

Cloning of the XPD gene and its function in malignant melanoma cells

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Abstract. The xeroderma pigmentosum group D (XPD) gene is a member of the transcription factor IIIH complex and serves an important role in gene repair. Previous studies have suggested that genetic variants of the XPD gene may be associated with an increased risk of cutaneous melanoma. However, the exact mechanism remains unclear. In the present study, the XPD gene was cloned, and its localization and function in malignant melanoma cells were investigated. The human full length XPD gene was cloned via reverse transcription-PCR using the total RNA extracted from human cervical squamous cell carcinoma epithelial HeLa cells. Subsequently, the gene was inserted into a plasmid fused to green fluorescent protein (GFP; pEGFP-N1/XPD), and pEGFP-N1/XPD and pcDNA3.1(+)/XPD were transfected into human malignant melanoma A375 cells using Lipofectamine[®] 2000. The expression levels of XPD were detected by western blotting. The Golgi marker GM130 and the endoplasmic reticulum membrane protein marker KDEL were used for immunofluorescence staining, and the subcellular localization of XPD was observed under a fluorescence microscope. Cell proliferation was measured using an MTT assay. The recombinant pEGFP-N1/XPD plasmid expressing the human wild-type XPD gene was successfully constructed by restriction enzyme digestion and assessed by gene sequencing. XPD was localized in the endoplasmic reticulum of malignant melanoma A375 cells, as confirmed by immunofluorescence staining. Furthermore, MTT assays indicated that XPD inhibited the proliferation of malignant melanoma A375 cells. The present study provides a basis for further investigation of the biological effects and functions of XPD in malignant melanoma cells.

Introduction

UV radiation, smoking and various other environmental factors can cause DNA damage (1). In the human body, DNA damage can be corrected by direct repair, base excision repair and nucleotide excision repair (NER) to maintain genome stability. Among these, the NER system is the primary repair method (1). If DNA damage is not removed or repaired in time, it can lead to various diseases, such as cancer and neurodegenerative diseases, Parkinson's disease, Alzheimer's disease, Huntington's disease, aging (2). Xeroderma pigmentosum group D (XPD) is a major protein involved in NER. As an important DNA repair gene, it serves an essential role in transcription factor IIIH (TFIIH) protein complex-mediated NER and its transcription process. Mutations in the human embryonic XPD gene can result in xeroderma pigmentosum, Cockayne syndrome and fur dystrophy syndrome (3). The risk of skin cancer in individuals with xeroderma pigmentosum is 1,000 times higher than that in normal individuals. From a molecular basis, xeroderma pigmentosum is a birth defect of NER in which UVB-associated DNA damage cannot be repaired (4). Previous studies have revealed associations between XPD gene polymorphisms and prostate cancer, lung cancer, bladder cancer, basal cell carcinoma and malignant melanoma (5-8). Investigations into the molecular mechanism of the susceptibility to cutaneous melanoma has revealed that mutated XPD is a biomarker of skin malignant melanoma (2). However, to the best of our knowledge, the role of the XPD gene and its protein product in malignant melanoma cells has not yet been reported.

In the present study, the XPD gene was cloned and the recombinant pEGFP-N1/XPD and pcDNA3.1(+)/XPD expression plasmids were constructed and transfected into human malignant melanoma cells. By observing XPD expression, its intracellular localization and its effects on cellular proliferation, the biological effects of XPD on malignant melanoma cells were analyzed.

Materials and methods

Reagents and antibodies. Human cervical squamous cell carcinoma epithelial HeLa cells and malignant melanoma A375 cells were purchased from The Cell Bank of Type

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Culture Collection of the Chinese Academy of Sciences. pEGFP-N1 and pcDNA3.1(+) plasmids were obtained from Clontech Laboratories, Inc. and Invitrogen (Thermo Fisher Scientific, Inc.), respectively. Restriction enzymes, RNAiso extraction reagent, reverse transcription reagent part of the PrimeScript™ RT reagent and PrimeSTAR high fidelity enzyme were purchased from Takara Bio, Inc. Lipofectamine® 2000 was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). T4 DNA ligase and the DNA gel recovery and plasmid extraction kits were obtained from Promega Corporation. The XPD antibody was ordered from Abcam (cat. no. ab102682), while GM130 (cat. no. sc-55590) and KDEL antibodies (cat. no. sc-58774) were obtained from Santa Cruz Biotechnology, Inc. Goat anti-rabbit IgG-HRP-labeled (cat. no. 4030-05), goat anti-rabbit IgG-FITC-labeled (cat. no. sc-2012) and goat anti-mouse IgG-TRITC-labeled (cat. no. 4030-03) antibodies were provided by OriGene Technologies, Inc. DMEM and FBS were purchased from Gibco (Thermo Fisher Scientific, Inc.). RIPA buffer (cat. no. P0013C) was purchased from (Beyotime, Institute of Biotechnology). Primers were synthesized by Shanghai Shengong Biology Engineering Technology Service, Ltd., and the enhanced chemiluminescence (ECL) reagent was obtained from Merck KGaA.

Construction of the pEGFP-N1/XPD recombinant plasmid. HeLa cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin) at 37°C (5% CO₂) for 2 weeks. Total RNA was extracted using the RNAiso reagent and reverse transcribed at 37°C for 15 min into cDNA. The primers were designed according to the human XPD intact mRNA sequence published on GenBank (NM_001130867) (9), and the restriction endonuclease sites of *Hind*III and *Sal*I were added to the 5'-ends of the forward and reverse primers, respectively. The primer sequences were as follows: External nest primer XPD2-F1, 5'-TCAACGTGGACGGGCTCCTGGTCTA-3' and XPD2-R1, 5'-TATTTGGCTGCATCTTTGCTACTGG-3'; nested primer XPD2-F2, 5'-CCC AAGCTTATGCGGGAGCTCAAACGCACGCTG-3' (*Hind*III restriction site underlined) and XPD2-R2, 5'-GCGTCTGACTGGGATGAGATCTTTTTGGTTCCTG-3' (*Sal*I restriction site underlined). The reaction system of the PrimeSTAR enzyme was configured according to the manufacturer's protocol, and the target gene was amplified by nested PCR using the thermocycling conditions of one cycle at 55°C for 15 sec followed by 38 cycles at 72°C for 90 sec. The pEGFP-N1 empty vector and the purified PCR product were double-digested with *Hind*III and *Sal*I sequentially. After purification and recovery of the digestion products by DNA gel recovery kit, the XPD gene fragment and the pEGFP-N1 vector fragment were ligated at a ratio of 5:1 in the presence of T4 DNA ligase, at 16°C for 12 h. The ligation product was transformed into competent *Escherichia coli* DH5α cells (cat. no. RR420A; Takara Bio Inc.) and cultured overnight on LB solid medium containing a final concentration of 30 μg/ml kanamycin. The resulting colonies were individually selected, added to Luria-Bertani medium (cat. no. CM0007; Beijing Leagene Biotech Co., Ltd.) and shaken at 37°C for 16 h; the recombinant plasmid was extracted using a plasmid extraction kit and identified by *Bgl*II digestion. The positive plasmid was sequenced by Shanghai Shengong Biology Engineering Technology Service, Ltd.

Liposome-mediated transfection. The constructed recombinant pEGFP-N1/XPD expression vector was validated by DNA sequencing and subsequently transfected (1 μg/ml) into malignant melanoma A375 cells which were cultured in DMEM supplemented with 10% FBS at 37°C in a 5% CO₂ incubator) using Lipofectamine® 2000.

Western blot analysis. Total protein of the A375 cells was extracted using the RIPA buffer. The protein content was measured using the bicinchoninic acid assay (Beyotime Institute of Biotechnology). Equal amounts of protein (40 μg) were separated via 12% SDS-PAGE, and the separated proteins were subsequently transferred onto a nitrocellulose membrane. Membranes were blocked with 5% milk in TBST. Next, the membrane was incubated with the XPD (dilution, 1:500; cat. no. ab102682; Abcam) and GAPDH (dilution, 1:3,000; cat. no. 60004-1-Ig; Proteintech Group, Inc.) primary antibodies and shaken for 2 h at room temperature. After washing 3 times with TBST (TBS containing 0.1% Tween-20), the membrane was incubated with a horse radish peroxidase goat anti-rabbit (IgG) secondary antibody (1:10,000; cat. no. ab6721; Abcam) and shaken for 2 h at room temperature. After washing 3 times with TBST, protein bands were visualized using an ECL reagent. GAPDH was used as the protein loading control. The experiment was repeated three times.

Immunofluorescence staining. The XPD2-F2 and XPD2-stop-R2 primers (5'-GCGTCTGACTTAGGGATGATCTTTTTGGTTC-3'; underlined for *Sal*I restriction site) were used to observe the localization of the XPD gene in A375 cells. The eukaryotic expression plasmid pcDNA3.1(+)/XPD was constructed by amplification, digestion, ligation and transformation, as aforementioned, and subsequently transfected (1 μg/ml) into A375 cells for 48 h using Lipofectamine® 2000. The Golgi membrane protein marker GM130 and the endoplasmic reticulum membrane protein marker KDEL were used for labeling, and the location of the gene was detected by immunofluorescence staining. Briefly, cells were fixed with freshly prepared 4% paraformaldehyde for 10 min at room temperature. The cells were washed with PBS thrice for 5 min each time and then permeabilized with 0.2-0.5% Triton-100 (prepared in PBS) for 10 min at room temperature. Next, the cells were washed again thrice in PBS and subsequently blocked with 2% BSA for 30 min at room temperature. Following blocking the cells were incubated with primary antibodies against GM130 (1:200) and KDEL (1:200) for 1 h at room temperature. The cells were again washed thrice with PBS and then incubated with secondary antibody (1:500) for 1 h at room temperature. Cells were visualized using a fluorescence microscope (magnification, x400).

MTT assay. Following pcDNA3.1(+) and pcDNA3.1(+)/XPD transfection into A375 cells for 24, 48 and 72 h, 5x10³ cells/well were seeded into 96-well plates. After 3 days of cell culture, 20 μl MTT (5 mg/ml) was added to each well for 4 h. The culture supernatant was subsequently discarded, and 150 μl triple solution of dimethyl sulfoxide was added to each well and shaken for 10 min to fully dissolve the purple formazan crystals. The light absorption value of each well was measured at a wavelength of 490 nm.

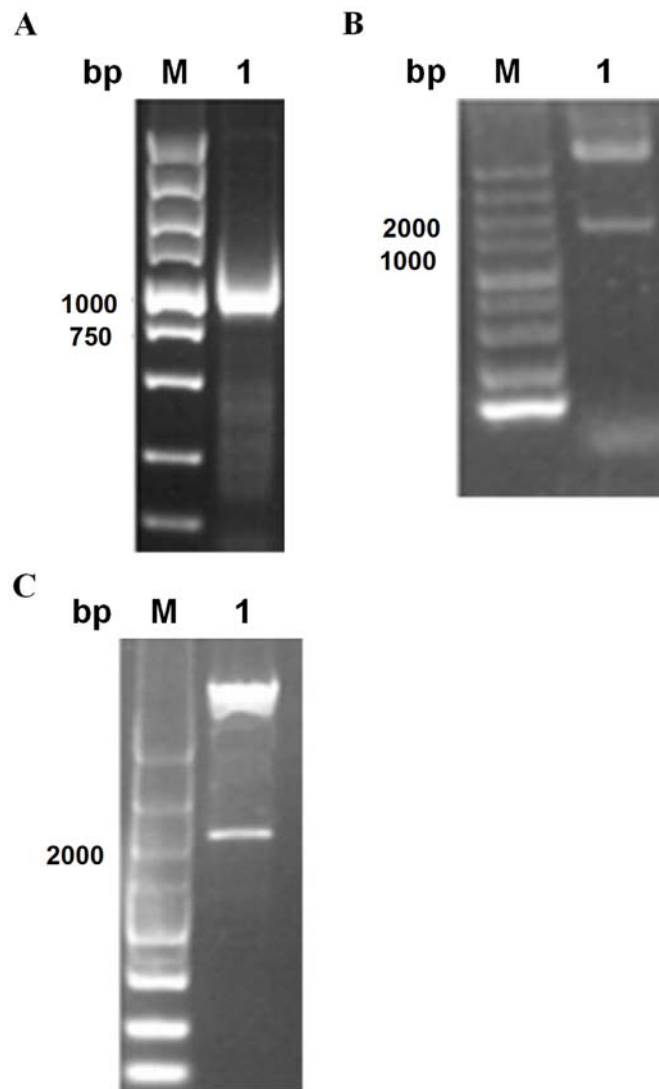


Figure 1. Cloning and identification of the XPD gene. (A) Following reverse transcription from total RNA extracted from HeLa cells, nested PCR was used to obtain a 1,234 bp product (M: DNA ladder; 1: HeLa cells PCR product). (B) *Bgl*II single digestion resulted in a 4,737 and 1,200 bp product (M: DNA ladder; 1: *Bgl*II single digestion product). (C) pcDNA3.1(+)/XPD was obtained and identified by *Hind*III and *Xba*I digestion. XPD, xeroderma pigmentosum group D. (M: DNA ladder; 1: *Hind*III and *Sal*I digestion products).

Statistical analysis. Each experiment was repeated three times and the data are presented as the mean \pm SD. Statistical analyses were conducted using SPSS v17.0 (SPSS, Inc.). A Student's unpaired t-test was used to compare differences between two groups, such as pcDNA3.1(+) 48 h and pcDNA3.1(+)/XPD 48 h groups, pcDNA3.1(+) 72 h and pcDNA3.1(+)/XPD 72 h groups. One-way ANOVA was used to compare differences among multiple groups, such as the DMEM, pcDNA3.1(+) and pcDNA3.1(+)/XPD groups. ANOVA was followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cloning and identification of the XPD gene. Following the reverse transcription of HeLa cell total RNA, a nested PCR method was used to obtain the expected 1,234 bp band (Fig. 1A). The PCR amplification products of pEGFP-N1 and XPD cDNA were digested using *Hind*III and *Sal*I, respectively. After gel

recovery, ligation, transformation and plasmid extraction, enzymatic digestion was performed. Due to the presence of the *Bgl*II restriction site at the multiple cloning site of pEGFP-N1 and the 3'-end of XPD, *Bgl*II single digestion resulted in 4,737 and 1,200 bp products, as shown in Fig. 1B. The eukaryotic expression vector pcDNA3.1(+) was digested with *Hind*III and *Xho*I, while the PCR products were digested with *Hind*III and *Sal*I. The digestion products were subsequently ligated using *Sal*I and *Xho*I sites (per the principle of homologous enzymes) to obtain the pcDNA3.1(+)/XPD plasmid. Following identification with *Hind*III and *Xba*I, the present results were consistent with the expectations (Fig. 1C). Additionally, the obtained sequencing results (Fig. 2) were consistent with the human XPD sequence reported on GenBank.

Recombinant plasmid transfection into A375 cells. The negative control (PBS), pEGFP-N1 and pEGFP-N1/XPD recombinant plasmids were transfected into malignant melanoma A375 cells using Lipofectamine® 2000. GFP expression was observed

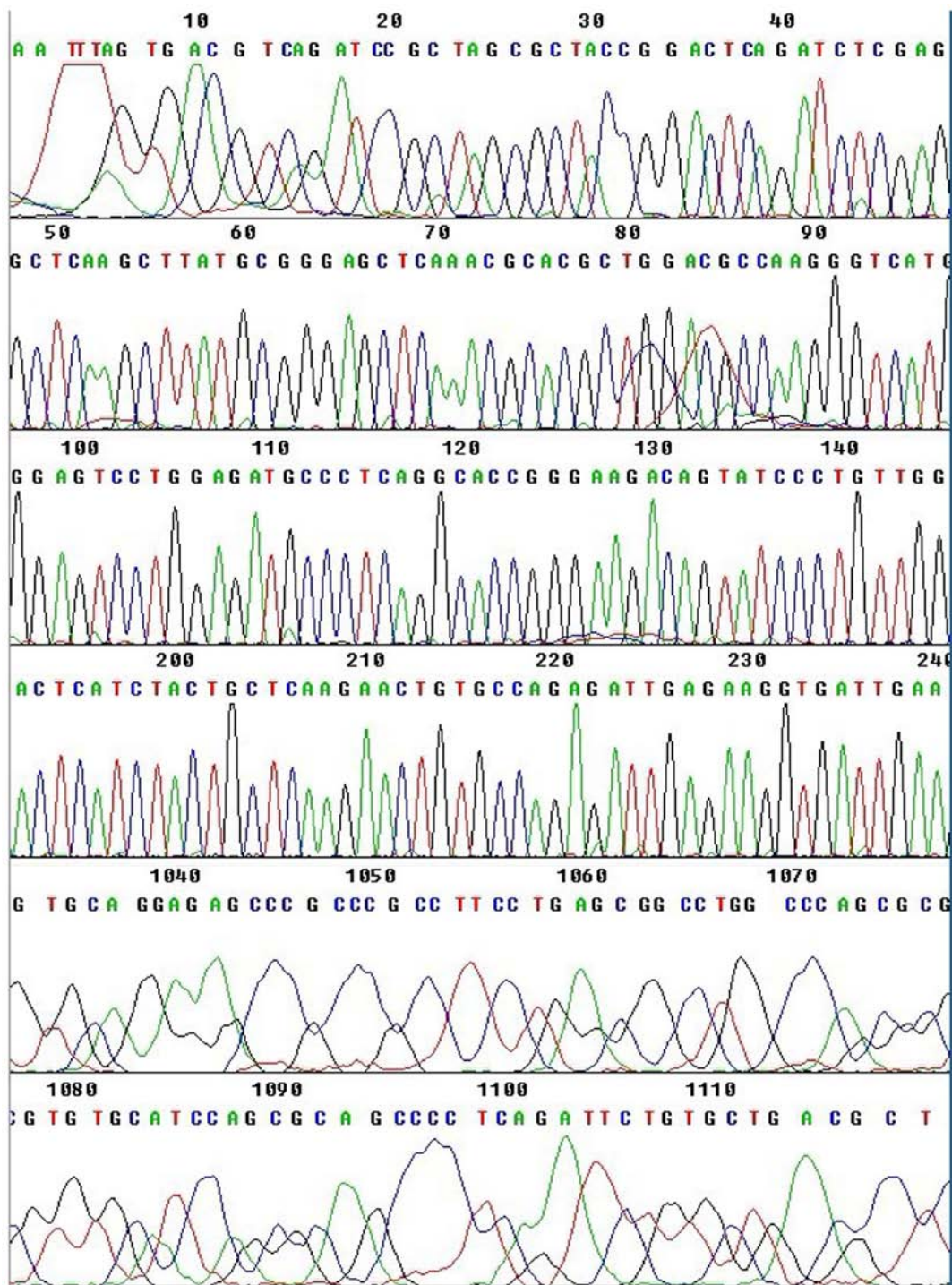


Figure 2. Recombinant plasmid sequencing results, consistent with the human xeroderma pigmentosum group D sequence on GenBank.

under a fluorescence microscope. The results revealed fluorescence in both the recombinant pEGFP-N1/XPD- and the empty pEGFP-N1 plasmid-transfected cells, while the negative control group exhibited no fluorescence, which proved that the recombinant plasmid was successfully transfected into A375 cells (Fig. 3).

Detection of XPD protein expression. Following transfection of pEGFP-N1/XPD and pcDNA3.1(+)/XPD into A375 cells, XPD expression was analyzed via western blot analysis. The results revealed that XPD was expressed in the

transfection groups (Fig. 4). The 46 kDa band corresponds to pcDNA3.1(+)/XPD, the 73 kDa band corresponds to pEGFP-N1/XPD. As pEGFP-N1/XPD has green fluorescent protein, it can present as a 73 kDa band which is larger than pcDNA3.1(+)/XPD.

Localization of XPD to the endoplasmic reticulum. Since pEGFP-N1/XPD-transfected A375 cells exhibited a clustered distribution of XPD-EGFP (as indicated by fluorescence microscopy), the subcellular location of XPD was analyzed. Using the Golgi membrane protein marker GM130 and the

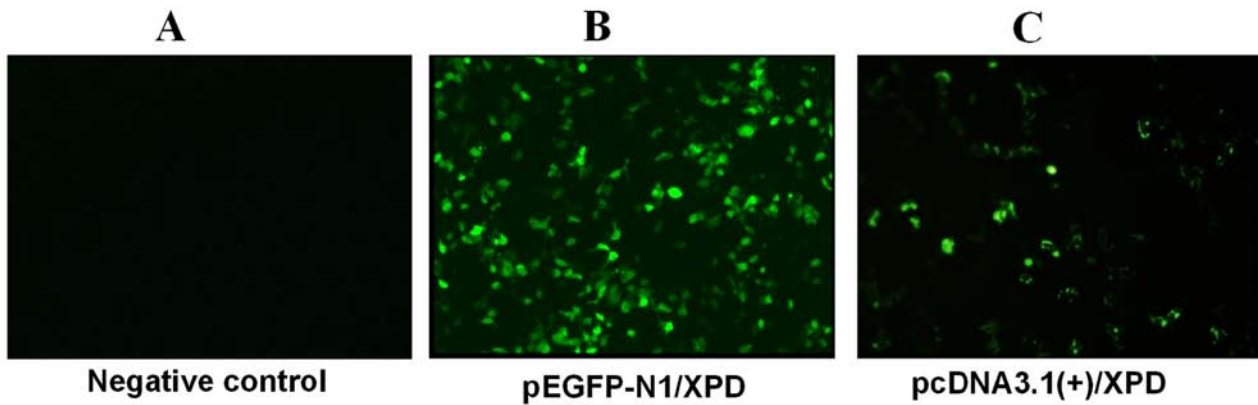


Figure 3. Recombinant plasmids transfected into A375 cells. GFP expression in A375 cells was observed under a fluorescence microscope (magnification, x200). (A) Negative control (A375 cells). (B) pEGFP-N1/XPD and (C) pcDNA3.1(+)/XPD transfected A375 cells. GFP, green fluorescent protein; XPD, xeroderma pigmentosum group D.

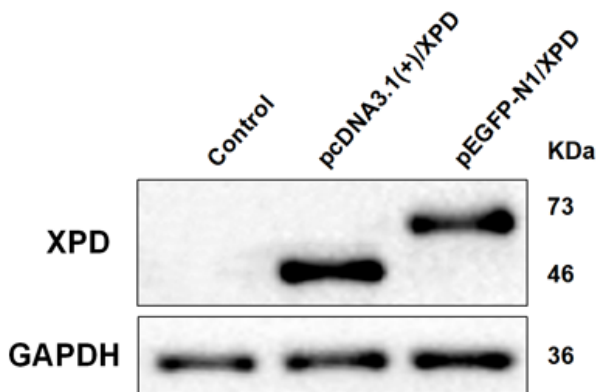


Figure 4. Detection of XPD protein expression in A375 cells transfected with pEGFP-N1/XPD and pcDNA3.1(+)/XPD. Western blot analysis Control consisted of A375 cells only. XPD, xeroderma pigmentosum group D.

endoplasmic reticulum membrane protein marker KDEL, immunofluorescence staining revealed that XPD was localized to the endoplasmic reticulum (Fig. 5).

Inhibition of A375 cell proliferation by XPD. Following transfection of pcDNA3.1(+) and pcDNA3.1(+)/XPD into A375 cells for 24, 48 and 72 h, cell proliferation was analyzed via MTT assay. The cell proliferation of the pcDNA3.1(+)/XPD group was significantly lower than that of the DMEM and pcDNA3.1(+) groups after 48 and 72 h of transfection ($P < 0.05$; Fig. 6), indicating that XPD inhibits the proliferation of A375 cells.

Discussion

In recent years, the incidence of skin cancer has been increasing worldwide, and this trend will continue to increase as the global population ages (10). This increase is primarily caused by the destruction of the ozone layer, which leads to an increase in the number of ultraviolet rays reaching the earth's surface (11). Previous study has demonstrated that UVB light can cause numerous types of DNA damage, leading to skin cancer (12). Therefore, DNA repair is important in maintaining the genetic integrity of the skin.

Normal human keratinocytes have a well-established DNA damage repair system to prevent gene mutations caused by UVB-associated DNA damage. The NER pathway is the primary means of repairing UVB-induced DNA damage, and the main line of defense against carcinogenesis caused by UVB rays (13). To date, two NER pathways have been discovered, including transcriptional coupling repair and whole genome nucleic acid excision repair, which are complex processes involving >30 gene products (14). XPD is the major protein of the nucleic acid excision repair process; the XPD gene is located on chromosome 19 and encodes a protein with ATP-dependent DNA helicase activity (7). The XPD protein is the second largest subunit of the TFIIH complex, which is composed of 9 subunits (namely XPB, XPD, p62, p52, p44, p34, cdk7, cyclinHT and MAT1); XPB, p62, p52, p44 and p34 form a core subcomplex object, while cdk7, cyclinHT and MAT1 form a subcomplex with the cdk active kinase (CAK) (3). XPD acts primarily as a scaffold that mediates CAK anchoring on the core subcomplex (5). During NER, XPD is responsible for opening the DNA duplex at the damaged position from the 5' to 3' direction, allowing damage-specific nucleases to cut the damaged DNA from both sides (5). During transcription, the role of XPD is to maintain the structural stability of the TFIIH complex and to promote transcription amplification (6). Therefore, XPD serves an important role in the TFIIH complex-mediated NER and its transcription process (3). In addition, XPD participates in various physiological and pathological processes, such as cellular proliferation, apoptosis and tumorigenesis (13,15-22). In the present study, the XPD gene was cloned into a eukaryotic expression vector to construct the pEGFP-N1/XPD recombinant plasmid; this was confirmed by sequencing, which was consistent with the XPD sequence published on GenBank.

Malignant melanoma is a type of malignant tumor originating from neural crest melanocytes (22). It mainly occurs in the skin and is the third most common skin malignant tumor (22). Additionally, it commonly results in metastasis and relapse, leading to a poor prognosis (11). The etiology of malignant melanoma is not fully understood, but an animal study has confirmed that UVB is closely associated with malignant melanoma formation (23). Indeed, UVB can cause various types of DNA damage, and can lead

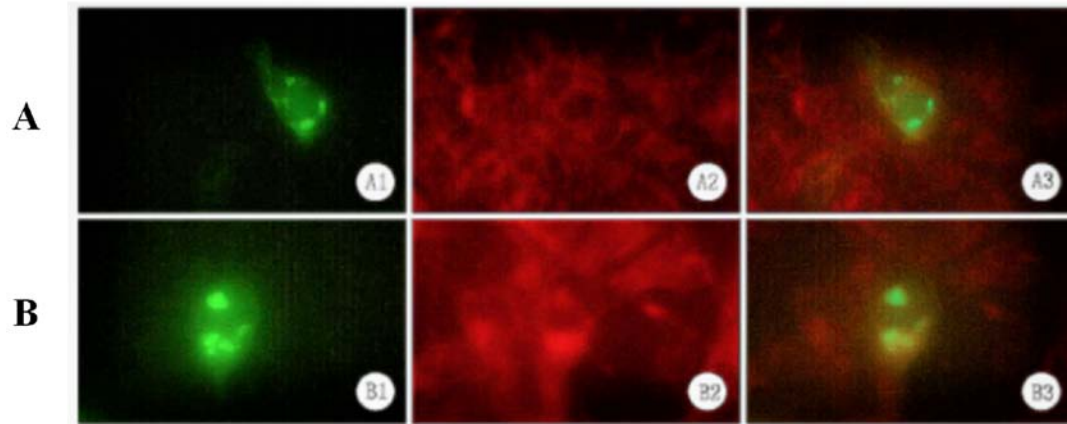


Figure 5. XPD is localized in the endoplasmic reticulum. (A) Immunofluorescence detection of XPD and the Golgi marker GM130, suggesting that there is no co-localization (A1, XPD; A2, GM130; A3, merge). (B) Immunofluorescence detection of XPD and the endoplasmic reticulum marker KDEL, suggesting that these 2 entities are colocalized coexist (B1, XPD; B2, KDEL; B3, merge). XPD, xeroderma pigmentosum group D.

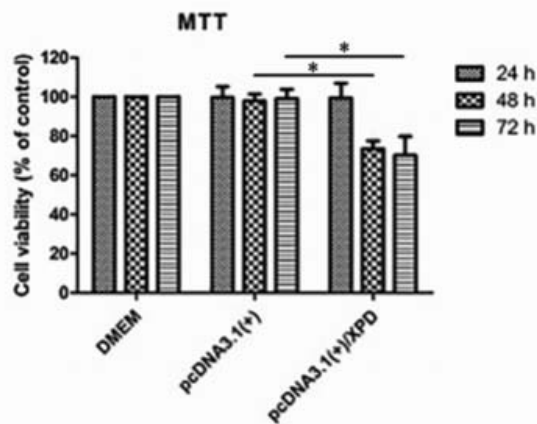


Figure 6. XPD inhibits the proliferation of A375 cells. Cellular proliferation was detected by MTT assay after transfecting A375 cells with pcDNA3.1(+) and pcDNA3.1(+)/XPD for 24, 48 and 72 h. Data are represented as the mean \pm SD (n=3). ANOVA was used to determine significant differences among the DMEM, pcDNA3.1(+) and pcDNA3.1(+)/XPD groups. * $P < 0.05$ compared with the control DMEM and pcDNA3.1(+) groups. XPD, xeroderma pigmentosum group D.

to the development of cutaneous malignant melanoma (13). Studies on the molecular mechanisms of the susceptibility to cutaneous malignant melanoma genes have suggested that this susceptibility is associated with sequence variation mutations in NER genes (2,24). Mutated XPD may be a biomarker of skin malignant melanoma (1). Kertat *et al* (25) revealed that the Gln/Gln genotype of the XPD 751 codon is a genetic marker of male malignant melanoma, and that the Lys/Gln genotype is important for predicting tumor progression.

To investigate the biological effects of XPD on malignant melanoma cells, the XPD gene was cloned and the recombinant pEGFP-N1/XPD plasmid was constructed and transfected into malignant melanoma A375 cells. GFP expression was confirmed by fluorescence expression of XPD-EGFP. Additionally, western blot analysis revealed that XPD was successfully expressed in transfected cells, indicating that an XPD eukaryotic expression system was successfully constructed. Furthermore, immunofluorescence analysis

was used to detect the location of XPD in the endoplasmic reticulum. An MTT assay revealed that the proliferative capacity of malignant melanoma A375 cells transfected with pcDNA3.1(+)/XPD was significantly lower than that of cells transfected with pcDNA3.1(+) or DMEM, suggesting that XPD inhibits the proliferation and metabolism of A375 cells. Therefore, the present study provides a basis to further clarify the function of XPD in malignant melanoma.

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Availability of data and materials

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

Authors' contributions

MG designed the study. YuW and YZ performed the experiments and wrote the manuscript. YaW and CP analyzed and interpreted data. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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