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RESEARCH

Nucleotide Excision Repair Gene Subunit XPD is Highly Expressed in Cervical Squamous Cell Carcinoma

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Abstract One of the subunits in the mammalian transcription factor II H complex, XPD (TFIIH p80), plays a significant role in the nucleotide excision repair pathway. Events such as abnormal DNA excision repair may be involved in the cervical carcinogenesis process. Expression of the human XPD protein was examined using immunohistochemistry in 84 normal cervical tissues and 148 cervical squamous cell carcinoma samples. Additionally, qRT-PCR was performed to analyse the XPD mRNA expression in 69 fresh normal cervical tissues and 110 cervical carcinoma samples. The relationships between XPD expression and various clinicopathological features (including age, FIGO stage, tumor size, stroma involvement, lymph node metastasis and histologic grade) were assessed. The XPD (TFIIH p80) protein was detected primarily in the cytoplasm. We found a statistically significant difference in XPD expression level in cervical carcinoma versus normal cervical tissue ($Z=-7.302$, $P=0.000$). Notably, XPD mRNA was significantly over-expressed in cervical carcinoma tissues but not in normal cervix tissues ($t=6.942$, $P=0.000$). However, no statistically significant relationship was found regarding XPD expression and age, FIGO stage, tumor size, stroma involvement, lymph node metastasis or histologic grade ($P=$ 0.089, 0.953, 0.809, 0.275, 0.421, 0.387 respectively). Our results showed that XPD was highly expressed in cervical squamous cell carcinoma tissues. A poorly understood change may occur during the XPD transcription process, resulting in the abnormal enrichment seen from mRNA to the protein level, thus leaving the protein unable to perform the normal function of excision repair. There is a need for further research in order to elucidate the specific mechanism involved.

Keywords XPD · Cervical squamous cell carcinoma · Nucleotide excision repair

Abbreviations

TFIIH	Transcription factor II H complex
XPD	Xeroderma pigmentosum D
qRT-PCR	Quantitative Real-Time PCR
FIGO	International Federation of Gynecology and Obstetrics
NER	Nucleotide excision repair
PBS	Phosphate buffered saline
CIN	Cervical intraepithelial neoplasia
HPV	Human papillomavirus
SNP	Single-nucleotide polymorphism

Introduction

Cervical carcinoma is a serious reproductive tract malignancy in females. It is well established that HPV infection is a key factor leading to the development of cervical carcinoma, a long-term, complex process. During the period of infection,

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cervical intraepithelial injury cannot be effectively repaired, which in turn may contribute to cervical cancer progression.

Neoplasm has been considered a type of multiple gene disease induced by cell cycle disorders as well as out of control cell growth. Many oncogenes and tumor suppressor genes are directly involved in the regulation of a cell cycle [1, 2]. Mutation of these genes may lead to the cell growth characteristics of excessive proliferation and reduced apoptosis [3]. Cell tumorigenesis is closely related to the abnormal change of regulation factors during the cell cycle G phase as well as the G/S translation phase. The cell cycle is regulated by the DNA damage 'check point' [4], which is involved in blocking the cell cycle and activating the DNA damage-repair signaling pathway. Nucleotide excision repair (NER) is a vital component in the DNA damage-repair signaling network [5]. Mammalian transcription factor II H (TFIIH) is a complex comprised of two sub-complexes, the core-TFIIH with the five subunits p34, p44, p52, p62 and XPB, and the CAK complex (Cdk-activating kinase) with Cdk7, Cyclin H and MAT1, subunits involved in general transcriptional regulation, nucleotide excision repair, and cell cycle control [6–8]. In the TFIIH complex, the remaining XPD (TFIIH p80) is similar to an organizing bridge that interacts with the core TFIIH complex and the CAK complex, and is located near the CAK complex. During NER, XPD acts as a 5′3′ATP-dependent helicase. The substrate of Cdk7 is altered by the combination of CAK and TFIIH, which contributes to the regulation of various intracellular functions of Cdk7 [9–11]. It has been reported that Cdk7 activity is crucial for the Cdk family operation during the key period of the cell cycle. As the catalytic subunit of the CAK complex, Cdk7 phosphorylation may activate other kinase regulations of the cell cycle [12, 13], which obviously influences the DNA duplication and damage-repair process. Nucleotide excision repair (NER) is the primary method of DNA repair used in mammalian cells, and the process also functionally removes any DNA spiral distortion caused by large-scale platinum-based compounds [14]. Moreover, it has been reported that over-expression of XPD could be related to drug resistance as DNA is induced to cross-link [14]. Mammalian transcription factor II H (TFIIH) is comprised of two sub-complexes that are involved in general transcriptional regulation of nucleotide excision repair and cell cycle control [6, 9]. The XPD (TFIIH p80) subunit functions as the bridge allowing for interaction between the main two sub-complexes that ultimately contribute to the regulation of group of diverse cyclin dependent kinases. Hence, the potentially important role XPD plays in tumorigenesis and tumor drug resistance has recently been recognized. The present study was designed to investigate the expression of XPD in human cervical cancer and to determine the correlation between XPD expression and various clinicopathological parameters.

Materials and Methods

Tissue Samples and Patients

Paraffin-embedded specimens (n=232) were randomly selected from patients in the Pathological Department of Women's Hospital, School of Medicine, Zhejiang University, China. Complete clinicopathological data was obtained for all subjects. Of the 148 cervical squamous cell cancer patients, 44 (29.73 %) had grade 1 disease, 77 (52.03 %) grade 2, and 27 (18.24 %) grade 3; 24 (16.22 %) had FIGO stage IA, 114 (77.03 %) stage IB, and 10 (6.76 %) had stage IIA. All of the patients had undergone radical hysterectomy and pelvic lymphadenectomy, and those who initially received preoperative chemotherapy and/or radiation therapy were excluded from the study. Meanwhile, 84 normal cervical tissue samples obtained from non-cancer patients (*e.g.*, uterine functional bleeding, uterine myoma, and uterine prolapse) were used as control samples. Two pathologists reconfirmed the histological diagnosis for each specimen. In addition, 110 fresh cervical squamous cell cancer tissue samples and 69 normal cervix tissue samples were obtained after hysterectomy and were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. This study was approved by the Ethics Committee of the Faculty of the School of Medicine at Zhejiang University. Informed consent was obtained from all patients.

RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and subjected to reverse transcription using RT reagent (Takara Biotechnology, Japan) after digestion with RNase free DNase I. Real-time PCR was carried out using The SYBR® Premix Ex Taq TM (perfect real-time) (Takara Biotechnology, Japan) with an Applied Biosystems 7900HT Fast real-time PCR system according to the manufacture's protocol. The melting curve of each primer pair was commonly constructed to confirm product specificity. All of the reactions were run in triplicate and no template control reactions were included in each assay run. The mRNA level of beta-actin was used for control. The relative mRNA expression levels of the target gene were calculated according to the $2^{-\Delta ct}$ method, in which Δct represents the difference in value between each ct value of target gene minus that of beta-actin.

PCR Primers

Primers for human XPD and beta-actin genes were designed to be intron-spanning. The following primers were used for quantitative RT-PCR and the program for Real-time PCR system was set: 95 °C for 30 s, then 40 cycles at 95 °C for

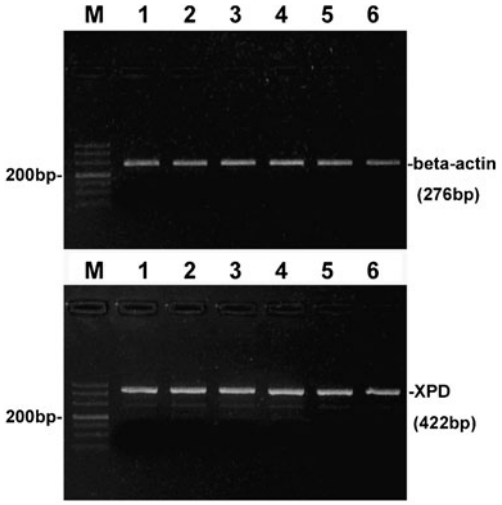

Fig. 1 The validation of PCR products by electrophoresis. The length of XPD PCR product was 422 bp. The length of beta-actin PCR product was 276bp. **M:** 100bp marker(500,400,300,200,150,100,50); **1,2,3:** Normal cervical tissue; **4,5,6:** Cervical squamous cell cancer

5 s and followed with 60 °C for 30 s.

XPD, forward5′ – CCACAACATTGACAACGTC – 3′, reverse5′ – CAGCACAGAATCTGAGGG – 3′

beta – actin, forward5′ – GTGGACATCCGCAAAGAC – 3′, reverse5′ – GTCCGCCTAGAAGCATTT – 3′

In order to ensure that these primers give rise to just a single and specific PCR-product with the expected molecular weight, we performed a a standard (not real-time) PCR assay with the indicated primers ,followed by resolution of PCR products on an agarose gel. The length of XPD PCR product was 422 bp,while beta-actin PCR product was 276 bp.

Immunohistochemical Analysis and Evaluation

All paraffin-embedded tissues were routinely fixed with formalin and embedded in paraffin. Prior to staining, 4-nm thick sections mounted on poly-L-lysine–coated slides were deparaffinized in xylene for 10 min and rehydrated through a gradient ethanol series (100–70 %) to water. For antigen retrieval, the sections were exposed to 10 mM sodium citrate buffer (pH 6.0, Maxin, Fujian Province, China) at 120 °C for 2.5 min in an autoclave. To inhibit endogenous peroxide activity, sections were immersed in Peroxidase inhibitor (Maxin, Fujian Province, China) for 15 min. After washing with phosphate buffered saline (PBS, pH 7.6), samples were incubated at room temperature (RT) for 2 h with rabbit anti-human TFIIH p80 polyclonal antibodies (Santa Cruz, USA) diluted to 1:50 with the blocking solution. After the incubation, sections were washed three times with PBS followed by incubation with EnVision-HRP complex (Dako,Carpinteria, CA) at RT for 1 h. Finally, 3′3-diaminobenzidine tetrahydrochloride (Dako) was added to each slide for visualization. After chromogen development, sections were washed with distilled water and counterstained with hematoxylin. Neutral resin was used to mount the sections.

Any discrepancies were resolved by consensus review. Expression of XPD was scored 'positive' when at least 10 % of neoplastic cells showed cytoplasmic staining of at least moderate intensity. Positive cells were indicated by the presence of brown staining in the cytoplasm. We counted the number of positively stained cells out of 100 in 10 random fields (400-fold magnification). The fraction of positive cells was estimated using a four-tiered scale (≤9 %=1, 10~49 %=2, 50~79 %=3, ≥80 %=4). The staining intensity was also scored on a four-tiered scale (negative 0, low intensity positive staining 1, moderate intensity positive staining 2, strong intensity positive staining 3). The overall score for each case represented the cross product of the staining intensity score and the positive fraction score; thus total scores ranged from 0 to 12. The semiquantitative immunoreactive scoring system was evaluated based on the cross product as follows: –,0~ ; +,4~; ++, 6~; +++, 8~. Each section was evaluated two times by two independent observers who were blinded to the clinical outcome.

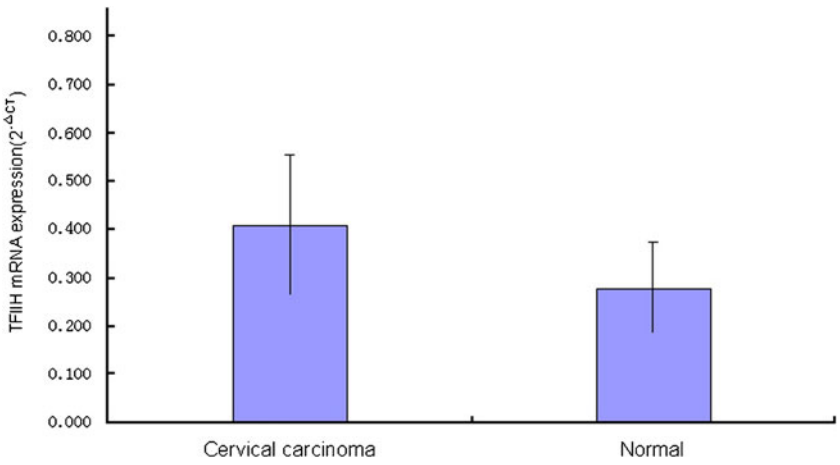

Fig. 2 XPD mRNA expression in cervical carcinoma vs. normal cervical tissue. The average value of normal cervix tissues upon normalization with beta actin was 0.280±0.093, while the value of cervical carcinoma tissues was 0.409 ±0.145. The difference was observed statistically significant (t=6.942, P=0.000)

Table 1 XPD protein expression in cervical squamous cell cancer vs. normal cervical tissue

	total	(−)	(+)	(++)	(+++)	Mean Rank	Z	P value
Normal cervical tissue	84	49	19	9	7	75.57	−7.302	0.000*
Cervical squamous cell cancer	148	17	54	15	62	139.73		

Statistical Analysis

The data were summarized and analyzed using SPSS 15.0 software. The Kruskal–Wallis H and the Mann–Whitney U tests were used to evaluate expression differences between the various groups. The Spearman and Kendall tests were applied to analyze the association between expression and clinical pathological factors. The t-test was used to analyze the relative mRNA expression level differences between the two groups. A result was considered to be statistically significant when the P value was less than 0.05.

Results

XPD mRNA Notably Over-Expressed in Cervical Carcinoma Tissues

Shown in the ordinary electrophoresis figure, each of the beta-actin and XPD had their own single and specific electrophoresis band (Fig. 1), as we expected.

We determined the XPD mRNA expression of 110 cervical carcinoma samples and 69 normal cervix samples using qRT-PCR. Upon normalization with the Ct value of beta actin, the average $2^{-\Delta ct}$ value of normal cervix tissues

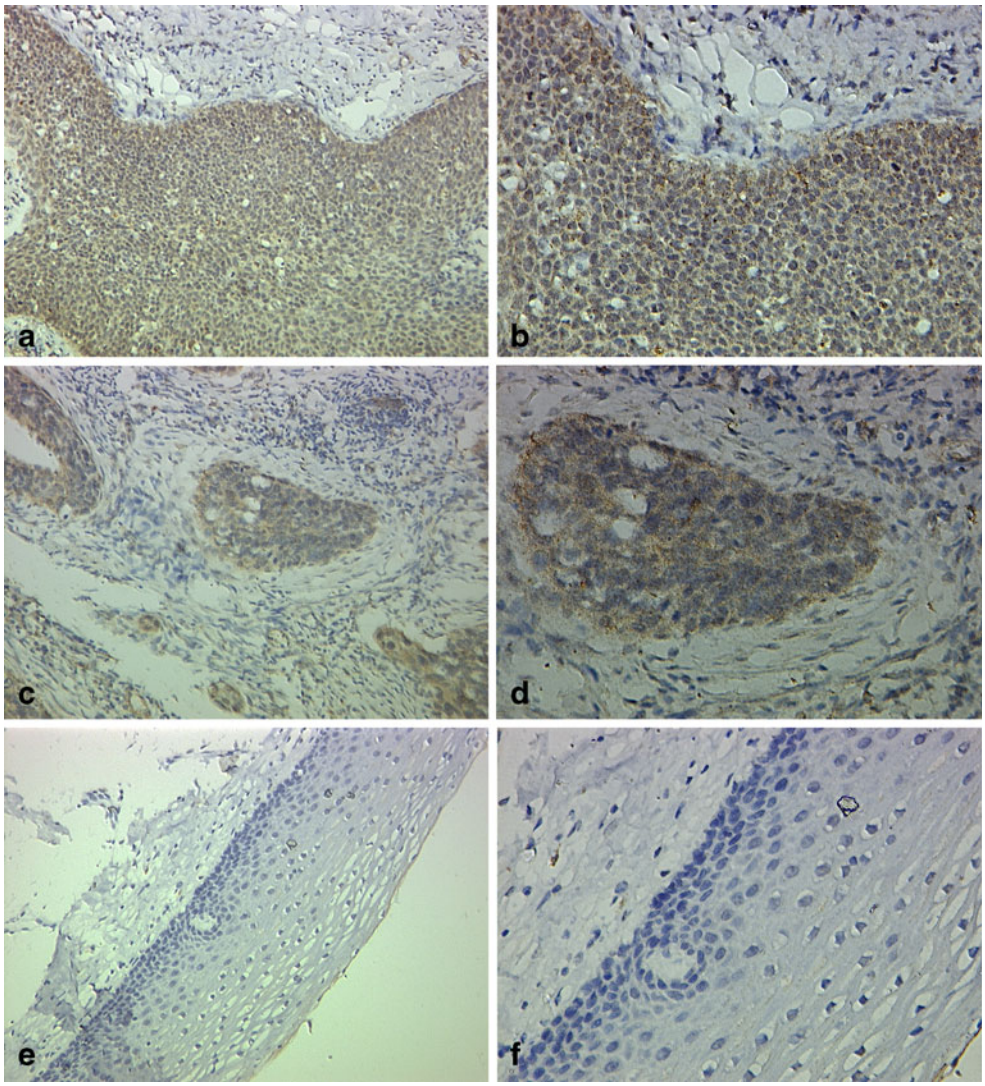

Fig. 3 XPD protein expression in cervical squamous cell cancer vs. normal cervical tissue. The XPD mainly located in the cytoplasm. **a**~**b**, negative control (200×,400×);**c**~**d**, normal cervical tissue (200×,400×); **e**~**f**, cervical squamous cell cancer tissue (200×,400×)*

was 0.280±0.093, while the average $2^{-\Delta ct}$ value of cervical carcinoma tissues was 0.409±0.145. Both sets of data were tested to determine the normality of the two distributions using the One-Sample Kolmogorov-Smirnov Test. The XPD mRNA was notably over-expressed in cervical carcinoma compared to normal cervix tissues when analyzed by the t-test; a statistically significant difference was observed (t=6.942, P=0.000) (Fig. 2).

XPD Protein Over-Expressed in Cervical Carcinoma Tissues

The positive reaction of XPD protein was measured by the amount of brown-yellow particles observed and was found to be primarily located in the cytoplasm. Positive XPD expression was detected in 131 (88.51 %) of 148 cervical carcinoma cases, while 35 (41.67 %) of 84 normal cervical tissues were observed positive. There was a statistically significant trend of XPD expression toward cervical carcinoma as compared to normal cervical tissues (Z=−7.302, P=0.000).(See Table 1 and Fig. 3).

Relationship Between XPD Expression and Clinicopathological Parameters

The correlation between XPD expression and clinicopathological variables is shown in Table 2. There was no statistically significant relationship between XPD expression and age, FIGO stage, tumor size, stroma involvement, lymph node metastasis or histologic grade (P=0.089, 0.953, 0.809, 0.275, 0.421, 0.387 respectively).

Discussion

It is well established that cervical carcinoma develops through a multistep process. Our previous results support the hypothesis that genetic variation in DNA repair genes may contribute to an inherited genetic susceptibility to cervical carcinoma [15]. This indicates that HPV infection alone is not sufficient to develop cervical intraepithelial neoplasia (CIN) or cancer, and that other cofactors such as variations or mutations in DNA repair genes should be considered. There are several studies demonstrating that XPD helicase activity and the corresponding ATP enzyme activity is essential for the function of nucleotide excision repair [11, 16, 17]. XPD mutation may not only disrupt the XPD interaction with P44, but moreover may cause XPD to be unable to function at Cdk7, resulting in potential inhibition of the ability to regulate the entire CAK sub-complex cell cycle [16].

It has been previously reported that, as a nucleotide excision repair gene, XPDs expression in cancer tissue is lower than the level found in paraneoplastic tissue. Researchers concluded that the DNA repair mechanism of XPD appeared to undergo an abnormal change during the tumorigenesis process, hence XPD was then unable to play its role in DNA repair. Moreover, the base mutation of XPD

Table 2 The correlation analysis between XPD expression of 148 cervical squamous cell cancer and clinicopathological data

	Total N=148	− N=17	+ N=54	++ N=15	+++ N=62	r	P
Age							
<=35	40	6	16	7	11	0.131	0.089
>35	108	11	38	8	51		
FIGO staging							
IA	24	3	6	6	9	−0.004	0.953
IB	114	13	46	6	49		
IIA	10	1	2	3	4		
Tumor size							
<=2cm	49	7	15	8	19	0.019	0.809
>2cm	99	10	39	7	43		
Invasive depth							
Superficial(<=1/2)	104	13	40	10	41	0.084	0.275
Deep(>1/2)	44	4	14	5	21		
LNM							
negative	138	16	52	13	57	0.062	0.421
positive	10	1	2	2	5		
Histologic grade							
Grade 1	44	5	16	4	19	0.066	0.387
Grade 2	77	10	25	8	34		
Grade 3	27	2	13	3	9		

have been proven to be correlated with the genetic susceptibility of various kinds of tumors through the single-nucleotide polymorphism(SNP) research. Therefore, it is generally accepted that XPD is down-regulated in carcinoma *in-situ*. There have been some studies that report a link between the XPD SNPs and cancers of various types, including basal cell carcinoma, head and neck carcinoma, hepatocellular carcinoma and cancer of the bladder, lung, and prostate [18–22]. In our previous study we determined that XPD SNPs correlated with genetic susceptibility for cervical carcinoma [15], hence, we hypothesized that XPD expression may be correlated with the process of cervical carcinoma. As demonstrated in our present study, the XPD expression level in cervical carcinoma is dramatically stronger than that in normal cervical tissues; not only on the mRNA level but also on the protein level ($P<0.001$). Researchers have proven that expression of the HPV E6 protein promotes the degradation of wild type p53 [23], however inhibition of XPD promoter activity does not follow, on the contrary, HBV replication does not interfere with the known cellular functions of p53, but may suppress XPD helicase activity [16]. The research of Gao's group suggests that individuals who have the XPD751 Gln allele may be at an increased risk for bladder cancer, although this may not lead to an increased risk for mutations in the p53 gene [18]. XPD expression may be inconsistent with p53 expression. Therefore, wild type p53 may be degraded in cervical carcinoma, whereas XPD may appear to exhibit abnormally high expression without normal excision repair function. In turn, as the incorrect accumulation increases, the accompanied expression of XPD increases abnormally as well.

We did not find any correlation between XPD and the clinicopathological variables. On the one hand, the sample population was small, however, it appears that the single nucleotide polymorphism of XPD was clearly related to the genetic susceptibility to cervical carcinoma, meaning that XPD may be involved in the accumulation of DNA mutable points and the early onset of disease, as a start, not in the progress and prognosis of the disease. On the other hand, XPD may be abnormally enriched as a consequence of the cervical cancer progress in return, which may, furthermore, aggravate the effect of cancer development. This kind of conflict about XPD's function showed as a double-edged sword presenting a challenge for study of cervical or other kind of carcinomas. Accordingly, there may be a poorly understood change that occurs during the XPD transcription process and results in the abnormal enrichment observed from the mRNA to the protein level. This XPD would not be able to perform the normal function of excision repair in which the specific mechanism remains unclear and deserves further study.

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Author Contribution Statement FY and HC conceived and carried out experiments and analysed data. JJ conceived experiments and analysed data. CZ and QC carried out experiments. All authors contributed to the writing of the manuscript and had final approval of the submitted and published versions.

Conflict of Interest Statement The authors have no conflicts of interest to declare.

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