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Functional Significance of XPD Polymorphic Variants: Attenuated Apoptosis in Human Lymphoblastoid Cells with the XPD 312 Asp/Asp Genotype[1]

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Abstract

Recent molecular epidemiological studies have identified polymorphisms in the *XPD* gene that are associated with increased risk of brain gliomas and head, neck, lung, and skin cancers. However, the functional significance of these polymorphic variants in altering cell processes such as cell cycle checkpoints, DNA repair, and apoptosis is uncertain. We have cloned the XPD variants Lys751Gln, Asp312Asn, and Lys751Gln-Asp312Asn into a pcDNA-3.1-expression vector. Using these constructs, we did not find any detectable difference in either *in vitro* binding with wild-type p53 or in DNA repair proficiency as measured by host cell reactivation assay. We then genotyped 34 different lymphoblastoid cell lines from six Centre d'Etude du Polymorphisme Humaine (CEPH)/Utah pedigree families and a CEPH/French pedigree family for polymorphisms at codons 751 and 312 and assessed their apoptotic response after either UV or ionized radiation exposure. The lymphoblastoid cell lines with homozygous or heterozygous Asp at codon 312 have similar apoptotic rates, whereas cell lines with homozygous Asn at codon 312 showed a 2.5-fold increased response to UV ($P = 0.005$; Student's *t* test). This is the first report known to us of a functional polymorphism in a gene involved in DNA damage-induced apoptosis. However, the presence of Lys or Gln at codon 751 did not influence the apoptotic response to UV. The diminished apoptotic response of cells containing the 312 Asp allele could both allow the survival and selective clonal expansion of carcinogen-damaged cells and be a mechanistic explanation for the increased risk of cancer at diverse tissue sites.

Introduction

NER[4] is the major cellular repair pathway by which a variety of DNA lesions in the form of UV-induced photoproducts and bulky chemical adducts are eliminated from the genome (1–3). NER involves ≥25 proteins that locate the damaged strand, introduce incisions on each side of lesions, excise an oligonucleotide of 24–32 residues, and fill in the gap by repair synthesis and ligation. The DNA helicases XPD and XPB, subunits of the basal transcription factor TFIIH that are required for transcription initiation by RNA polymerase II, are two of the major proteins involved in NER (4). Germ-line mutations in the coding region for XPD are correlated with xeroderma pigmentosum, trichothiodystrophy, and Cockayne's syndrome (2). It has been proposed that sequence variations in DNA repair genes might modulate cancer susceptibility (5–9). Several polymorphisms in the *XPD* gene including the most common Lys751Gln and Asp312Asn have been reported (10). The XPD 312 polymorphism is characterized by a G to A substitution causing an Asp (D)→Asn (N) amino acid exchange at codon 312. The XPD 751 polymorphism is characterized by an A to T substitution causing a Lys (K)→Gln (Q) amino acid exchange at codon 751. Epidemiological studies have indicated that these sequence variations may influence cancer risk (8, 11–14). Lunn *et al.* (12) reported that the XPD 751Lys/Lys genotype is associated with suboptimal DRC. Lymphocytes homozygous for the Lys allele showed reduced repair in an X-ray-induced chromosome aberration assay. Dybdahl *et al.* (14) also reported that individuals with the 751Lys/Lys genotype were at a higher risk of basal cell carcinoma and that the variant allele might be protective. Another study claimed a specific association with melanoma suggesting that the 751Lys/Lys allele was associated with greater risk for cancer than 751Gln/Gln (9). In contrast, the 751Gln/Gln genotype is more common in patients with squamous cell carcinoma of the head and neck and is associated with a modest increased risk (13). Although one study found no relationship between Lys751Gln polymorphism and DRC (15), Spitz *et al.* (8) have reported that the 751Gln/Gln and 312Asp/Asn genotypes were associated with less optimal DRC. Finally, a study of glioma patients indicated that cases were less likely than controls to carry the variants at codons 751 or 312 (16). These latter findings are consistent with our data reported previously stating that the 312 variant (312Asn/Asn) is protective against lung cancer when compared with the wt allele, and the codons 312 and 751 are in linkage disequilibrium (11).

We and others have shown earlier that p53 can modulate NER by protein-protein interactions with XPB and XPD (17–19). Moreover, p53-induced apoptosis can be mediated by these helicases. We have reported previously that p53-mediated apoptosis is attenuated in primary human fibroblasts from xeroderma pigmentosum individuals that harbor germ-line mutations in XPD or XPB (20, 21). To further investigate the mechanistic significance of the amino acid substitutions in XPD at codons 312 and 751, we performed functional assays to assess their effects on p53 binding, DNA repair proficiency, and UV- or IR-induced apoptosis.

Materials and Methods

Plasmids. PcDNA3.1-XPD was provided by Dr. Jan Hoeijmakers (Erasmus University, the Netherlands) and used as a template for generating Lys751Gln, Asp312Asn, and Lys751Gln-Asp312Asn by site-directed mutagenesis (Stratagene, La Jolla, CA).

Purification of Recombinant Proteins and *in Vitro* Protein Binding and Analysis of Protein Complexes. Purification of recombinant proteins and *in vitro* protein binding were performed as described previously (20). Experiments were repeated at least three times. PcDNA-XPD cDNA fragments 1–732, 733-1432, and 1433–2281 cDNA-XPD (representing the NH_2 termi-

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[4] The abbreviations used are: NER, nucleotide excision repair; DRC, DNA repair capacity; IR, ionizing radiation; GST, glutathione *S*-transferase; CTD, COOH-terminal domain; CAT, chloramphenicol acetyltransferase; ATM, ataxia telangiectasia mutated; wt, wild-type.

nus, central domain, and COOH-terminus, respectively) were amplified by PCR in combination with a specifically tailed forward primer enabling *in vitro* transcription by T-7-RNA polymerase and the reverse primer encoding a stop codon (22). The products were analyzed by agarose gel to verify the amplification and gel purified (Stratagene). For generation of *in vitro*-translated ^{35}S-labeled proteins, the PCR-products, encoding the corresponding protein fragments, were incubated at 30°C for 90 min with [^{35}S]methionine (Amersham, Piscataway, NJ) in a TNT *in vitro* transcription and translation system (Promega, Madison, WI).

Genotyping. Detection of polymorphisms was performed as described previously (11).

Apoptosis Assay after UV-C and IR Treatment. Lymphoblastoid cells (34 cell lines from CEPH/Utah pedigree families 1331, 1333, 1340, 1345, 1420, and 13291, and a CEPH/French pedigree family, 23; Coriell Cell Repositories, Camden, NJ; 2×10^6 each) in 2 ml of RPMI 1640 were placed into 60-mm polystyrene dishes (Falcon, Bedford, MA). Three dishes of each cell line were irradiated with 20 J/m^2 of UV-C irradiation or 4 Gy of IR, respectively. Three of the dishes were treated in an identical manner but without irradiation. Fresh medium (2 ml) was added to the plates and incubated for 48 h. After incubation, 5×10^5 cells were collected and treated with an Annexin-V FITC Apoptosis kit according to the manufacturer's instructions (Biosource International, Camarillo, CA). A total of 34 cell lines were analyzed with lymphoblastoids from an XPD patient used as a positive control (GM 02653F; Coriell Cell Repositories). Uptake of the stain was assessed on a Beckman Coulter fluorescence-activated cell sorter analysis cell reader. Specific apoptosis was determined by the formula [100 × (mean experimental apoptosis − mean spontaneous apoptosis)/100 − mean spontaneous apoptosis]. All of the experiments were done in triplicates.

ELISA for Detection of Protein-Protein Interactions. Plates (96-well) were coated with 1–5 ml of *in vitro*-translated protein overnight at 37°C and washed three times with washing buffer (PBS and 0.5% Tween 20). Blocking buffer (PBS, 0.5% Tween 20 and 3% BSA) was added to the wells and incubated for 2 h at 24°C. Wells were aspirated and washed with blocking buffer and p53 protein diluted to 1 ng/ml. Binding buffer [50 mM Tris/HCL (pH 7.4), 5 mM $MgCl_2$, 5 mM ATP, 100 mg/ml BSA, and 50 mM NaCl] was added to the appropriate wells and incubated for 1 h. The wells were washed three times with washing buffer. Primary anti-p53 antibody [rabbit polyclonal IgG 1:4000 (Signet Laboratory, Dedham, MA) in blocking buffer] was added to the wells and incubated for 1 h at 24°C. Wells were aspirated and washed six times with washing buffer. Horseradish peroxidase-conjugated secondary antibody (goat antirabbit; Jackson Immuno Research, West Grove, PA) was diluted to 1:10000 in blocking buffer, added to the wells, and incubated for 1 h at 24°C. Wells were aspirated and washed six times with 0.2 M diethanolamine buffer. Binding was detected using K-Blue substrate (Biogen Corp., Cambridge, MA). Absorbance readings were taken at 450 nm at different time intervals with a microplate reader (Molecular Devices, Sunnyvale, CA). The binding experiments were repeated four times.

Host Cell Reactivation Assay. Twenty-nine lymphoblastoid cell lines from CEPH/Utah pedigree families (1331, 1333, 1340, 1345, 1420, and 13291) and a CEPH/French pedigree family (23) were transfected in triplicate (2×10^6 cells each) with either a UV-damaged plasmid (700 J/m^2) or an undamaged pCMVLUC with DEAE-Dextran (23). Transfection was stopped after 15 min, and the cells were incubated with fresh RPMI 1640 with 15% fetal bovine serum for 40 h. Then the cells were lysed for 1 h with Passive Lysis Buffer (Promega), and 20 μl of the cleared cell supernatant was used to measure luciferase activity (Promega). The activity of the damaged plasmid was compared with the transfection of an undamaged plasmid. The experiments were done in triplicates.

Host Cell Reactivation Assay after Cotransfection of XPD Plasmids Containing Varying Genetic Polymorphisms. Luciferase plasmid (irradiated with 700 J/cm^2 to cause damage or nonirradiated) was cotransfected in XPD patient cells GM02253 with either the wt XPD or the codon 312 variant, codon 751 variant, or the plasmid with both variants. Cells were lysed after 40 h of incubation. The luciferase activity was measured and compared with the undamaged plasmid to determine the percentage of repair. The experiments were repeated three times.

Results

We have performed different assays to reveal the functional effects of sequence variations at codons 751 and 312. We have genotyped various EBV-immortalized lymphoblastoid cell lines for their status at codons 751 and 312. Cell lines with different genotype combinations for codons 312 and 751 were used in experiments with UV-irradiation. Fig. 1 shows the apoptotic response of lymphoblastoid cell lines with different genotypes at codons 312 and 751 to UV irradiation. The results illustrate that lymphoblastoid cell lines with homozygous or heterozygous Asp at codon 312 have similar apoptotic rates. However, cell lines with homozygous Asn genotype at codon 312 demonstrated almost a 2.5-fold higher response to UV. The tested genotypes at codon 751 did not influence the UV response. Fig. 1*B* summarizes the results by comparing the homozygous Asp lymphoblastoid cells with the Asn homozygous cells. A *P* value of 0.005 as assessed by Student's *t* test indicates a statistically significant effect.

Similar experiments with the same cell lines shown in Fig. 2 have been performed using IR to induce apoptosis (Fig. 2). The apoptotic responses induced by IR are within the range that was observed for the 312 Asp variants treated with UV. Fig. 2*B* summarizes the results presented in Fig. 2*A* by comparing the homozygous Asp and Asn lymphoblastoid cells. The *P* of 0.13 determined by Student's *t* test indicates no statistical difference between these two genotypes.

p53 mediates apoptosis by both transcription-dependent and transcription-independent pathways (reviewed in Refs. 20, 24). The CTD of p53 is involved in the apoptotic function by protein-protein interactions (17). The p53 CTD can bind directly to XPD and induce apoptosis. To test whether the sequence variation at codons 312 and 751 can influence p53 binding, we identified the p53-binding site of

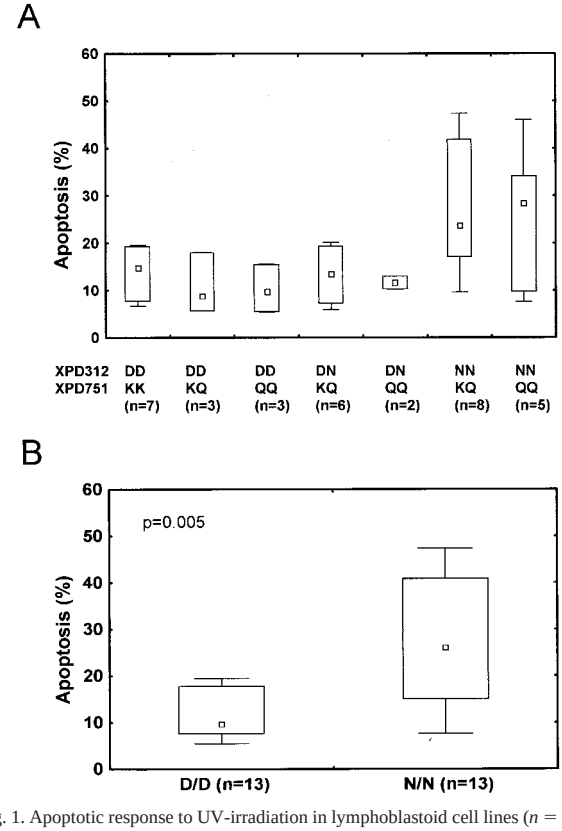

Fig. 1. Apoptotic response to UV-irradiation in lymphoblastoid cell lines ($n = 34$) with the genotype 312Asn/Asn. One letter amino acid nomenclature has been used, Asn (*N*), Asp (*D*), Lys (*K*), and Gln (*Q*). The *P*s have been calculated by summarizing the data for homozygous Asp (*D*) and homozygous Asn (*N*) using Student's *t* test. *B* summarizes the results of *A*; *bars*, ± SE.

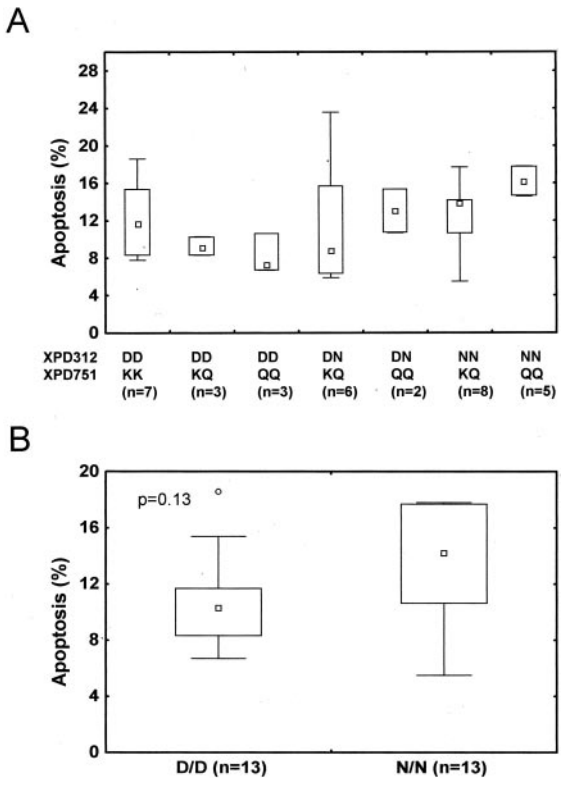

Fig. 2. Apoptotic response of lymphoblastoid cells ($n = 34$) with different genotypes after IR treatment. *B* summarizes the results of *A*. *P*s have been calculated by summarizing the data for homozygous Asp (*D*) and homozygous Asn (*N*) using Student's *t* test; *bars*, ± SE.

XPD. PCR-generated cDNA fragments corresponding to the NH_2 terminus, the central domain, and the CTD of XPD, were *in vitro*-translated in the presence of [^{35}S]methionine and tested for their ability to bind to purified GST-p53 protein. Only purified GST protein was used instead of GST p53 as a negative control for binding. *Lanes 9–12* represent 20% of the amount of *in vitro*-translated and [^{35}S]-methionine-labeled proteins that have been used for these binding essays. Consistent with previous reports, Fig. 3*A* shows binding of *in vitro*-translated and ^{35}S-labeled XPD (full length) to GST p53 *(Lane 1*). Binding was also observed for the 478–759 fragment *(Lane 4*), which represents the XPD CTD. No binding was observed for fragments 1–244 and 245–477 representing the central region and the NH_2 terminus *(Lanes 2* and *3*) or with GST alone *(Lanes 5–8*). These results indicate that p53 binds to the CTD of XPD. Furthermore, these results demonstrate that the common XPD variant Lys751Gln is within this binding region. Therefore, it was of interest to determine whether the polymorphisms influence the binding. Thus, we cloned the XPD variants Asp312Asn and Lys751Gln/Asp312Asn into a pcDNA3.1-expression vector, *in vitro*-translated them, and tested their p53-binding capacity using an ELISA-based method (Fig. 3*B*). Within the sensitivity of this assay, these three variants bind to p53 with the same efficacy as wt XPD. Similar results were obtained when we performed *in vitro* binding experiments with GST p53 and ^{35}S-labeled XPD variants (data not shown).

Several laboratories (8, 12) have reported that XPD variations at codons 312 and 751 are linked to decreased DRC. To test this hypothesis, we performed host cell reactivation assays on the same 29 lymphoblastoid cell strains used in the apoptotic experiments. Although we observed differences in the DRC of cells categorized by their genotype, there was no apparent correlation with XPD genotype (Fig. 4*A*). To further address the role of these XPD polymorphisms with DRC, we investigated an isogenic system in which XPD patient lymphoblastoid cells were cotransfected with the repair reporter plasmid and a plasmid containing the XPD variants at codon 312 and/or 751. Each of the plasmids complemented the mutated *XPD* gene and restored the DRC of the cell strain, but the differences among wt XPD and the variants were not statistically significant (Fig. 4*B*).

Discussion

Epidemiological studies have indicated that sequence variations within XPD may influence cancer risk (8, 11–14). To provide additional evidence of how subtle sequence variations can influence cellular processes, we tested the significance of the polymorphic variants Asp312Asn and Lys751Gln on p53 binding, UV- or IR-induced apoptosis, and DRC. Our data present, for the first time, the apoptotic consequences of these variants. Lymphoblastoid cell lines with homozygous Asn genotype at codon 312 showed a nearly 2.5-fold higher apoptotic response to UV and represent a direct link between a polymorphism at codon 312 and its biological significance. Sequence variation at codon 751 seems not to influence the response to UV indicating that amino acid exchanges within codon 312 are of

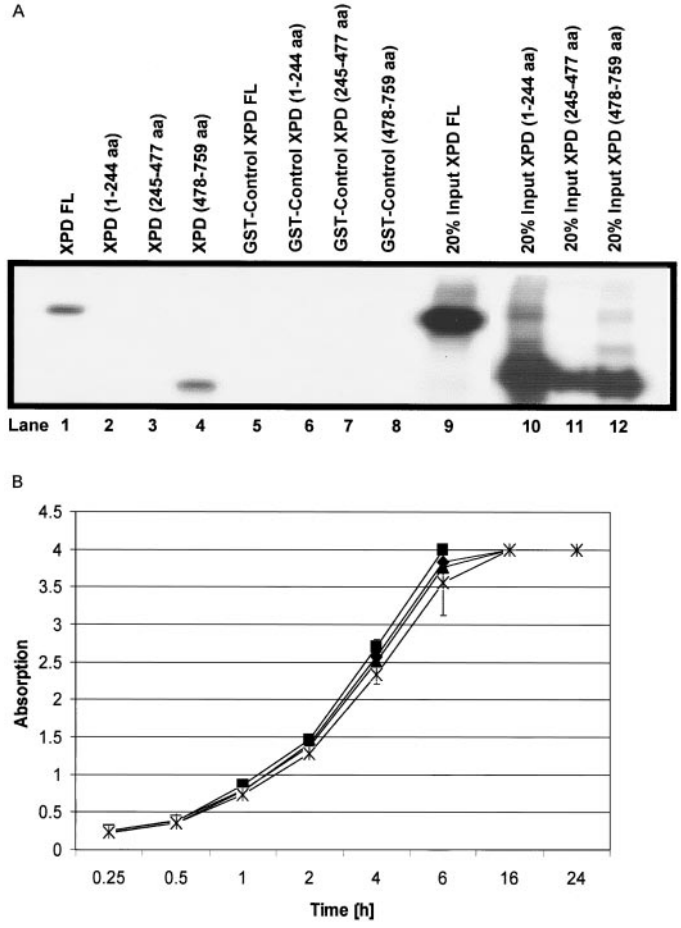

Fig. 3. Analysis of putative binding sites of p53 to XPD. *A*, pCDNA3.1-XPD cDNA fragments 1–732, 733-1432, and 1433–2281 cDNA-XPD were amplified by PCR, *in vitro*-translated, and ^{35}S-labeled. ^{35}S-labeled pcDNA3.1-XPD fragments (5 μl) were mixed with either Sepharose beads loaded with GST-p53 fusion protein or only GST protein for 3 h at 4 C. Then beads were washed with a binding buffer and analyzed by SDS-page. *Lanes 1–4*, the binding of pcDNA-XPD full length and cDNA fragments 1–732, 733-1432, and 1433–2281. No binding could be observed with GST alone using the same XPD fragments (5–8). *B*, ELISA analysis of *in vitro* binding of wt XPD (♦) and the polymorphic variants Lys751Gln (▲), Asp312Asn (■), and Lys751Gln/Asp312Asn (×) to p53; *bars*, ± SE.

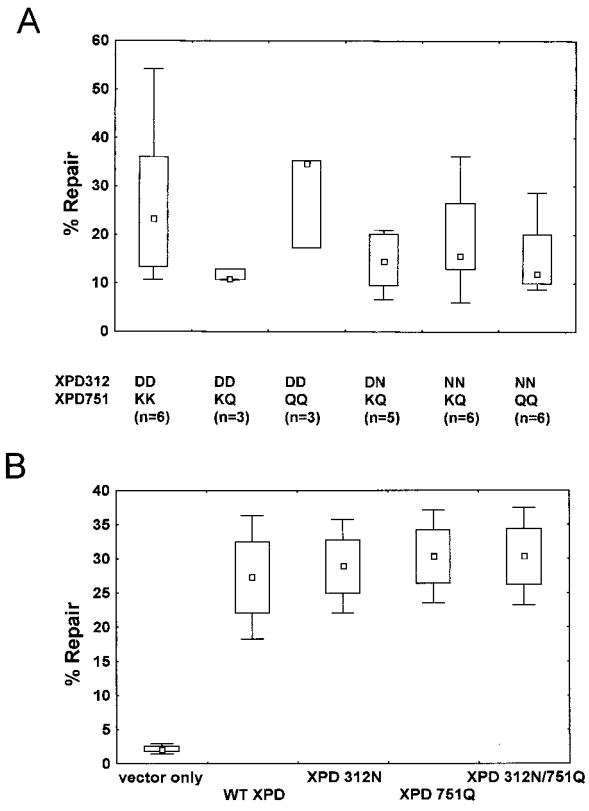

Fig. 4. *A*, host cell reactivation assays using lymphoblastoid cell ($n = 29$) lines categorized by their XPD genotype at codons 312 and 751 to assess DRC. *B*, HCR assay using the wt XPD plasmid and its variants in an XPD mutant cell line; *bars*, ± SE.

more biological significance and is consistent with the finding that this residue is highly conserved through evolution (11). Furthermore, this is in alignment with our previous study showing that individuals homozygous for Asn in codon 312 (312Asn/Asn) have a lower risk for cancer susceptibility (11). Thus, a higher apoptotic response that can effectively eliminate mutation-prone cells may be the mechanistic explanation as to why individuals with a homozygous Asn allele are less susceptible to cancer.

Various explanations are possible for the differences in apoptotic response to IR and UV. IR generates single- and double-strand DNA breaks and oxidative base damage, whereas UV radiation produces the typical NER substrates consisting of cyclobutane dimers and 6-4 photo products. Cells can respond to DNA damage by activating a highly complex DNA damage-response pathway that includes cell-cycle arrest, transcriptional and posttranscriptional regulation of genes associated with repair, and under certain circumstances, the triggering of apoptosis. The members of the phosphoinositide kinase-related kinase family, which includes ATM and ATR (ATM- and Rad3-related), play important roles in signaling the presence of damage and activating responsive pathways (25). It has been shown that the immediate early response to IR is largely ATM-dependent, whereas ATR largely mediates the UV-induced rapid phosphorylation of p53 (26). Furthermore, ATM is required for the immediate phosphorylation of BRCA1 after IR, whereas the phosphorylation of BRCA1 after UV is ATM independent (25). In addition, UV irradiation causes the activation of several transcription factors collectively called the UV response (27). The fact that cells derived from AT patients are hypersensitive to agents that cause double-strand breaks such as IR but retain normal resistance to UV-C and other UV-like DNA-damaging agents demonstrates that the choice of DNA repair or apoptotic pathways depends on the nature of the damage. All of these differences also could explain the differences in the apoptotic response to IR and UV.

As a member of the multiprotein complex TFIIH, XPD is involved in multiple cellular functions and, therefore, interacts with various proteins (2). Thus, amino acid variations at codons 751 and 312 of XPD may affect different protein interactions, resulting in the expression of different functional phenotypes (12). In addition, it is possible that the sequence variation at codon 312 could affect RNA stability or influence protein synthesis leading to differences in apoptosis (8). The apoptotic response to IR or UV treatment indicates that this process is linked directly to NER, because UV damage is repaired primarily by NER, whereas IR-induced damage is repaired mainly by double-strand break repair (28). Spitz *et al.* (8) have reported recently that the genotypes 751Gln/Gln and 312Asp/Asn are associated with decreased DRC. Interestingly, although there is a significant difference in apoptotic response among the variants, our HCR studies did not detect a statistically significant difference in DRC (8). This divergence between our study and the study by Spitz *et al.* (8) might also be caused by the differences in the experimental approach and the sensitivities of the assays. For example, Spitz *et al.* (8) used a BPDE-treated *CAT* gene as a reporter plasmid, representing a different source of DNA damage. It also is possible that differences in the cellular expression level of CAT and LUC proteins exist and that the radioactive CAT assay might be more sensitive than the Luciferase assay. Therefore, the differences in UV-induced apoptosis could reflect subtle differences in DRC.

UV-induced DNA damage leads to p53-mediated cell-cycle arrest at the G_1-S and G_2-M checkpoints. G_1-S checkpoint arrest prevents the initiation of replication on a damaged template, which can result in the fixation of mutations and cancer (29). XPD physically interacts with p53. It has been shown that p53 can bind through its CTD to XPD and modulate its helicase activity (18, 19). This is important for p53-mediated apoptosis and DNA repair. We investigated the hypothesis that p53 binding to XPD is affected by these sequence variations. Although our mapping studies revealed that XPD interacts through its CTD with p53 and that Lys751Gln is within this binding region, we could not detect any effect on p53 binding. Besides a possible sensitivity problem in the methodological approach it is also possible that the variant 751 has only a minor effect on p53 binding, because it has not demonstrated any biological significance in the apoptosis assays. It has been shown that the p44 subunit of the core TFIIH interacts with and modulates XPD helicase activity (30). Interestingly, our data show that both p53 and p44 bind to the CTD of XPD indicating a highly complex regulated interaction mechanism, which is not well understood, yet. Furthermore, in addition to its enzymatic activity, XPD is needed to stabilize the quaternary structure of TFIIH (30, 31). Therefore, it also is possible that these amino acid variations might have a higher impact on p44 binding and the stabilization of the TFIIH structure, and only minor differences in p53 binding.

Taken together, our results indicate that polymorphisms at codon 312 of the *XPD* gene have a functional consequence in UV-induced apoptosis and suggest a possible mechanistic explanation for the reported differences in cancer risk.

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