

Research article

A FUNCTIONAL ANALYSIS OF G23A POLYMORPHISM AND THE ALTERNATIVE SPLICING IN THE EXPRESSION OF THE XPA GENE

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Abstract: The *XPA* gene has a commonly occurring polymorphism (G23A) associated with cancer risk. This study assessed the functional significance of this polymorphism, which is localised near the translation start codon. Lymphoblastoid cell lines with alternative homozygous genotypes showed no significant differences in their XPA levels. The luciferase reporter assay detected no functional difference between the two sequences. Unexpectedly, we found that the alternatively spliced form of *XPA* mRNA lacked a part of exon 1. Only the reading frame downstream of codon Met59 was preserved. The alternative mRNA is expressed in various human tissues. The analysis of the 5' cDNA ends showed similar transcription start sites for the two forms. The *in vitro* expression of the alternative XPA labelled with the red fluorescent protein (mRFP) showed a lack of preferential nuclear accumulation of the XPA isoform. The biological role of the alternative *XPA* mRNA form remains to be elucidated.

Key words: Single nucleotide polymorphism, XPA, Kozak sequence, Alternative splicing, Cancer risk, Reporter assay

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Abbreviations used: DAPI – 4',6-diamidino-2-phenylindole; DMEM – Dulbecco's Modified Eagle Medium; DMSO – dimethylsulfoxide; dNTP – deoxyribonucleotide triphosphate; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; mRFP – monomeric red fluorescent protein; PCR – polymerase chain reaction; RACE – rapid amplification of cDNA ends; RPA – replication protein A; SNP – single nucleotide polymorphism; TDG – thymine-DNA glycosylase

INTRODUCTION

The nucleotide excision repair (NER) pathway removes DNA lesions induced by ultraviolet light (UV), tobacco smoke and other chemicals, including environmental pollutants or chemotherapeutic agents, such as cisplatin. The XPA protein participates in DNA damage recognition and recruits other NER components to the lesion site [1]. In humans, defects in NER, which are caused by mutations in the NER genes, are associated with a rare, cancer-prone syndrome called *xeroderma pigmentosum* (XP). This syndrome is characterized by an impaired DNA repair capacity, extreme UV sensitivity, skin abnormalities, skin cancer, and in some cases, neurodegeneration. XP complementation group A patients, who have a mutated *XPA* gene, show the most severe symptoms of all the XP types [2-4].

Variations in the DNA repair capacity caused by functional polymorphisms of DNA repair genes may result in the modulation of an individual's susceptibility to cancer [5, 6]. So far, reports on four infrequent single nucleotide substitutions in the coding sequence of the *XPA* gene have been published. These were not associated with XP [7-9]. However, the G23A polymorphism (rs1800975) present in the *XPA* 5' untranslated region has proven to occur commonly enough that population-based, case-control studies could be performed.

The G23A polymorphism is characterized by a G-to-A substitution within the Kozak sequence in the fourth nucleotide upstream from the initiation codon (ATG; Fig. 2A). The carriers of the two G alleles are wild-type homozygotes. This polymorphism has been associated with lung, endometrial, oesophageal and oral carcinoma risk in several studies [10-15]. Kiyohara and Yoshimasu wrote a meta-analysis on genetic polymorphisms and lung cancer risk, concluding that the *XPA* 23 GG genotype protects against lung cancer [16]. This polymorphism was also shown to influence the course of the disease or therapy response in bladder, ovarian and non-small cell lung cancers [17-19]. Healthy individuals with one or two G alleles exhibited a more efficient DNA repair capacity than AA genotype carriers [20].

Although the functionalities of the two *XPA* coding sequence polymorphisms have been studied [9, 21], there is no data on the functional significance of the more common one, the G23A polymorphism. As this polymorphism appears to be an important modulator of both the cancer risk and the outcome of cancer patients' treatment, we attempted to examine the functional significance of the G-to-A substitution. Interestingly, during this project we identified alternatively spliced *XPA* mRNA and investigated its expression pattern in a panel of RNAs from different human tissues. Moreover, we assessed the cellular localisation of the alternative mRNA-coded XPA isoform in the human osteosarcoma cell line U-2 OS.

MATERIALS AND METHODS

Cell culture and Western blotting for XPA

To identify Epstein-Barr virus-immortalized human lymphoblastoid cell lines (from Centre d'Etude du Polymorphisme Humain-CEPH and Utah pedigree families, Coriell Cell Repositories, Camden, NJ) as having the *XPA AA* or *GG* homozygous genotypes, DNA was isolated from cell lines via standard methods, and the samples were used as the templates for the genotyping reaction described below. Subsequently, three selected cell lines with the *AA* genotype and three with the *GG* genotype were grown on RPMI 1640 medium (Gibco BRL) supplemented with 15% heat-inactivated foetal calf serum at 37°C in 5% CO₂. Logarithmically growing cells were centrifuged, and the cell pellet was washed with phosphate-buffered saline (PBS) and snap-frozen. The cells were lysed via treatment with a RIPA buffer supplemented with protease inhibitors. The total cell lysate was obtained by incubating the cells in the buffer for 20 min on ice and centrifuging (14000 rpm, 20 min, 4°C). The supernatant was saved and an equal amount of protein extract (70 µg) was loaded and separated on a 12% denaturing polyacrylamide gel. After the electrotransfer, the nitrocellulose membrane was blocked with a 5% skim milk in PBS/0.1% Tween 20 solution and incubated with mouse monoclonal anti-XPA antibody, diluted to 1:400 (Ab-1; Lab Vision Corp.) at 4°C overnight. To verify that there was equal protein loading in each lane, the nitrocellulose membrane was reprobbed with the monoclonal anti-β-actin antibody (Chemicon Int.). To calculate the relative levels of the XPA protein in the studied cell lines, the pictures of the blots were scanned, and the areas of the peaks were measured in arbitrary units via semi-quantitative densitometric analysis (One-Dscan, Scanalytics). The levels of the XPA protein in each cell line were normalised to the corresponding β-actin levels.

Genotyping of the *XPA G23A* polymorphism

The polymorphism was detected via a PCR assay combined with restriction enzyme digestion (PCR-RFLP). The PCR primer sequences used were: (sense) 5' TCAGAAAGGCCGCTGGGT 3' and (anti-sense) 5' CTGGCGCAGCAT CAGTGC 3'. For PCR, 50 ng of genomic DNA was used with 1 x PCR Buffer II (PE Applied Biosystems), 1.5 mM MgCl₂, 0.2 mM of each dNTP (Pharmacia), 12.5 pmol of each primer (BioTez, Berlin), 2 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems) and 5% DMSO in a total volume of 25 µl. The reaction was performed as follows: pre-denaturation and enzyme activation at 94°C for 12 min; 30-35 cycles of denaturation at 94°C for 30 sec; primer annealing at 63°C for 45 sec; primer extension for 45 sec at 72°C; and then a final extension at 72°C for 4 min. The PCR yielded a 233-bp *XPA* DNA fragment. Next, 10 µl of the PCR product was digested with 6 U of *MspI*, according to the manufacturer's instructions (New England Biolabs). The digestion products were separated in 4% NuSieve:SeaKem (3:1) agarose gels

(BMA). The *XPA* G23A polymorphism results in a loss of the *MspI* restriction site, and the digestion pattern observed was: 108-, 95-, and 30-bp fragments in homozygote *GG* (an additional control digestion site was present within the PCR product); 138-, 108-, 95-, and 30-bp fragments in heterozygote *GA*; and 138-, and 95-bp fragments in homozygote *AA*.

Molecular cloning of plasmids with the *XPA* variant 23G and variant 23A promoter

The *XPA* polymorphism is located within the Kozak consensus sequence. The wild-type and polymorphic versions of the *XPA* promoter and the 5'UTR region were inserted into the pGL3-Basic plasmid, which contains the firefly luciferase gene *luc*⁺ (GenBank U47295; Promega, Madison). This allows for reporter luciferase gene expression under the transcriptional and translational control of the *XPA* promoter, and 5'UTR in either the wild-type or polymorphic version using a reporter assay described below under "Functional assay for the *XPA* G23A polymorphism". The cloning was performed using the triple-ligation strategy, involving the ligation of two inserts (e.g. PCR products) into a plasmid at three mutually incompatible restriction sites (*HindIII*, *PpuMI* and *BstBI* in our experiment, Fig. 1). The first PCR fragment (from position 1228 to 1803 of the GeneBank U16815 sequence) was amplified from genomic DNA and encompassed the *XPA* promoter sequence and 5'UTR with the *PpuMI* restriction site located 17 residues upstream from the *XPA* translation start codon. This fragment was amplified using the primers: **TTTTAAGCTTAAGGCTGTGTC TCTAGGCCG** (sense), containing the *HindIII* restriction site (bold font), and **CTGGCGCAGCATCAGTGC** (anti-sense) located in the *XPA* exon 1. The fragment downstream of the *PpuMI* site was removed after the restriction digestion reaction and gel purification of the PCR product.

The second PCR product contained the fragment of the *XPA* 5'UTR downstream from and with the *PpuMI* restriction site and the polymorphic residue (either G or A), introduced by specifically designed sense primers, preceding the *luc*⁺ start codon. The sequences of the sense primers were: for the G variant, **GAGCTAGGTCCTCGGAGTGGGCCgGAGATGGAAGACGCCAAAAACA TAAAG** and for the A variant **GAGCTAGGTCCTCGGAGTGGGCCaGAG ATGGAAGACGCCAAAAACATAAAG**. The primers start with the sequence complementary to the *XPA* 5'UTR containing the *PpuMI* site (marked by bold characters) and the polymorphic residue (small characters). The sequence complementary to the luciferase sequence is underlined. The anti-sense primer (GTGATTTGTATTTCAGCCCATATCG) was located within the luciferase cDNA sequence and was complementary to the sequence downstream from the *BstBI* restriction site (Fig. 1). The pGL3-Basic DNA served as a template for the generation of these PCR products (G-luc or A-luc fragments, 255 bp each).

The PCR reactions were performed in 50 μ l with 100 ng of template DNA, 50 pmols of each primer (BioTez, Berlin), 1x PCR Buffer II (Applied Biosystems), 2.5 mM MgCl₂, 200-300 μ M of each dNTP (Pharmacia) and 2.5 U of AmpliTaq

DNA polymerase (Applied Biosystems). The amplification of the *XPA* promoter region required the use of 1 mM betaine and 2.5 U AmpliTaq Gold DNA polymerase. The temperature profile of PCR for the *XPA* promoter fragment was 95°C for 10 min (DNA denaturation and polymerase activation); then 40 cycles at 95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec; followed by a final extension in 72°C for 7 min. For the G-luc or A-luc fragments, pre-denaturation was at 95°C for 5 min; then 30 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec; and a final extension at 72°C for 4 min. Next, the PCR products were gel-purified with a QIAEX II kit (Qiagen), and digested with *Hind*III and *Ppu*MI (*XPA* promoter) or with *Ppu*MI and *Bst*BI (G-luc, A-luc), according to the manufacturer's instructions (New England BioLabs), then gel purified again. The pGL3-Basic plasmid was digested with *Hind*III and *Bst*BI, and also gel purified. The digested PCR products were ligated into the *Hind*III and *Bst*BI sites located in the plasmid. The ligation products were transferred into competent DH5 α bacteria. After recombinant plasmid purification, the clones carrying the vectors with either the G variant (pGL3-XPApromG) or A variant (pGL3-XPApromA) were identified. The inserts of the selected clones were sequenced with a BigDyeTM Terminator Cycle Sequencing kit (Applied Biosystems), using the primers GTGATTTGTATTCAGCCCATATCG and TCTTCCAGCGGATAGAATG to check for the presence of the *XPA* G or A sequence and to verify that the inserts did not contain any mutations that may have been introduced during the PCR. Subsequently, the G and A clones were used to produce four batches of each plasmid DNA using a Qiagen QIAprep Spin Miniprep kit (Qiagen). After measuring the concentration of the plasmid solutions (Beckman DU 640B spectrophotometer), the DNA concentrations in all of the batches were equalised using Tris-HCl buffer at pH 8.0.

The DLR assay for the *XPA* G23A polymorphism

To study the influence of the *XPA* G23A polymorphism on the activity of the *XPA* promoter/5'UTR region, we used the Dual-Luciferase ReporterTM (DLR) assay system (Promega), which measures the reporter gene (*luc*⁺) activity under the transcriptional control of an inserted promoter. Two plasmid versions, containing the *XPA* promoter sequence with either the G or A nucleotide in the fourth position before a start codon of the *luc*⁺ gene (i.e., pGL3-XPApromG or pGL3-XPApromA constructs, respectively), were transfected separately into human NCI H1299 cells in culture together with a control vector, pRL-TK (Promega). The pRL-TK control plasmid contains the herpes simplex virus thymidine kinase promoter, controlling the expression of the *Rluc* gene, which codes for a luciferase from *Renilla reniformis*. Both the firefly and *Renilla* luciferase activities were measured together in the same cell lysate in a single tube, using the Lumat LB 9501 luminometer (Berthold Technologies). The activity of the *Renilla* luciferase served as an internal control. The firefly luciferase activity in each experiment was normalised to the activity of the internal control to minimise experimental variability caused by differences in the

transfection and cell lysis efficiency. In each measurement, the normalised firefly luciferase activity (NFLA) was calculated by dividing the firefly luciferase activity reading by the *Renilla* luciferase activity reading. Four transfections and luciferase assays were performed for each of the plasmids, pGL3-XPA_{promG} and pGL3-XPA_{promA}. The control transfection with the pGL3-Basic plasmid was also performed. This experiment was repeated twice, using different batches of the plasmid preparations to avoid the variability that could be introduced due to differences in the DNA quality. The means and standard deviations for the NFLA were calculated for the experiments, and the statistical significance of the NFLA difference between cells transfected with G or A vectors was evaluated using the *t*-test.

The cell line used in this study (lung carcinoma cell line NCI H1299, from the American Type Culture Collection) was grown in DMEM medium supplemented with 10% foetal bovine serum (Gibco BRL), and was maintained at 37°C in 5% CO₂. The cells were grown in 1 ml of medium on 12-well plates (NUNC Brand Products). All of the transfections were performed on subconfluent cells (40% confluence) with the FuGene 6 reagent, according to the manufacturer's instructions (Roche Applied Sciences). The experimental/control vector ratio was 10:1. The activity of the reporter gene was measured 24 h after the transfection.

RT-PCR of XPA

The amplification of the 5' fragment of *XPA* cDNA was performed using the XPA12 anti-sense primer (GCTTGTGTTTATCATCAGCATC), located at the beginning of exon 4, and the XPA14 sense primer (TAGGTCCTCGGAGTG GGC), located in exon 1 upstream of the translation start site (Fig. 2C). The PCR was performed in a reaction volume of 25 µl, with 1x PCR buffer II (Applied Biosystem), 1.5 mM MgCl₂, 10 pmols of each primer 200 µM of each dNTPs, and 1 unit of AmpliTaq Gold polymerase (Applied Biosystems). The enzyme was activated by 10 min incubation at 95°C, which was followed by 35 cycles at 94°C for 30 sec, 60°C for 20 sec, and 72°C for 40 sec, and a final extension at 72°C for 7 min. Under these conditions, only the alternatively spliced *XPA* sequence was amplified. To amplify the major sequence, the reaction mixture was supplemented with a 1 M betaine solution (Aldrich Chemical Co). The other reaction conditions were not changed, except for the number of PCR cycles, which was lowered to 30. All of the PCR reactions were performed in 200-µl reaction tubes in a Mastercycler (Eppendorf). The templates were commercially available cDNA samples synthesized from RNA isolated from various human tissues (Human MTC Panel I; BD Biosciences), and the control PCR reaction was performed according to the manufacturer's protocol with primers amplifying the *GAPDH* housekeeping gene, provided in the Human MTC Panel I kit (BD Biosciences).

The 5'-RACE analysis of XPA

The analysis was performed using the Gene Racer kit (Invitrogen). The synthesis of cDNA was primed by XPA-SEQ2 oligonucleotide (AATTTAAGAGGTG GCTCTC) using, as a template, RNA isolated from normal human fibroblasts GM08402, obtained from Coriell Cell Repositories. The first round of 5'-RACE PCR was performed using GeneRacer 5 primer from the kit and XPA-RACE2 primer (TTTACATTAGCCATGCCGAG) spanning the exon 1-exon 2 junction of the alternative transcript. This primer pair can only amplify the alternative sequence. The nested PCR was performed using the GeneRacer 5 Nested primer and XPA-RACE3 primer (TCCGCGGGTTGCTCTAAAGC) complementary to the exon 1 fragment. The product of the nested PCR was directly sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) after purification by exonuclease I and shrimp alkaline phosphatase. Alternatively, the two rounds of PCR were performed with primers: GeneRacer 5 and XPA 11 (AAGATATTCTTGTGTTTGCCTCTG); GeneRacer 5 Nested and XPA12 (Fig. 2C). These primer pairs can amplify both major and alternatively spliced sequences. The amplification reactions using these primers were performed either with or without 1 M of betaine solution. The resulting PCR products were cloned using the TOPO cloning kit for sequencing (Invitrogen) and the inserts of plasmid DNA isolated from several clones were sequenced.

The molecular cloning of XPA-mRFP and protein localisation analysis

The *XPA* cDNA was amplified using the primers XPA-Afl-S (TTTTCTTAAGCTGGGAGCTAGGTCCTCGGAG) and XPA-Afl-A (TTTTCTTAAGCATTTTTTCATATGTCAGTTCATGGCC), containing the *Afl*III restriction sites (underlined). The PCR product was gel purified and ligated into the *Afl*III site of the pcDNA3.1/HisC expression vector (Invitrogen) yielding the XPA-AFL-HisC plasmid. Subsequently, the *Hind*III-*Bam*HI fragment of the previously made XPA-EGFP plasmid was cut and gel-purified. The *Hind*III site is located in *XPA* cDNA (Fig. 2C and 3E), and the *Bam*HI site was placed just downstream of the last amino acid codon of XPA. This fragment was ligated to the *Hind*III and *Bam*HI sites of the XPA-AFL-HisC vector. This step removed the second *Afl*III site, downstream of the *XPA* cDNA from the XPA-AFL-HisC vector, and the part of the pcDNA3.1/His C polylinker between the *Afl*III and *Bam*HI sites (the fragment between *Bam*HI and *Apa*I was left). The resulting plasmid XPA-AFL-BAM vector contained a single *Afl*III site joining the vector with the *XPA* cDNA and *Bam*HI site downstream of the *XPA* cDNA sequence, suitable to join other protein coding sequences in-frame with the *XPA* open reading frame (ORF). The *mRFP1* sequence preceded by the Xpress antibody epitope coding sequence was PCR-amplified from the plasmid provided by Roger Tsien [22] with primers containing the *Bam*HI (upstream) and *Not*I (downstream) sites, and ligated into the *Bam*HI and *Not*I sites of the XPA-AFL-BAM plasmid, resulting in the XPA-mRFP plasmid (shown in Fig. 3E). The XPA and mRFP1 are linked by the Xpress antibody epitope sequence, suitable

for fusion protein detection via Western blotting. The plasmid, with the alternative form of *XPA* cDNA was constructed via PCR amplification, without the betaine of the alternative cDNA sequence but with the primers, XPA-Afl-S and XPA11, and ligation of the PCR product into the *Afl*III and *Hind*III sites of the XPA-mRFP, ultimately generating the Alt-XPA-mRFP vector. All of the PCR-generated sequences were sequenced to ensure that there were no mutations in the plasmids.

The cellular localisation of the mRFP-labelled XPA forms was examined after the transfection with FuGene6 (Roche) of the expression vectors into the U-2 OS human osteosarcoma cell line cultured in DMEM medium supplemented with 10% FBS and penicillin-streptomycin solution. The cells were fixed in 3.7% formalin 28 h after transfection, and after ethanol dehydration, they were mounted in Vectashield with DAPI (Vector Laboratories). The cells were photographed using a Nikon Eclipse fluorescent microscope.

Statistical analysis

The Mann Whitney U-test and Student's t-test were respectively used to compare the differences in the XPA protein levels between the cell lines with the *AA* and *GG* genotypes, and in the mean NFLA values between the cell lines transfected with the pGL3-XPApromG and pGL3-XPApromA reporter vectors. Differences were considered statistically significant at $P < 0.05$. All of the statistical analyses were performed using STATISTICA 6.0 software.

RESULTS

The functional analysis of G23A polymorphism

To find out whether the *XPA* polymorphism located within the Kozak consensus sequence in the 5'UTR had any influence on the *XPA* promoter/5'UTR activity, we inserted the relevant *XPA* region into the pGL3-Basic plasmid (Fig. 1A). The firefly *luc*⁺ gene carried by the plasmid was under the transcriptional and translational control of the *XPA* promoter/5'UTR with or without the polymorphism (versions G or A, i.e. the pGL3-XPApromG or pGL3-XPApromA vectors, respectively). The activity of the reporter *luc*⁺ gene is presented as normalised firefly luciferase activity (NFLA), which is calculated by dividing the reporter (firefly) luciferase activity reading by the control *Renilla* luciferase activity reading (pRL-TK plasmid co-transfection – see the Materials and Methods section). The reporter gene activities were similar for the G and A versions of the sequence (respectively 5.10 ± 0.66 versus 4.91 ± 0.57 , $P = 0.67$ by *t*-test; Fig. 1B). The results demonstrate that the G23A substitution does not influence the reporter gene expression in this assay.

Then, to test the hypothesis that the *XPA* polymorphism may modulate the protein level in other cell types and under conditions that were closer to physiological, we determined the amount of XPA protein in extracts derived from three different lymphoblastoid cell lines with *AA* genotypes and three lymphoblastoid cell lines with *GG* genotypes via densitometric analysis of

Western blots. As illustrated in Fig. 1C, the two groups of cell lines had similar XPA protein levels (GG: 94.1 ± 1.5 versus AA: 89.8 ± 1.3 , $P = 0.13$, via Mann Whitney U-test). Thus, in the examined cells growing under standard conditions, the polymorphism was not associated with detectable changes in the XPA protein level.

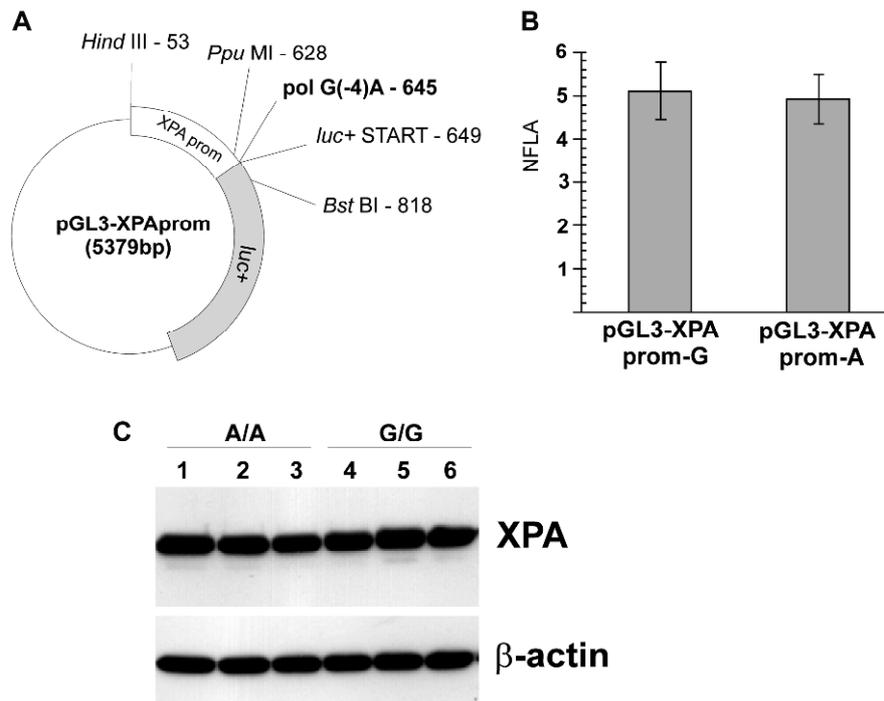


Fig. 1. The functional analysis of G23A polymorphism. A – The plasmid map showing the *XPA* promoter inserted upstream of the *luc*⁺ gene, the position of the polymorphic nucleotide *pol G(-4)A*, and the restriction sites used for the cloning. The start codon of the *luc*⁺ open-reading frame is also marked. For image clarity, other plasmid features are not shown. B – The normalised firefly luciferase activity (NFLA) in the NCI H1299 cell line transfected with the pGL3-XPApromG or pGL3-XPApromA reporter vectors, together with the control pRL-TK vector. Each bar shows the mean NFLA value from four separate transfections with the respective reporter plasmid and the control plasmid, while the whiskers show the standard deviation of the mean (\pm SD). C – Western blot analysis of XPA protein expression levels in three different lymphoblastoid cell lines with the *XPA* G23A homozygous AA genotype and three lymphoblastoid cell lines with the GG genotype. Lines: 1 – #6992, 2 – #7005, 3 – #6988, 4 – #7000, 5 – #7062, 6 – #7053 (CEPH lymphoblastoid cell lines).

The alternatively spliced form of *XPA* mRNA

Unexpectedly, the amplification of the 5' end fragment of the *XPA* cDNA by RT-PCR, using XPA14 and XPA12 primers, yielded a single DNA fragment that

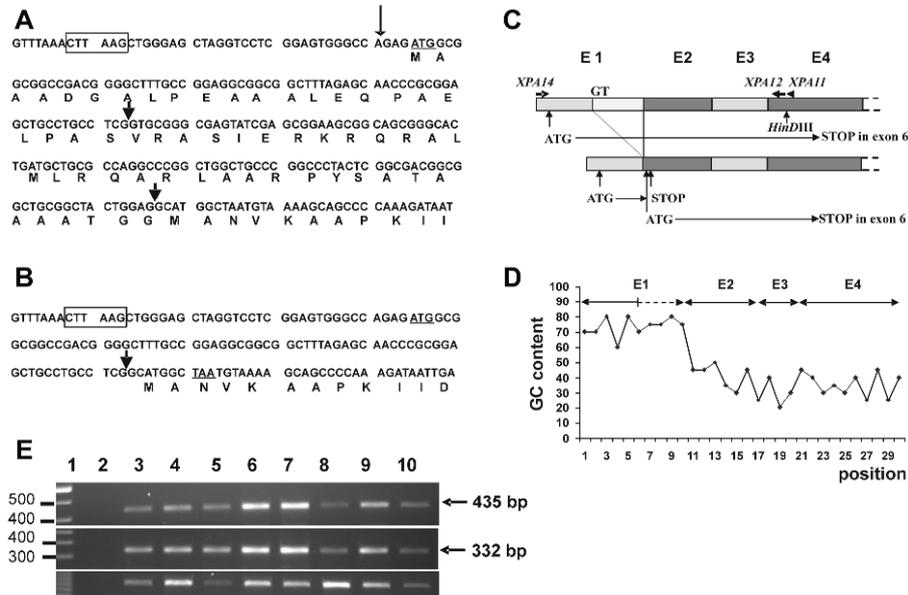


Fig. 2. The alternative splicing of XPA mRNA. A – The 5' end sequence of the major *XPA* cDNA form. The *Afl*III cloning site of the pcDNA3.1/HisC expression vector is marked by the rectangle. The XPA amino acid sequence is shown below the cDNA sequence. The arrowheads flank the sequence spliced out from the alternative mRNA. The polymorphic residue is marked by the thin arrow. B – The 5' end of the alternatively spliced form of *XPA* cDNA. The alternative site of the exon junctions is marked by an arrow. The ATG translation start codon of the major *XPA* mRNA is underlined as well as the in-frame stop codon TAA. This mRNA can produce part of the XPA protein lacking the first 58 amino acids, provided that the translation starts from the second ATG codon corresponding to codon 59 of the major *XPA* mRNA. C – The positions of the exon-coded fragments of the major (longer) and alternatively spliced form (shorter) of *XPA* mRNA. E1-E4 represent exons 1-4. The last two exons are not shown. The XPA12 and XPA14 arrowheads mark the position of the PCR primers used to detect the mRNA forms by RT-PCR. The GT represents the position of the alternative splicing 5' donor site. The ATG in exon 1 represents the first translation start codon of the *XPA* open-reading frame (ORF). This reading frame ends in exon 2 (STOP) in the alternative mRNA. The position of the methionine codon (ATG) in exon 2 that may drive the translation of the XPA protein without the first 58 amino acids is also shown. D – The GC content in the major form of *XPA* cDNA calculated for consecutive 20-bp fragments. E1-E4 represent exons 1-4. The dashed arrow represents the part of exon 1 that is spliced out in the alternative mRNA. E – The RT-PCR products amplified with the XPA12 and XPA14 primers from cDNA derived from various human tissues. Top panel – the major *XPA* cDNA form (PCR performed with betaine, product length 435 bp). Middle panel – the alternative *XPA* cDNA (PCR performed without betaine, product length 332 bp). Bottom panel – the cDNA fragment of the *GAPDH* housekeeping gene amplified using gene specific primers. Lanes: 1 – 100-bp ladder, 2 – blank control, 3 – brain, 4 – heart, 5 – lung, 6 – liver, 7 – pancreas, 8 – skeletal muscle, 9 – kidney, and 10 – placenta.

was about 100 bp shorter than expected (Fig. 2E, middle panel). The sequencing revealed that this DNA fragment is derived from *XPA* cDNA with a missing fragment of exon 1 (Fig. 2B). The analysis of the missing sequence indicated that the shorter fragment was formed by the use of the alternative 5' donor splicing site located in exon 1, 70 residues downstream of the first nucleotide of the start codon (Fig. 2A, C). The major form of *XPA* mRNA could be amplified if 1 M betaine was present in the PCR reaction. In the presence of betaine, the amplification of the minor splicing form was not detectable. The betaine helps to amplify GC-rich sequences. The GC content in exon 1 is higher than in the rest of the *XPA* cDNA (Fig. 2D). The analysis of the alternatively spliced form indicated that the reading frame defined by the first ATG codon ends 27 codons downstream, at the beginning of exon 2-coded fragment (Fig. 2B, C). The alternative mRNA could direct the production of XPA if the translation started from codon 59, coding for methionine in the context of the Kozak sequence (Fig. 2A, B, C). In the alternatively spliced mRNA, there was no other upstream start codon in frame with the *XPA* ORF, assuming that both forms had the same 5' end. To find out if this assumption was correct, we performed a 5'-RACE analysis of the alternative mRNA on RNA isolated from normal human fibroblasts. The first RACE-PCR amplification was performed with the primers GeneRacer 5 and XPA-RACE2 (a primer complementary to the alternative junction of exon 1-exon 2, amplifying only the alternative transcript) and the second, nested PCR was performed using the GeneRacer 5 Nested and XPA-RACE3 primers. The direct sequencing of the nested PCR product revealed that the major transcription start is located 32 nucleotides upstream from the translation site (TGGAGCTGGGAGC, transcription start site is underlined). When the 5'-RACE analysis was performed using XPA11 and XPA12 as anti-sense primers, and the betaine was not used in the PCR reaction mixture (Fig. 2C), the alternative mRNA end was preferentially amplified and the longest transcript did not go beyond the 35th residue upstream from the translation start codon. However, when the same primers were used as the anti-sense primers and the betaine was added to the PCR reaction mixtures at a concentration of 1 M, the two transcripts were amplified. This RACE-PCR product was cloned into the pCR4-TOPO vector and the inserts of six clones were sequenced. Two clones represented the alternative sequence starting 28 and 57 residues upstream from the start codon and four clones represented the major *XPA* mRNA form starting 27, 28, 35 and 87 residues upstream from the start codon. Thus, our 5'-RACE analysis showed transcription start heterogeneity and did not detect any alternative transcript that would go upstream of the 57th residue of the major translation start codon.

The expression of the alternative *XPA* mRNA and localisation of its protein

The presence and the cellular level of the alternative *XPA* mRNA sequence were determined via RT-PCR. The amplification was performed using the XPA14 and XPA12 primers located as shown in Fig. 2C. The 35 cycles of PCR were

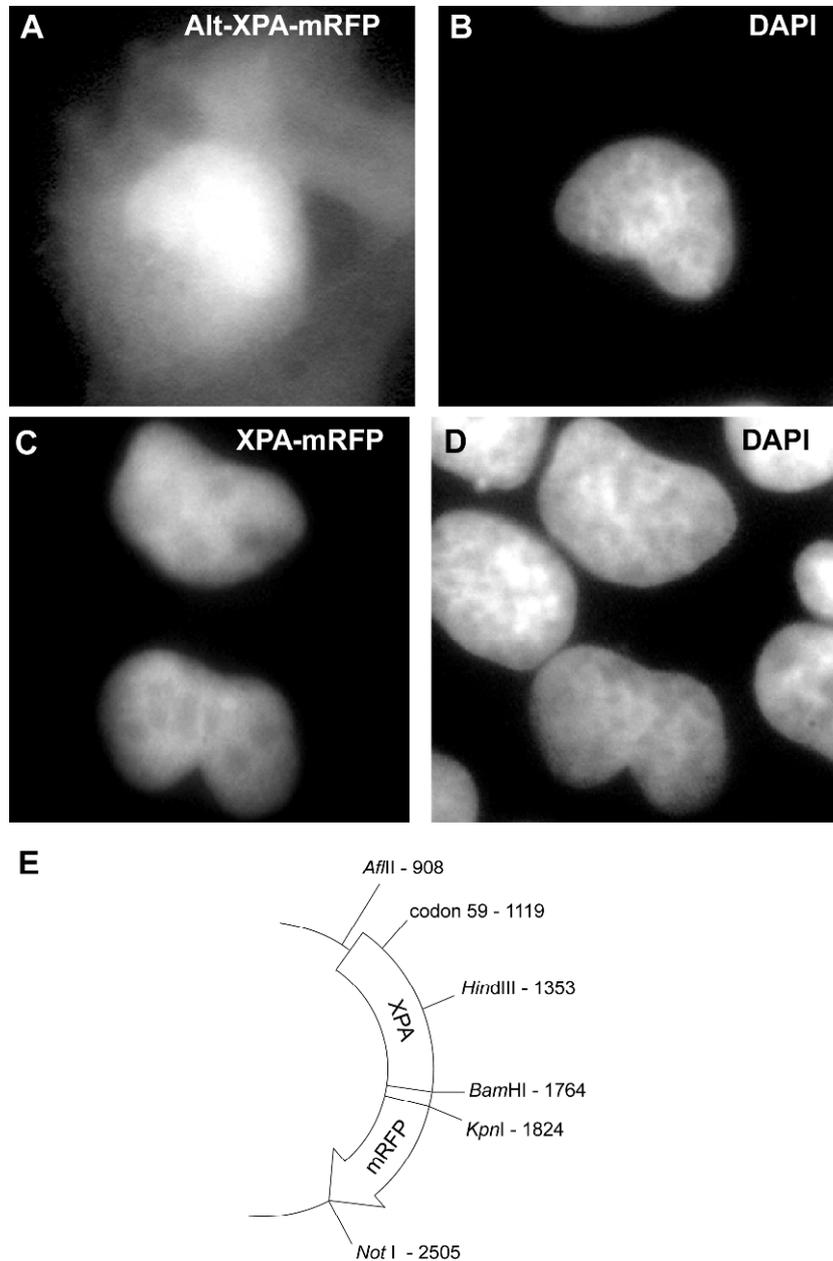


Fig. 3. The cellular localisation of mRFP-tagged XPA in U-2 OS cells. A – The localisation of the tagged XPA coded by the alternative mRNA; the cell nucleus is stained with DAPI (B). C – The localisation of the major XPA form (two visible cells express the recombinant protein); the nuclei are counterstained with DAPI (D). E – The structure of the cloned cDNA sequence coding for the major form of XPA fused to the mRFP by a short linker between the *Bam*HI and *Kpn*I sites.

performed without betaine. As a template, we used a panel of first-strand cDNA solutions purchased from BD Biosciences, derived from RNA samples isolated from brain, heart, lung, liver, pancreas, skeletal muscle, kidney, and placenta tissue. Four independent amplifications were performed, and the amount of PCR product on the agarose gel is shown in Fig. 2E (middle panel). All of the amplifications yielded a similar pattern of the relative *XPA* expression, i.e. the highest expression of the alternative form can be detected in the RNA isolated from the liver and pancreas, whereas the lowest level is detected in the skeletal muscles. The expression pattern of the major *XPA* form is very similar to the expression of the alternative form, indicating that the expression of both mRNAs is coordinated (Fig. 2E, top panel). The control, housekeeping gene (*GAPDH*) also shows variable expression in the examined human organs (Fig. 2E, bottom panel). This is not unusual and was observed by others: the relatively high expression of the gene in the heart and skeletal muscles and low expression in the lung and placenta is consistent with the relative abundance of *GAPDH* mRNA molecules in the respective human tissues [23].

Next, we cloned the *XPA* cDNA sequence into an expression vector and fused it with the cDNA coding for the red fluorescent protein (mRFP). The map of the expression vector, named XPA-mRFP, is shown in Fig. 3E. The *XPA* cDNA sequence together with the 31 nucleotides upstream from the translation start site was inserted using PCR into the *Afl*III site of the pcDNA3.1/HisC vector (Invitrogen). The six nucleotides forming the *Bam*HI site were used to join the last codon of *XPA* with the sequence coding for the anti-Xpress antibody epitope, which was followed by the cDNA of the mRFP1 sequence ending with the *Not*I site of pcDNA3.1/HisC. The identity of the alternative sequence was confirmed via the sequencing of the *XPA* cDNA. The vector was named Alt-XPA-mRFP.

The localisation of XPA-mRFP- and Alt-XPA-mRFP-coded proteins was examined in the U-2 OS cells 28 h after the start of transfection. As expected, the major form of the XPA protein showed conspicuous nuclear localisation (Fig. 3C, D). However, the mRFP-labelled protein expressed from the Alt-XPA-mRFP vector showed both cytoplasmic and nuclear localisation patterns (Fig. 3A, B), which is not surprising considering the presence of the nuclear localisation signal within the first 58-amino acid sequence of XPA [24], i.e. the fragment that is lost due to the alternative splicing (Fig. 2A, B).

DISCUSSION

The *XPA* G23A polymorphism significantly modulates the risk of various cancers [10-16], the DNA repair capacity [20], the level of DNA damage [25] and the course of the disease [17-19]. However, the mechanistic basis of these epidemiological observations is elusive. The *AA* genotype is associated with a lower DNA repair capacity [20], and a higher level of DNA damage [25], and it is believed that suboptimal DNA repair associated with the *AA* genotype leads

to an increased risk of cancer [16]. This may also modulate the response of an individual to the therapy of cancer [18]. Although the epidemiological data is quite consistent, especially in relation to the lung cancer risk [16], the mechanistic explanation of this phenomenon is only a matter of speculation. Our study was an attempt to better understand the molecular basis of the epidemiological observations. One report showed an increased cancer risk in heterozygous carriers of the *xeroderma pigmentosum* mutation [26]. We hypothesized that the *XPA* polymorphism could impair the expression of the *XPA* gene, leading to a decreased DNA repair capacity and an increased cancer risk. However, in our experiments, we did not find evidence that the polymorphism modulated the expression level of the XPA protein. The G23A substitution is localised within the sequence surrounding the translation start codon in the 5'UTR, and it may influence the translation and/or transcription efficiency. Moreover, this substitution destroys a CpG site, and may interfere with proper methylation of the *XPA* promoter and proper regulation of *XPA* expression. Those observations prompted us to study the influence of this SNP on the *XPA* promoter/5'UTR activity using the DLR assay, and its influence on the amount of XPA protein in cell lines exhibiting homozygous *GG* or *AA* genotypes.

The sequence context of the start codon is assumed to affect the protein translation initiation process. Therefore, changes in translation efficiency may cause a variation in protein expression between individuals [27]. Although the XPA protein expression levels measured in the two *XPA* genotype groups of the lymphoblastoid cell lines were similar in our study, others observed that the polymorphism in the Kozak sequence of different genes modulated the amount of the expressed protein [28, 29].

Moreover, tissue and organ-specific differences in the mRNA levels observed for various NER genes including *XPA* [30, 31] may influence the repair rate of a particular tissue. Also, in this study, we detected differences in the expression levels of *XPA* mRNA between various human organs (Fig. 2E). Thus, a possible modulating effect of the *XPA* polymorphism may be dependent on the tissue type and may be different in white blood cells compared with lung epithelial cells. Alternatively, this *XPA* polymorphism may be in linkage disequilibrium with another functional polymorphism that is as yet unknown, and may also explain why we observed no effect of the *XPA* G23A polymorphism on the activity of the reporter *luc*⁺ gene.

Unexpectedly, we observed the existence of alternatively spliced form of *XPA* mRNA. It was preferentially amplified in PCR without betaine. This substance is frequently employed to amplify GC-rich sequences [32]. The 5'-RACE analysis showed that both major and alternative forms of *XPA* mRNA had heterogenous transcription start sites, a common phenomenon in the housekeeping genes. However, this heterogeneity could be clearly seen only if the RACE-PCR was performed under the conditions allowing for the amplification of GC-rich sequences. Moreover, the analysis of the longest 5' fragment of the alternative

mRNA indicated that in the alternative mRNA sequence, there was no translation start site upstream from codon 59 that would be in frame with the *XPA* ORF downstream from codon 59. Thus, if the alternative form is translated, it produces XPA molecules with 58 amino acids deleted from the N-terminus. This truncated XPA isoform lacks a nuclear localisation signal and the RPA p34 subunit binding site [24]. Fused to the mRFP and expressed in cells, it shows both nuclear and cytoplasmic localisation (Fig. 3A, B). It remains to be determined whether it is produced in cells from the endogenous *XPA* locus, and if so, what function it has.

Previously, we showed the presence of the alternative splicing form of the *TDG* gene with missing exon 2. Analysis of this alternative *TDG* sequence did not show clearly what protein it encodes [33]. Alternative splicing is a common phenomenon changing the diversity of protein molecules [34]. It also is common among genes coding DNA repair proteins [35, 36], enabling them, for instance, to code for protein isoforms able to enter the mitochondria and repair mitochondrial DNA [37]. To the best of our knowledge, the alternative splicing of *XPA* has not been reported so far. Frequently, the alternatively spliced mRNA molecules are less efficiently translated than the major mRNA forms [38], and sometimes they produce protein molecules showing a dominant-negative effect [39] or are aberrantly localised [40]. It remains to be determined whether the alternatively spliced form of *XPA* mRNA has physiological significance or if it is merely a product of the imperfect splicing. Interestingly, exon 1 is the only *XPA* exon where *xeroderma pigmentosum* mutations have not yet been found. It is likely that the truncated protein produced from the alternative mRNA and translated from the Met59 coded by exon 2 is able to provide enough XPA activity that the exon 1 mutations do not show severe phenotypic effects. Thus, some alternative mRNA forms may be a source of residual protein activity protecting cells from the deleterious effects of mutations. A case of *xeroderma pigmentosum* group A (XP-A) was reported with a novel *XPA* mutation destroying the splice donor site in intron 1 [41]. The RT-PCR did not reveal the existence of the major *XPA* mRNA in samples isolated from the cells of this patient. Instead, the researchers could amplify only the truncated form of the *XPA* mRNA sequence. This form (named def E1A) is identical to the alternatively spliced form that we amplified from normal human fibroblasts and other sources. Tanioka *et al.* [41] considered this form as aberrantly spliced, which was justified because under the PCR conditions used to amplify *XPA* mRNA from normal, control fibroblasts, the alternative *XPA* mRNA did not show up. However, we amplified this form from many different normal tissues indicating its non-pathological origin. Thus, what they detected [41] is apparently not aberrantly spliced, but an alternatively spliced mRNA form which is present in the cells of the examined XP-A patient. This alternative form may provide some residual XPA protein activity. This hypothesis is supported by the observation that the examined XP-A patient did not show the severe neurological symptoms that are characteristic for patients with mutations that

destroy XPA activity (usually mutations affecting the DNA-binding domain of XPA) [3]. Thus, some residual XPA activity is probably preserved in the cells of the newly detected XP-A case. This supposition is not contradicted by the lack of detection of the truncated XPA protein by the monoclonal antibody [41]. This form may be expressed below the detection threshold level of the antibody in the Western blot.

The results presented in this study and the observations reported by others [41] warrant further research on the alternatively spliced *XPA* form. This research may improve our understanding of XPA functioning and may help to explain the molecular pathology of some *xeroderma pigmentosum* cases.

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