014	0.483±0.018
siRNA-FANCD2	0.268±0.006	0.263±0.004[***]	0.263±0.004[***]	0.369±0.017[*]	0.384±0.009[***]

[*]$P<0.05$ and [***]$P<0.001$ vs. Control.

Table 2. Cell cycle distribution of MG-63 cells after siRNA-FANCD2 interfere ($\bar{x}\pm s$, n = 4)

Groups	Positive cell percentage (%)		
	G_0/G_1	S	G_2/M
Control	61.11±1.56	33.22±1.60	5.67±0.32
siRNA-control	62.14±0.87	31.81±0.35	6.08±0.58
siRNA-FANCD2 24 h	72.16±0.31[***]	16.19±0.32[***]	11.64±0.54[***]
siRNA-FANCD2 48 h	77.25±1.52[***,##]	12.66±2.71[***]	10.09±1.55[**]

[*]$P<0.05$, [**]$P<0.01$ and [***]$P<0.001$ vs. Control; [##]$P<0.01$ vs. siRNA-FANCD2 24 h.

Table 3. Apoptotic percentages of MG-63 cells after FANCD2 siRNA interfere by Flow Cytometry ($\bar{x}\pm s$, n = 4)

Groups	Positive percentage, %		
	Normal	Necrosis	Apoptosis
Control	86.68±0.35	0.31±0.12	13.01±0.36
siRNA-control	85.56±0.45[*]	0.29±0.01	14.15±0.46[*]
siRNA-FANCD2 24 h	77.45±0.69[***]	0.36±0.23	22.17±0.81[***]
siRNA-FANCD2 48 h	64.00±1.71[**,###]	0.53±0.30	35.47±1.42[***,###]

[*]$P<0.05$, [**]$P<0.01$ and [***]$P<0.01$ vs Control; [###]$P<0.01$ vs. FANCD2 siRNA 24 h.

Cell cycle

The siRNA-control group had similar percentage of cells in G_0/G_1, S and G_2/M phase as the control group. While after siRNA-FANCD2 transfection, cells in the G2/M and G_0/G_1 phase increased significantly, and the percentage of S phase was significantly lower than the control group ($P<0.001$); and the percentage of cells in G_0/G_1 phase at 24 h after siRNA interference was significantly higher than that of the 48 h group ($P<0.01$) (**Table 2**). These results indicated that the RNAi resulted in G_0/G_1 and G_2/M phase arrest.

siRNA-FANCD2 induced apoptosis of MG-63

Apoptotic percentages of MG-63 cells after FANCD2 siRNA transfection were detected by Flow cytometry (**Table 3**). Compared to the control group, siRNA-Control group had a higher percentage of apoptosis, which may be caused

by the affects of transfection reagents. The percentage of normal cells were significantly decreased, while the percentage of apoptotic cells was significantly increased in the siRNA-FANCD2 24 h and siRNA-FANCD2 48 h group ($P<0.01$, $P<0.001$, **Table 3**).

Gene expression profiles of MG-63 cells after siRNA interference by gene microarray

Compared with the control group, 35 genes were up-regulated and 53 were down-regulated in the FANCD2 siRNA 24 h group, involving 37 pathways according to the KEGG database. And 103 genes were up-regulated and 30 were down-regulated in the FANCD2 siRNA 48 h group, involving 54 pathways according to the KEGG database. These differentially expressed genes were involved in many aspects, including cell proliferation, apoptosis, cell cycle control, DNA damage repair and signal transduction.

mRNA expression related to the p53 pathway

Compared to the Control group, TP53INP1 mRNA expression in the siRNA-control group was similar, while the p53, p21, caspase-9 and caspase-3 mRNA expression increased. For the siRNA-FANCD2 24 h and siRNA-FANCD2 48 h group, TP53INP1, p53, p21, caspase-9 and caspase-3 mRNA expression were significantly increased (**Table 4**), up to 25 times, and this effect increased with time.

Protein production related to the p53 pathway

As shown in **Figure 2**, compared to the control group, the production of p53, phos-p53, p21,

Table 4. Relative expression of mRNAs in MG-63 cells after FANCD2 siRNA interfere ($\bar{x}$±s, n = 3)

Groups	mRNA relative expression				
	TP53INP1	p53	p21	caspase-9	caspase-3
Control	1	1	1	1	1
siRNA-control	1.10±0.02	1.34±0.04[*]	1.63±0.02[*]	1.24±0.04[*]	1.29±0.03[*]
siRNA-FANCD2 24 h	5.41±0.29[**]	6.64±0.32[**]	4.23±0.11[**]	12.42±0.54[***]	14.88±0.60[***]
siRNA-FANCD2 48 h	13.00±0.21[***,#]	13.40±1.60[***,#]	11.14±0.21[***,#]	21.19±0.23[***,##]	25.05±1.52[***,##]

[*]$P<0.05$, [**]$P<0.01$ and [***]$P<0.001$ vs. Control; [#]$P<0.05$, [##]$P<0.01$ vs. FANCD2 siRNA 24 h.
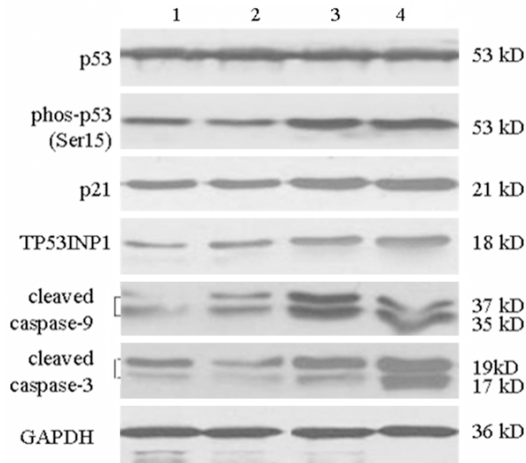
Figure 2. Western blotting picture of p53, phos-p53, p21, TP53INP1, cleaved caspase-9 and-3 protein expression after RNAi. Lane 1: Control; Lane 2: siRNA-Control; Lane 3: siRNA-FANCD2 24 h; Lane 4: siRNA-FANCD2 48 h.

TP53INP1, cleaved caspase-9 and cleaved caspase-3 were similar in the siRNA-control group. Production of p53 in the siRNA-FANCD2 24 h and siRNA-FANCD2 48 h group did not change significantly, but phos-p53, p21 protein and TP53INP1 increased significantly. Caspasae-9 was cleaved into 37 and 35 kD, caspase-3 was cleaved into 19 and 17 kD, and the expression was significantly increased, especially the cleaved caspase-3.

Discussion

In previous studies, it has been found that FANCD2 plays an important role in the FA/BRCA pathway. Other studies have shown that FANCD2 is involved in DNA damage repair, cell cycle regulation, chromatin remodeling, DNA methylation and apoptosis, and it is essential to cell growth, differentiation and maintaining normal function of the body [14-16]. However, its associations with osteosarcoma remain

unclear. In this study, we successfully constructed an efficient FANCD2 targeting siRNA, almost no expression of FANCD2 was observed after transfection into the MG-63 cells. And it caused suppressed cell proliferation, cell cycle arrest and apoptosis. Down-regulated or absent of the FANCD2 gene seriously altered the biological processes of tumor cell proliferation. Numerous studies have showed that tumor cells have unlimited proliferation, migration, invasion [17, 18]. Thus, induction of tumor cells apoptosis is the usual method to control tumor. After interference of FANCD2 in MG-63 cells, apoptosis was observed.

In order to illuminate the involved signal pathways, the gene expression changes in MG-63 cells were further detected using gene chip method. There are three major apoptotic pathway, namely the mitochondrial pathway, endoplasmic reticulum pathway and death receptor pathway. Mitochondrial pathway is activated when the Bcl-2 family members (including Bcl-2, Bax, Bak and other molecules) received the death signal of the cell, then the mitochondrial membrane permeability is changed and the transmembrane potential is lost, finally leads to apoptosis. p53 is a transcription factor located on the mitochondrial, which can interact directly with Bax and induce change of mitochondrial permeability, then cause the release of various pro-apoptotic factors into the cytoplasm, finally leads to apoptosis [19-21]. Endoplasmic reticulum path way is mainly mediated by caspase family members. Caspase 12 is located on the endoplasmic reticulum, whose over-expression results in transfer of caspase 7 from cytoplasm to the endoplasmic reticulum surface, and then further cleave caspase 3 and induce apoptosis [22]. Death receptor pathway is mediated by the death receptors, which are a class of transmembrane proteins belonging to the tumor necrosis factor receptor super family [23-25].

All these three pathways in general depend on the final caspase-mediated apoptosis.

After FANCD2 siRNA interference for 48 h, 103 genes were up-regulated, 30 were down-regulated, involving 54 pathways in KEGG database. In these differentially expressed genes, up-regulation of XAF1 and TP53NP1 attracted our attention, because these two genes are closely related to p53 pathway. It seemed that the siRNA-FANCD2 induced apoptosis is mediated by the p53 signaling pathway, which was confirmed by the results of Q-PCR and western blot.

Mutant p53 gene was found in a variety of cancers, such as breast cancer, colorectal cancer, lung cancer, liver cancer, osteosarcoma, the mutation rate of up to 50% [26]. Wild-type p53 plays a role in monitoring cell growth, cell cycle regulation, cell cycle, DNA damage repair and induction of apoptosis [27, 28]. TP53 protein is encoded by the p53 gene, which can regulate the cell cycle and prevent transformation of cancer cells. TP53INP1 is a target gene of p53, whose expression is regulated by the transcription factor p53, p73 and E2F1, and its activation can induce cell cycle arrest and p53-mediated apoptosis [29]. Studies have shown that over expression of TP53INP1 can cause inhibition of cell proliferation and apoptosis, and cells arrest in the G1 and G2 [30, 31]. XAF1 is a negative regulatory protein of the apoptosis inhibitor, which widely present in normal human tissues, but low or absent [32] in most cancer cells, which is located on chromosome 17p13.2 locus, adjacent to p53. Studies have shown that, XAF1 gene was down-regulated in a variety of malignancies, such as gastric cancer, non-small cell lung cancer, liver cancer and other tumors [33-35]. In recent years, researchers have found that XAF1 is a p53 target gene, wild-type p53 gene can down-regulate the expression of XAF1 mRNA, and accumulation of protein p53 in the nucleus leads to apoptosis, indicating that there is a feedback loop between the p53 and XAF1 genes [36]. TP53INP1, XAF1 and p53 are closely related, and therefore we believe that the apoptosis of MG-63 by siRNA-FANCD2 is mediated by the p53 signaling pathway.

TP53INP1 (upstream protein of p53), phosphorylation of p53 (phos-p53), p21 protein (downstream protein which is involved in regulation of G1 phase), activation of caspase-9 and caspase-3 (apoptosis related proteins) were detected by western blot methods. After siRNA-FANCD2 was transfected into MG-63 cells for 48 hours, TP53INP1, p53, p21, caspase-9 and caspase-3 mRNA expression were significantly elevated; and production of phos-p53, p21 and TP53INP1 proteins were also increased. The experimental results showed clearly the MG-63 cell apoptosis was mediated by p53 signaling pathway. After transfection of siRNA-FANCD2 into MG-63 cells, FANCD2 gene expression was suppressed, and TP53INP1 was activated in the transcription level. Overexpression of TP53INP1 promoted phosphorylation of the Ser15 site of p53 protein; the phosphorylated p53 activated p21, and then started the p53 apoptotic signaling pathway. p21 gene is at the downstream of the p53 gene, which is a cyclin dependent kinase inhibitor, whose interaction with p53 leads to cell cycle arrest. Interaction of activated p53 with p21 inhibits tumor cell proliferation, and keeps the tumor cells arrest in the G1 phase. In addition, phosphorylation of p53 mediates the mitochondrial pathway of apoptosis. It induces change of the mitochondrial permeability, and then cause release of a variety of pro-apoptotic factors into the cytoplasm, finally induces apoptosis in MG-63 cells.

p53 also can activate the caspase pathway, and the activation of caspase associated proteins would promote apoptosis. Caspase belongs to cysteine protease family, and plays a key role in the process of apoptosis [37]. In the caspase pathway, caspase 7 is firstly activated, then caspase 12, and the activated caspase 12 further cleaves caspase 9 and caspase 3, finally triggering apoptosis [38-40]. Caspase 3 is in the downstream of this process, which is the key enzyme of caspase family. It is widely expressed in various tumor tissues [41, 42]. This study showed that caspase 3 was cleaved and activated after RNAi. The activated caspase 3 finally induced the apoptosis.

In summary, after silence of the FANCD2 by siRNA-FANCD2 in MG-63 cells, inhibited cell proliferation, cell cycle arrest and induction of apoptosis were detected. The apoptosis was likely mediated by p53 signaling pathway. After FANCD2 expression was inhibited, TP53INP1

gene expression was promoted and then further enhanced phosphorylation of p53, thereby activated the p21 protein, leading to cell cycle arrest in G1, finally induced caspase-dependent cell apoptosis. It was suggested that FANCD2 plays an important role in the development of osteosarcoma, and inhibition of FANCD2 gene expression can effectively promote apoptosis of osteosarcoma cells, which providing new target for the treatment of osteosarcoma.

Acknowledgements

Disclosure of conflict of interest

None.

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