Role of Xeroderma Pigmentosum Group D in Cell Cycle and Apoptosis in Cutaneous Squamous Cell Carcinoma A431 Cells

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support:

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The study was supported by the Jiangxi Province Natural Science Fund Project (20142BAB205051)

Background: Cutaneous squamous cell carcinoma (cSCC) is the second most widespread cancer in humans and its incidence is rising. Novel therapy with better efficacy is needed for clinical treatment of cSCC. Many studies have shown the importance of DNA repair pathways during the development of cancer. A key nucleotide excision repair (NER) protein, xeroderma pigmentosum group D (XPD), is responsible for the excision of a large variety of bulky DNA lesions.

Material/Methods: To explore the role of XPD in A431 cells, we overexpressed XPD in A431 cells and performed MTT assay, flow cytometry, and Western blot analysis to examine cell proliferation, cell apoptosis, and genes expression.

Results: We found that the overexpression of XPD suppressed cell viability, induced cell cycle arrest at G1 phase, and promoted cell apoptosis. Additionally, XPD blocked the expression of c-myc, cdc25A, and cdk2, and improved the levels of HIPK2 and p53.

Conclusions: These results provide new evidence to reveal the role of XPD in cSCC A431 cells and suggest that XPD may serve as an anti-oncogene during cSCC development.

MeSH Keywords: Apoptosis • Carcinoma, Squamous Cell • Cell Cycle • Cell Proliferation

Abbreviations: **cSCC** – cutaneous squamous cell carcinoma; **HIPK2** – homeodomain-interacting protein kinase 2; **MTT** – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide; **NER** – nucleotide excision repair; **PBS** – phosphate-buffered saline; **PI** – propidium iodide; **XPD** – xeroderma pigmentosum group D

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Background

Cutaneous squamous cell carcinoma (cSCC) originates from stem/progenitor cells of the basal cell layer of the epidermis and cutaneous basal cell carcinoma [1], which is mostly common in skin epidermis or appendages [2]. cSCC is a serious threat to human health. Clinicians have developed some common treatments according to individual characteristics of patients, including radiotherapy, surgery, and chemotherapy [3]. However, these therapies have several drawbacks and limited treatment efficacy [4]. Therefore, novel therapy with better efficacy is needed for clinical treatment of cSCC.

DNA repair pathways play an essential role in cancer susceptibility by maintaining genomic integrity [5]. Studies have demonstrated that defects in DNA damage repair lead to accumulated DNA damage [6], which confers a higher risk of cSCC cancer [7]. As a research focus and problem in recent studies, molecular targeting treatment is a potential therapy for cSCC, but its efficacy largely depends on the selection of highly specific molecular targets [8]. The most important DNA damage repair mechanism in humans is nucleotide excision repair (NER). A key NER protein, Xeroderma pigmentosum group D (XPD), as a subunit of transcription factor IIH, has ATP-dependent 5'-3' helicase activity and is involved in gene transcription with the action of RNA polymerase II [9,10]. Some reports revealed that genetic polymorphisms of XPD in the nucleotide excision repair pathway affected the capacity for DNA repair to influence susceptibility to many cancers [6], such as hepatocellular carcinoma [11], esophageal cancer [6], and squamous cell carcinoma [12]. Also, ectopic expression of XPD was associated with the occurrence of angioderma pigmentosum and Cockayne's syndrome [13]. However, a few studies have explored the relationship between XPD and cSCC. Furthermore, the studies of XPD were mainly focused on the association between XPD polymorphisms and various cancers [14,15]. The mechanism by which XPD is involved in cancer development is unclear.

In the present study, we used cSCC A431 via the expression of XPD to investigate the role of XPD in cSCC development. We found that the overexpression of XPD repressed cell proliferation, blocked cell cycle, and enhanced cell apoptosis in A431 cells. Furthermore, ectopic expression of XPD regulated the expression of cell proliferation-related, cell cycle-related, and cell apoptosis-related genes. We conclude that XPD regulates expression of cell proliferation-related, cell cycle-related, and cell apoptosis-related genes to modulate the development of cSCC.

Material and Methods

Cell culture and transfections

Human cutaneous squamous cell carcinoma A431 cells were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A431 cells were seeded into 6-well plates incubated with Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) and incubated in a 5% CO_2 cell culture incubator at 37°C for 48 h. When the density reached 90%, cell transfection was carried out with Lipofectamine 2000 (Invitrogen, USA). Cells were divided into 4 groups and the control group (Control group) was administered the same amount of medium. The other 3 groups were transfected with only Lipofectamine (LF group), pEGFP-N2 (empty vector) + Lipofectamine (EM+LF group), or pEGFP-N2-XPD + Lipofectamine (XPD+LF group) in FBS-free medium. After 6 h, the old media were removed and cells were incubated with fresh DMEM containing 10% FBS for 40 h for further experiments.

MTT assay for cell proliferation

After cell transfection, A431 cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetrazolium bromide (MTT, 5 mg/mL) with fresh culture medium at the concentration of 100 µg/well for 4 h. After removing the supernatant, formazan crystal formed were dissolved in 100 µL of dimethyl sulfoxide and the absorbance was measured at 492 nm using a multifunction microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Six-well replication was used to calculate the cell relative viability. Data were analyzed from 3 independent experiments.

Determination of cell apoptosis by flow cytometry with Annexin V-FITC/PI reagent

After different treatments, A431 cells were digested with trypsin (Sigma, USA) and washed with phosphate-buffered saline (PBS) twice. Then, cells were incubated with 100 µL of binding buffer and 10 µL of FITC-labeled Annexin V. After being incubated at room temperature for 5 min in the dark, cells were analyzed by fluorescence-activated cell sorting (FACS) by flow cytometry (BD FACSCalibur, USA). Cells that stained positive for early apoptosis markers (annexin V-FITC stained only) and for late apoptosis markers (annexin V-FITC and PI stained) were combined for analysis.

Cell cycle distribution measured using flow cytometry assay

To analyze the cell cycle distribution, the transfected A431 cells were digested with trypsin and fixed with 70% cold

Table 1. The primers for qRT-PCR.

Genes	Forward primers(5'-3')	Reverse primers (5'-3')
XPD	gcccgctctggattatacg	ctatcatctcctggccccc
c-myc	aacccttgccgcatccac	cctcctcgtcgcagtagaaa
cdc25A	tgatgaggatgatggcttcg	ctgcacccttgat-gtggc
cdK2	actggcattcctcttccc	ctggcttggtcacatcctg
p53	ctacaagcagtcacagcacatga	tcattcagctctcggaacatctcg
HIPK2	cctaccttacgagcagaccatc	acgcttgagtccacatttttg
β-actin	gggcacgaaggctcatcatt	agcgagcatcccccaaagtt

ethanol overnight at 4°C. After being washed with PBS twice, the cells were subsequently stained with propidium iodide (PI, 50 μg/mL) and RNase A (100 μg/mL). One hour later, cells were subjected to flow cytometry analysis with flow cytometry (BD FACSCalibur, USA).

Quantitative real-time PCR (qRT-PCR)

After cell transfection, cells were collected and total RNA was isolated with an RNA Extraction Kit (Cwbiotech, Beijing, China). The quality and quantity of RNA were detected by measuring the absorbance at 260 nm and 280 nm using a Nano Drop-2000 ultramicrospectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). One μg of RNA was reverse transcribed into cDNA, and qRT-PCR was carried out with SYBR Green qPCR SuperMix (Invitrogen, USA) with the Real-Time PCR system (Bio-Rad, USA). The relative expression of genes was normalized to β-actin with the method of $2^{-\Delta\Delta CT}$. The primers for qRT-PCR are listed in Table 1.

Western blot

Proteins were extracted by using RIPA lysis buffer (Santa Cruz Biotechnology, USA) and centrifuged at 10 000 rpm at 4°C for 20 min. The concentration of protein was detected by bicinchoninic acid kit (Pierce, Germany) according to the manufacturer's protocol. Equal amounts of protein were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After being blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST), the membrane was incubated with primary anti-c-myc mouse monoclonal antibody (D199941, 1: 5000 dilution), anti-cdc25A rabbit polyclonal antibody (D120394, 1: 1000 dilution), anti-cdk2 mouse monoclonal antibody (D199431, 1: 1000 dilution), anti-HIPK2 (D161742, 1: 1000 dilution), anti-p53 rabbit polyclonal antibody (D220082, 1: 1000 dilution), or anti-XPD mouse monoclonal antibody (D198536, 1: 5000 dilution) (Sangon, Beijing, China) at 4°C overnight. The next morning, the membrane was probed with the corresponding secondary antibody at room temperature for 1 h. After being washed

with TBST 3 times, the blot was developed with electrochemiluminescence solution (Pierce, Germany). The target bands were normalized to β-actin using Quantity One software v4.1 (Bio-Rad, USA).

Statistical analysis

Statistical analysis was conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All experiments were carried out at least 3 times using independent samples. All analytical data are expressed in mean values ± standard deviation, and differences were analyzed using the *t* test. *P*<0.05 was considered to indicate a statistically significant difference.

Results

The overexpression of XPD suppressed cell proliferation.

Firstly, we transfected A431 cells with recombined vector targeting XPD to explore the role of XPD. The transfection efficiency in A431 cells was examined by the signals of green fluorescence intensity. In Figure 1A, in pEGF-N2-transfected (EM+LF) or pEGF-N2-XPD-transfected (XPD+LF) groups, more than 70% of total cells were green. However, no signal was observed in the control group (Control) or Lipofectamine only transfected group (LF group). Figure 1B and 1C show there was no marked difference among Control, LF, and EM+LF groups; however, the mRNA and protein levels of XPD in the XPD+LF group were significantly enhanced compared to those in the EM+LF group (*P*<0.01). These data show that the recombined pEGF-N2-XPD was successfully overexpressed in A431 cells for further experiments.

To examine the impact of XPD overexpression on cell proliferation, we transfected cells with pEGF-N2-XPD and performed MTT assay. In Figure 1D, there was no significant difference among Control, LF, and EM+LF groups. Compared with the EM+LF group, cell viability in the XPD+LF group was markedly suppressed (*P*<0.01). These results reveal that XPD obviously repressed cell proliferation in A431 cells.

XPD induced cell cycle arrest in G1 phase

To explore the role of XPD on cell cycle, we incubated cells with pEGF-N2-XPD and examined cell cycle distribution by PI staining and flow cytometry. There was no significant difference in the percentages of A431 cells at G1, S, or G2 phases among the Control group, LF group, or EM+LF group (*P*>0.05, Figure 2A–2C), suggesting that LF only had no significant effect on cell cycle. However, compared with the EM+LF group, the percentages in the XPD+LF group at G1 phase were markedly increased, and the percentages at S phase were significantly
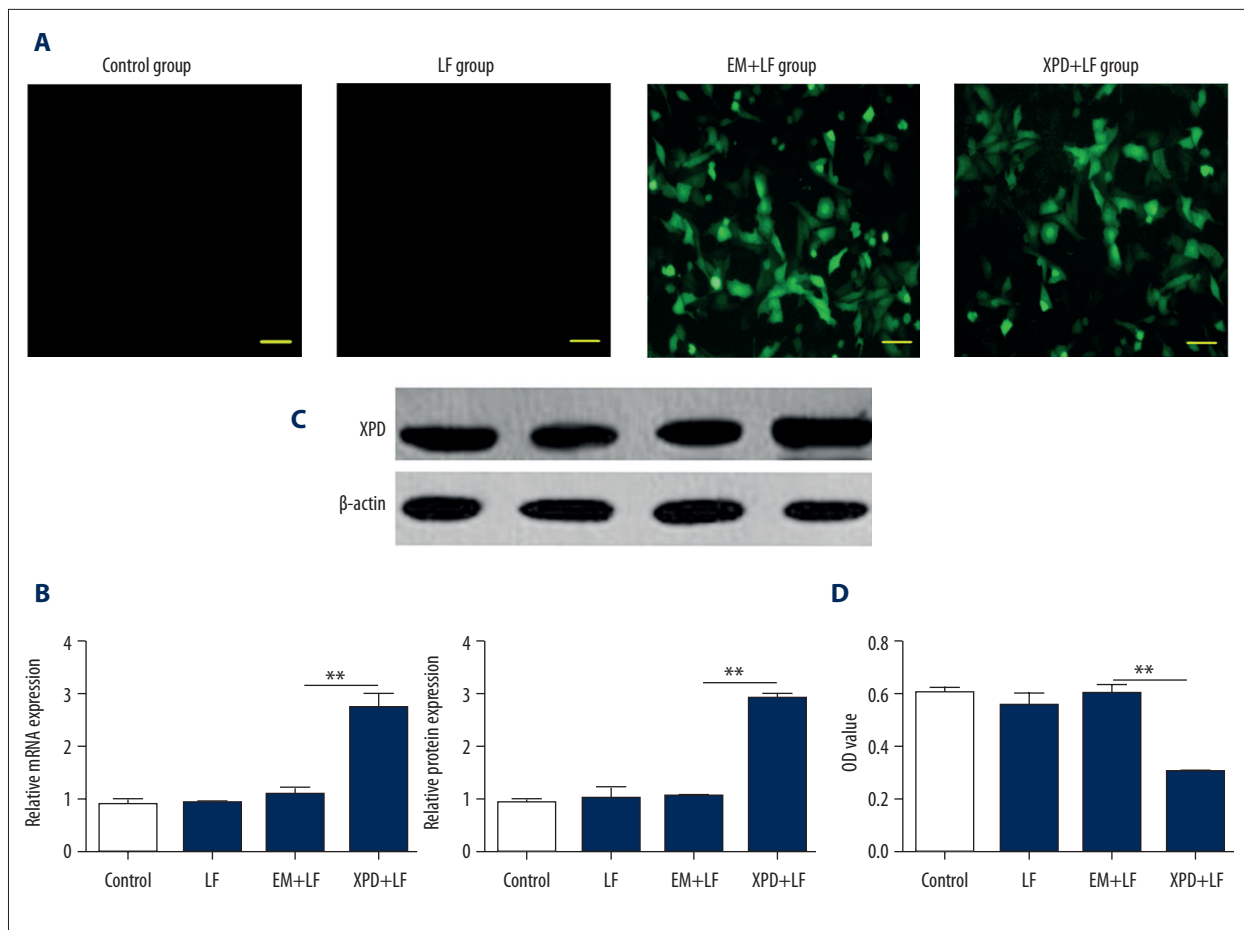
Figure 1. XPD repressed cell proliferation. A431 cells were divided into 4 groups, and the control group was administered the same amount of medium (Control group). The other 3 groups were transfected with Lipofectamine (LF group), pEGFP-N2 (empty vector) + Lipofectamine (EM+LF group), or pEGFP-N2-XPD + Lipofectamine (XPD+LF group). (**A**) The signals of XPD were detected after pEGFP-N2-XPD transfection. After cell transfection, A431 cells were observed with an inverted fluorescence microscope. Scale bar=100 μm. (**B**) The mRNA level of XPD was increased in the XPD+LF group. Total RNA was isolated from 4 groups for QRT-PCR analysis. * $P<0.05$, ** $P<0.01$. (**C**) The protein level of XPD was enhanced in the XPD+LF group. After cell transfection, proteins were extracted for Western blot analysis. * $P<0.05$, ** $P<0.01$. (**D**) The overexpression of XPD blocked cell proliferation. After cell transfection, cell proliferation was examined by MTT assay. * $P<0.05$, ** $P<0.01$.

decreased (Figure 2D), indicating that overexpression of XPD led to the G1 arrest of A431 cells. Table 2 shows the percentages of A431 cells in G1, S, and G2 phases from different groups as detected by flow cytometry. These data reveal that XPD induced cell cycle arrest at G1 phase in A431 cells.

XPD enhanced cell apoptosis

We analyzed cell apoptosis in A431 cells after pEGF-N2-XPD transfection to detect the effect of XPD on cell apoptosis. As accessed by flow cytometry with Annexin V-FITC/PI reagent, very few cells were apoptotic in Control, LF, and EM+LF groups (Figure 3A, 3B). However, the cell apoptosis in the XPD+LF group was nearly 4-fold greater than that in the EM+LF group ($P<0.01$, Figure 3C, 3D). These data indicate that the overexpression of XPD promotes cell apoptosis in A431 cells.

XPD regulated relates genes expression for cell proliferation and cell apoptosis

To determine how XPD modulates cell proliferation, cycle, and apoptosis in A431 cells, we detected the effect of XPD overexpression on cell proliferation-, cell cycle-, and cell apoptosis-related genes expression. In Figure 4A, no significant differences were observed on the mRNA expression of c-myc, cdc25A, cdk2, homeodomain-interacting protein kinase 2 (HIPK2), p53, and XPD among Control, LF, and EM+LF groups ($P>0.05$). However, the overexpression of XPD suppressed the mRNA levels of c-myc, cdc25A, and cdk2, but promoted the expression of HIPK2, p53, and XPD. A similar trend in the protein levels is shown in Figure 4B and 4C. Taken together, XPD monitored the expression of cell proliferation-related, cell cycle-related, and cell apoptosis-related genes.
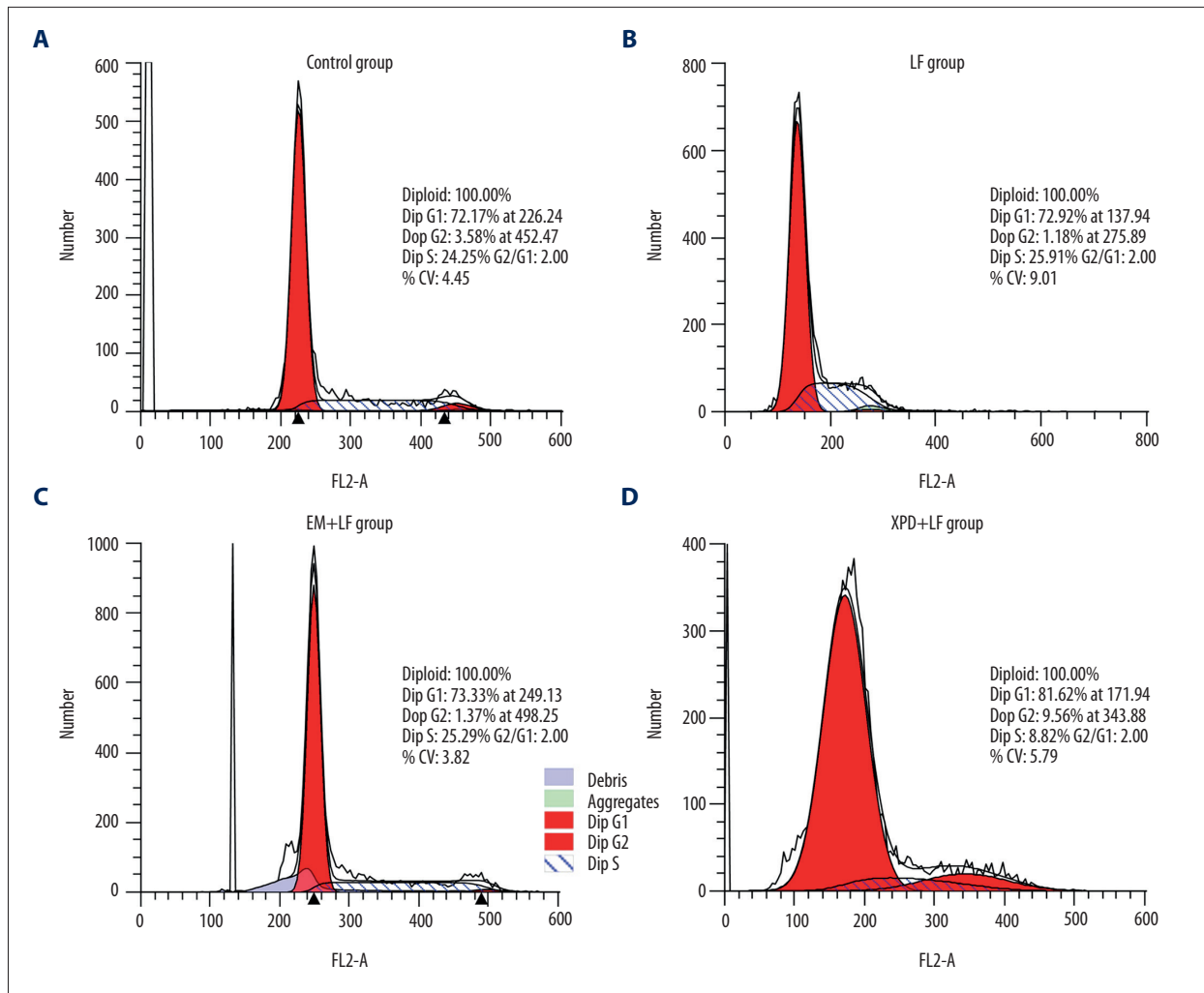
Figure 2. XPD induced cell cycle arrest in G1 phase. A431 cells were transfected with the same amount of medium (Control, **A**), Lipofectamine (LF, **B**), pEGFP-N2 (EM) + Lipofectamine (EM+LF group, **C**), or pEGFP-N2-XPD + Lipofectamine (XPD+LF group, **D**). Cell cycle distribution was measured using flow cytometry assay.

Table 2. Percentage of A431 cells in each cell cycle phase detected by flow cytometry.

Groups	G1	S	G2
Control	72.17±3.42	24.25±4.51	3.58±2.42
LF	73.00±4.61	26.92±5.48	0.08±1.26
EM+LF	73.83±3.63	26.04±4.97	0.14±1.76
XPD+LF	81.62±5.75*	8.82±4.58**	9.56±2.1**

Compared with EM+LF group, * $P<0.05$, ** $P<0.01$.

Discussion

cSCC is the second most widespread metastatic skin cancer, with increasing incidence, which has a more invasive growth pattern and higher potential to metastasize [16]. Although great research efforts have been made, the outcome of cSCC remains poor because of a lack of knowledge regarding molecular markers. Some evidence indicates the importance of DNA repair pathways in cancer genetic processes, such as in prostate cancer [17]. Indeed, deficient DNA repair may lead to deregulation of cell growth, imbalance of cell cycle control, and development of many diseases, including cancers [18]. The highly conserved NER pathway is responsible for the excision of a large variety of bulky DNA lesions [19]. XPD, encoded by the ERCC2 gene, is an ATP-dependent helicase enzyme involved in the NER pathway [20]. In our study, we found that the overexpression of XPD in A431 cells plays an important role in the progression of cSCC through the cell cycle, cell proliferation, and cell apoptosis pathways. XPD may potentially act as an anti-oncogene in cSCC and have clinical value as a prognostic marker for SCC.
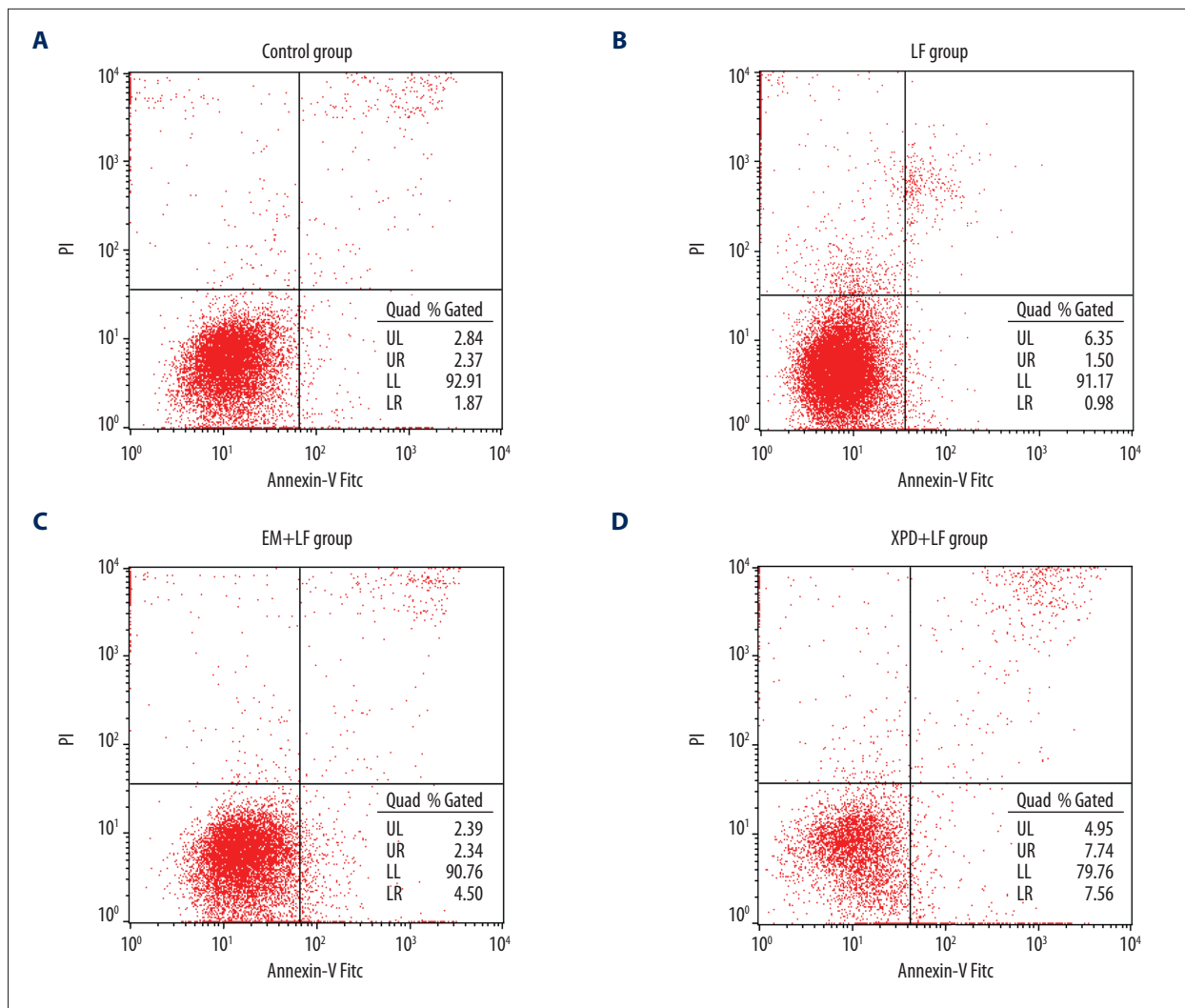
Figure 3. The overexpression of XPD promoted cell apoptosis. A431 cells were transfected with the same amount of medium (Control, **A**), Lipofectamine (LF, **B**), pEGFP-N2 (EM) + Lipofectamine (EM+LF, **C**), or pEGFP-N2-XPD + Lipofectamine (XPD+LF, **D**). Cells that stained positive for early apoptosis markers (annexin V-FITC stained only) and for late apoptosis markers (annexin V-FITC and PI stained) were combined for analysis.

In our subjects, we found that the overexpression of XPD was involved in cell proliferation, cell cycle, and cell apoptosis. C-myc is the master transcription factor for cell proliferation and is involved in numerous hematological and solid cancers [21]. In hepatoma cells, XPD functions in cell proliferation and apoptosis by suppressing the expression of c-myc and cdk2 [22]. The mutation of XPD affected proper regulation of c-myc expression and is involved in the development of malignancy [23]. Combined with our results, we conclude that XPD participates in cell proliferation via monitoring the expression of c-myc. In normal cells, progression through G1, S, and G2 phases of the cell cycle is dependent on temporal activation of cyclin-dependent kinases CDK1/2. The subsequent dephosphorylation and activation of CDK1/2 is mediated by 1 of 3 CDC25 phosphatases (A, B, or C) [24]. CDC25A regulation is complex, with input from multiple other kinases, resulting in either degradation or sequestration, depending on cell cycle phase [25]. CDK2 [26] and CDC25A [27] are important regulators of the G1/S phase transition. CDK2 is usually considered essential for progression through S phase [28]. Some reports indicated that overexpression of XPD leads to cell cycle arrest at G1 phase via suppression of CDK2 and CDC25A, which contributes to cell growth inhibition [29]. In our study, we observed that the overexpression of XPD increased the percentages of A431 cells at G1 phase and suppressed the mRNA and protein levels of CDK2 and CDC25A, which is in agreement with previous studies. In A431 cells, the overexpression of XPD induced cell cycle arrest at G1 phase by the blockage of CDK2 and CDC25A.
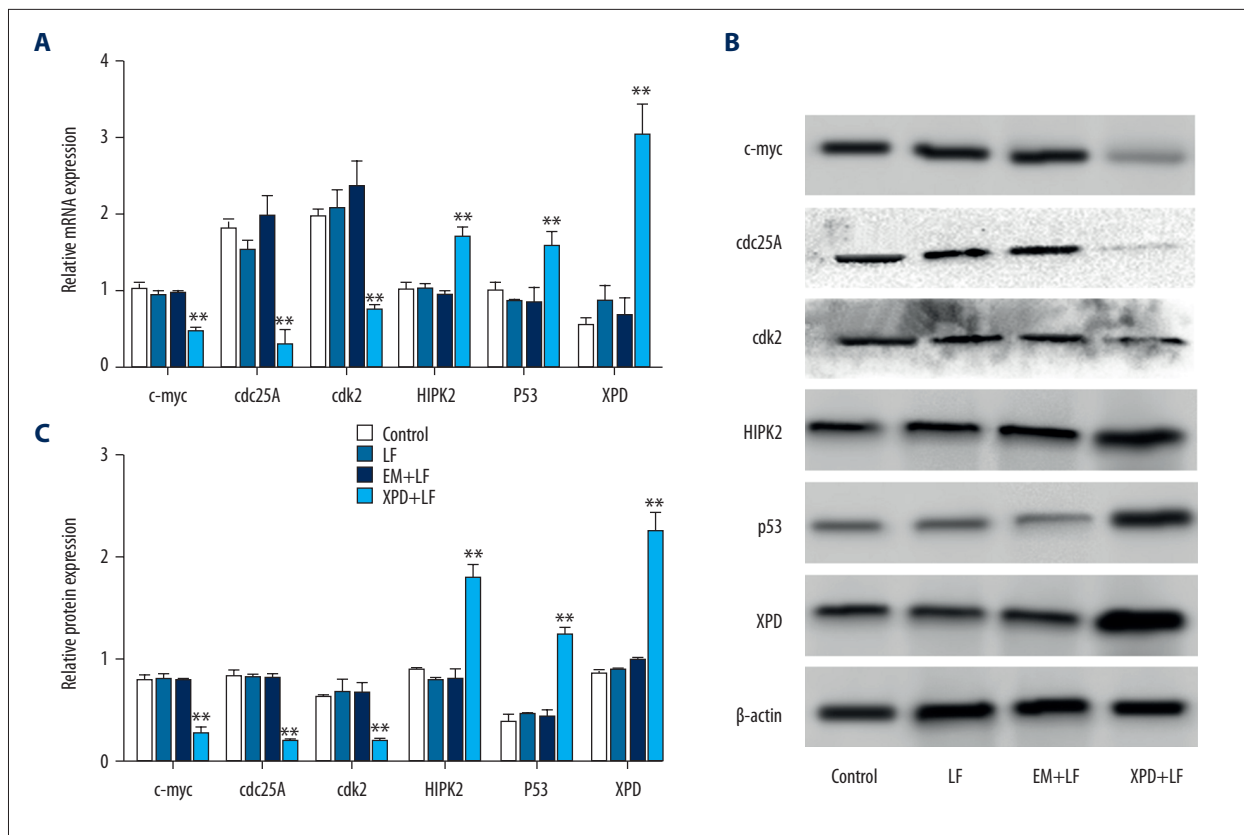
Figure 4. XPD monitored the related genes expression. A431 cells were transfected with the same amount of medium (Control), Lipofectamine (LF), pEGFP-N2 (EM) + Lipofectamine (EM+LF), or pEGFP-N2-XPD + Lipofectamine (XPD+LF). (**A**) The overexpression of XPD affected the mRNA levels of related genes for cell proliferation and cell apoptosis. After cell transfection, cells were collected and total RNA was isolated for qRT-PCR. (**B**) The effect of XPD overexpression on related proteins expression was analyzed by Western blot. (**C**) The target bands were normalized to β-actin using Quantity One software. * $P<0.05$, ** $P<0.01$.

HIPK2 is a member of the nuclear Ser/Thr kinase family, originally identified as a DNA damage-responsive cell fate regulator, which is negatively regulated by oncogenic signaling [30]. HIPK2 phosphorylates and activates the apoptotic program through interaction with diverse downstream targets [31]. HIPK2 activates tumor suppressor p53-dependent transcription and apoptosis by phosphorylating p53 at Ser46 [32]. P53 is stabilized, binds to specific target gene promoters, and regulates gene sets for cell fate towards apoptosis or DNA repairs [33]. In our study, the high expression of XPD significantly improved cell apoptosis and enhanced the expression of HIPK2 and p53. In hepatoma cells, XPD functions in cell apoptosis by inducing the expression of p53 [22]. A previous study reported that XPD interacts with p53 to regulate cell apoptosis [34] and the p53/HIPK2 connection by showing the role of HIPK2 in repressing activity in severe DNA damage response [35]. Our data were in agreement with a previous study reporting that XPD was involved in cell apoptosis by regulating the expression of HIPK2 and p53.

Conclusions

Taken together, XPD repressed cell proliferation by arresting cell cycle in G1 phase and induced cell apoptosis by suppressing the levels of c-myc, cdc25A, and cdk2 and enhancing the expression of HIPK2 and p53. These data provide new evidence that XPD serves as an anti-oncogene in cSCC. However, there also exist some limitations in our study. First, our study was mainly focused on the role of XPD in A431 cells *in vitro*. Further examinations need to be performed on the role of XPD *in vivo*, which would more comprehensively explain the function of XPD in cSSC. Second, in our study, we actually found that XPD affected the expression of c-myc, cdc25A, cdk2, HIPK2, and p53; however, the molecular mechanism of XPD regulating expression of these genes was unclear. Thus, more efforts need to be made for molecular basis exploration on gene regulation.

Conflicts of interest

None.

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