

A new xeroderma pigmentosum group C poly(AT) insertion/deletion polymorphism

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We found a common biallelic polymorphism (PAT) in the xeroderma pigmentosum complementation group C (XPC) DNA repair gene consisting of an insertion of 83 bases of A and T [poly(AT)] and a 5 base deletion within intron 9. We developed a PCR assay to resolve the XPC PAT+ and PAT- alleles and found that the PAT+ allele frequency was 0.44 in 156 cancer-free donors from the Johns Hopkins School of Public Health, 0.41 in 263 cancer-free donors from the Baltimore Longitudinal Study of Aging and 0.36 in samples from 216 unselected donors from NIH. We also found a single nucleotide polymorphism in exon 15 of the XPC gene (A2920C, Lys939→Gln) that creates a new enzyme restriction site. This XPC exon 15 single nucleotide polymorphism occurred at a frequency of 0.38 in 98 NIH donors and is in linkage disequilibrium with the PAT locus. We developed an allele-specific complementation assay utilizing post-UV host cell reactivation to assess DNA repair capacity of polymorphic alleles. We found similar DNA repair with XPC 2920A and XPC 2920C. These common polymorphisms in the XPC DNA repair gene may be useful for molecular epidemiological studies of cancer susceptibility.

Introduction

We have been searching for alterations of DNA repair genes (1,2) in the general population that may be associated with differences in cancer susceptibility. Defective DNA repair may lead to increased risk of skin cancer since patients with the rare recessive disorder xeroderma pigmentosum (XP) have a >1000-fold increase in sunlight-induced skin cancer and a smaller increase in internal cancers in association with defective DNA repair (3). It is possible that variations in DNA repair due to inherited polymorphisms of DNA repair genes might be associated with cancer susceptibility in the general population. Of the seven XP complementation groups, XP complementation group C (XPC) is relatively common in the USA.

Abbreviations: BLSA, Baltimore Longitudinal Study of Aging; CAT, chloramphenicol acetyl transferase; JHSPH, Johns Hopkins School of Public Health; NIH, National Institutes of Health; RFLP, restriction fragment length polymorphism; XP, xeroderma pigmentosum; XPC, xeroderma pigmentosum complementation group C.

The XPC gene contains 15 exons and codes for a 940 amino acid protein that plays a role in DNA damage recognition (4–7). We have found a common new biallelic polymorphism (PAT) in an intron in the XPC gene that is in linkage disequilibrium with a single nucleotide polymorphism in exon 15.

Materials and methods

DNA extraction and analysis of PAT polymorphism

DNA was extracted from lymphocytes and buccal swabs by alkaline lysis (8). The XPC PAT+/- polymorphism site within intron 9 of the XPC gene was PCR amplified using a forward primer at intron 9 (bp 1258–1276), designated N1m (5'-TAGCACCCAGCAGTCAAAG-3'), and a reverse primer at intron 9 (bp 1523–1504), designated N2n (5'-TGTGAATGTGCTTAATGCTG-3'). As an internal standard, sense and antisense β -actin primers were employed (9) that resulted in a fragment of 621 bp. Each PCR was performed in a total volume of 20 μ l containing 50 ng DNA, 50 ng N1m and N2n primers, 7 ng each β -actin primer, 1.6 μ l of 2.5 mM dCTG (dCTP, dTTP, dGTP), 0.05 μ l of 10 mM dATP, 0.06 μ l of [α -³²P]dATP (3000 Ci/mmol; NEN DuPont), 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 1.5 mM MgCl₂ and 1 U *Taq* DNA polymerase (Gibco BRL). Amplification conditions consisted of an initial denaturing step (94°C for 4 min) followed by 25 consecutive cycles of denaturation at 94°C for 20 s, annealing at 58°C for 30 s and extension at 72°C for 90 s, with a final elongation at 72°C for 10 min. The PCR products were diluted with sequencing dye solution (1:1) and 5 μ l were electrophoresed in a 6% polyacrylamide sequencing gel. The gels were dried and autoradiographed for 1–2 days with intensifying screens. Fragment sizes were measured relative to DNA sequence ladders derived from a known sequence. The XPC PAT+ fragment was 344 bp and the XPC PAT- fragment was 266 bp.

The same assay was modified for non-radioactive analysis using the Advantage cDNA PCR kit (Clontech). PCR was performed in a total volume of 25 μ l containing 50 ng DNA, 2.5 μ l of 10 \times PCR reaction buffer, 0.5 μ l of 50 \times dNTP mix, 0.5 μ l of polymerase mix, 50 ng N1m and N2n primers and 3.5 ng each β -actin primer. The PCR steps were as follows: 94°C for 3 min, then 40 cycles of amplification (94°C for 30 s and 66°C for 3 min), ending with 66°C for 3 min. The products were resolved on 2% agarose gels and photographed under UV light after staining the gel with ethidium bromide (0.5 μ g/ml).

Donors

Coded samples were obtained for analysis of DNA from three sources. (i) Frozen lymphocytes from 156 skin clinic volunteer cancer-free controls that had been collected as part of a skin cancer study (10) (age range 21–61 years, 52% men) at the Johns Hopkins School of Public Health (JHSPH), Baltimore, MD (10). (ii) Blood from 263 cancer-free men and women (age range 21–94 years, 73% men) enrolled in a cohort study at the Baltimore Longitudinal Study of Aging (BLSA) (11), National Institute of Aging, Baltimore, MD, which began in 1958. (iii) A sample of convenience of buccal swabs and lymphocytes from 216 unselected anonymous donors (men and women employees and unrelated children, age range 1–76 years, 78 men, 104 women and 34 gender unidentified) at the National Institutes of Health (NIH), Bethesda, MD. XP patients were excluded from all samples.

XPC exon 15 polymorphism restriction fragment length polymorphism (RFLP) assay

The A2920C polymorphism region in exon 15 of the XPC gene was amplified utilizing primers Exon15F (forward), 5'-GGAGGTGGACTCTCTCTGATG-3', and 3'ntcDNAR (reverse), 5'-TAGATCCCAGCAGATGACC-3'. The PCR reaction was performed in a 50 μ l mixture consisting of 1 \times PCR buffer, 1.5 mM MgCl₂, 200 μ M each dNTPs, 1 U *Taq* DNA polymerase (Gibco BRL), 100 ng each primer and 50 ng DNA. PCR was performed under the following conditions: 94°C for 60 s, then 35 cycles of amplification (94°C for 20 s, 59°C for 30 s and 72°C for 150 s), ending with 72°C for 10 min. The polymorphic position was analyzed by restriction enzyme digestion with *Pvu*II (New England Biolabs) of PCR products amplified from donor DNA

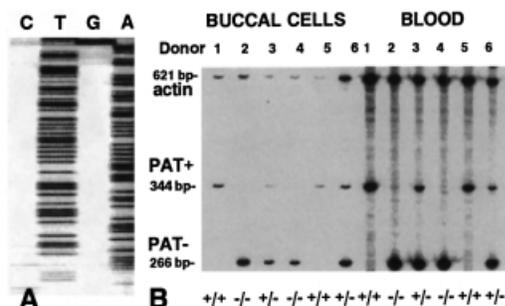


Fig. 1. XPC intron 9 PAT allele sequence determination and detection by PCR. (A) Portion of a DNA sequencing gel showing the region of XPC intron 9 containing 83 bp consisting of only A and T. (B) DNA extracted from buccal cells and blood from six donors was used in a PCR employing primers that flank the PAT region of XPC intron 9. Primers for β -actin were used as an internal standard for reaction efficiency. The reaction products were separated by PAGE and determined by autoradiography. The homozygous PAT+/+ genotype appears as a single band in the middle of the gel, the homozygous PAT-/- genotype appears as a single band at the bottom of the gel and the heterozygous PAT+/- genotype appears as two bands. The β -actin standard migrates near the top of the gel. The DNA from paired buccal cells and blood from each donor yielded the same XPC PAT genotype.

with primer pairs Exon15F and 3'ntcDNAR. The C at position 2920 of the XPC cDNA creates a *PvuII* site. *PvuII* digestion converts the 765 bp PCR product into two fragments of 180 and 585 bp, which were resolved on a 2% agarose gel.

XPC allele-specific complementation assay

In order to assess the functional activity of the XPC exon 15 polymorphism *in vivo* an expression vector [pXPC-3(2920C)] containing a C at position 2920 of the XPC cDNA was constructed. cDNA from an individual who was homozygous for the exon 15 polymorphism was subcloned into vector pCR 2.1-TOPO (TOPO TA Cloning Kit; Invitrogen). Restriction enzyme digestion (New England Biolabs) released an XPC (2920C) cDNA fragment from this construct which replaced a similarly released wild-type (2920A) XPC cDNA fragment in plasmid pXPC-3(2920A) (4) (a generous gift from Dr R. Legerski, M.D. Anderson Hospital, Houston, TX). We used a plasmid host cell reactivation assay (12) to measure functional correction of the XPC DNA repair defect by the wild-type and polymorphic forms of XPC cDNA as described previously (13). Briefly, either pXPC-3(2920A), the newly constructed pXPC-3(2920C) vector, or pEBS7 (pXPC3 without XPC cDNA) (14) were simultaneously co-transfected with a UVC-damaged reporter gene plasmid (pRSVcat) into SV40-transformed XPC (XP4PA-SV-EB) or normal (GM637) cells. DNA repair capacity is expressed as a percentage of chloramphenicol acetyl transferase (CAT) activity obtained from UV-treated plasmid pRSVcat compared with the control untreated pRSVcat. Correction of the DNA repair defect in the XPC cells results in increased post-UV CAT expression.

Results

Identification of the XPC PAT allele

In sequencing the 3.9 kb XPC intron 9 (GenBank accession no. AF076952) (13) we found an 83 bp insertion consisting only of A and T residues (ATA AATTTTTTAT AATAATTTAT AATATTTATA AATTATATAT ATTTATATAT ATAAATAAAT TTATAATATT TATAAATATT) along with a 5 bp deletion of GTAAC at positions 1457–1461 (Figure 1A). The XPC intron 9 allele containing this poly(AT) insertion is designated PAT+ (GenBank accession no. AF156539). The XPC PAT+/- polymorphism site within intron 9 of the XPC gene was PCR amplified using a forward primer at intron 9 (bp 1258–1276) and a reverse primer at intron 9 (bp 1523–1504) as described in Materials and methods. The XPC PAT+ fragment was 344 bp and the XPC PAT- fragment was 266 bp (Figure 1B). As an internal standard, sense and antisense β -actin primers were employed (9) that resulted in a fragment of 621 bp. The

intensity of the β -actin internal standard band serves as an indicator of the overall efficiency of the DNA extraction and PCR procedure for each assay.

Genotype analysis

DNA samples from different donors were found to have either XPC PAT+/+, -/- or +/- genotypes (Figure 1B). Paired buccal and blood DNA results from six donors representing each genotype were found to be consistent (Figure 1B). In all our studies ~1% of the samples did not yield sufficient signal to evaluate. We have also adapted this assay for non-radioactive genotype analysis (see Materials and methods).

XPC PAT allele frequency

We analyzed DNA from donors at JHSPH, BLSA and NIH. The XPC PAT+ allele frequency was 0.44 in DNA from 156 cancer-free donors from JHSPH, 0.41 in 263 cancer-free donors in BLSA and 0.36 in 216 unselected donors at NIH (Table I). Hardy-Weinberg theory predicts that for a two allele system where the frequency of one allele (PAT+) is p and the other (PAT-) is $q = 1 - p$, the expected frequency of the PAT+/+ homozygote is p^2 , the expected frequency of PAT+/- heterozygotes is $2pq$ and the expected frequency of the PAT-/- homozygote is q^2 . The PAT genotype distribution was not significantly different from that predicted by the Hardy-Weinberg theory (Table I).

XPC exon15 single nucleotide polymorphism

We found a change in the DNA sequence near the end of the XPC cDNA. Base number 2920 (the last translated base is 2925) was changed from A to C, resulting in the change Lys939→Gln. We developed a RFLP assay utilizing PCR with exon 15 primers and digestion with *PvuII*. *PvuII* digestion of C/C DNA yields two bands of 585 and 180 bp, while A/A DNA is resistant to digestion and gives a band at 765 bp. C/A heterozygotes yield all three bands (Figure 2A).

We tested DNA from 98 donors from the NIH group (Table II). The frequency of the 2920C allele was 0.38. The XPC exon 15 2920C allele is associated with the XPC intron 9 PAT+ allele and the XPC exon 15 2920A allele is associated with the XPC intron 9 PAT- allele (Table II). Only five of the 196 alleles tested were discordant. Thus the XPC intron 9 PAT and the exon 15 A2920C alleles are strongly associated. Since intron 9 and exon 15 are physically very close (~10 kb apart; S.G.Khan and K.H.Kraemer, unpublished results) this association is due to linkage disequilibrium.

Allele-specific complementation assay of XPC polymorphic alleles

The function of the XPC exon 15 A2920C alteration was assessed in an allele-specific post-UV host cell reactivation assay. XPC cDNA expressing the C at bp 2920 was equally as efficient as XPC cDNA expressing A at that position when the ability of these XPC cDNAs to complement the DNA repair defect in XPC cells was assessed (Figure 2B). Thus both forms of the XPC exon 15 A2920C polymorphism appear to be fully functional in this DNA repair assay.

Transfection of normal cells with XPC cDNA with C or A at bp 2920 did not alter their ability to repair a UV-damaged plasmid (data not shown). Thus overexpression of the XPC exon 15 A2920C polymorphism did not negatively or positively influence the rate of nucleotide excision repair in normal cells.

Discussion

DNA damage and repair

DNA repair plays an important role in protection against environmental and endogenous DNA damage. XP patients

Table I. XPC PAT allele frequency and genotype distribution

Group tested	Total (no.)	XPC PAT allele frequency		Observed genotype distribution (expected)			
		PAT+ (<i>p</i>)	PAT- (<i>q</i>)	PAT+/+ (<i>p</i> ²)	PAT+/- (<i>2pq</i>)	PAT-/- (<i>q</i> ²)	<i>P</i> ^a
JHSPH cancer-free donors	156	0.44	0.56	18.6% (19.0%)	50.0% (49.2%)	31.4% (31.8%)	0.83
BLSA cancer-free donors	263	0.41	0.59	17.9% (16.9%)	46.4% (48.4%)	35.7% (34.7%)	0.50
NIH unselected donors	216	0.36	0.64	15.7% (12.7%)	39.8% (45.9%)	44.4% (41.4%)	0.052

^a*P* value for observed population distribution versus Hardy-Weinberg (expected) distribution.

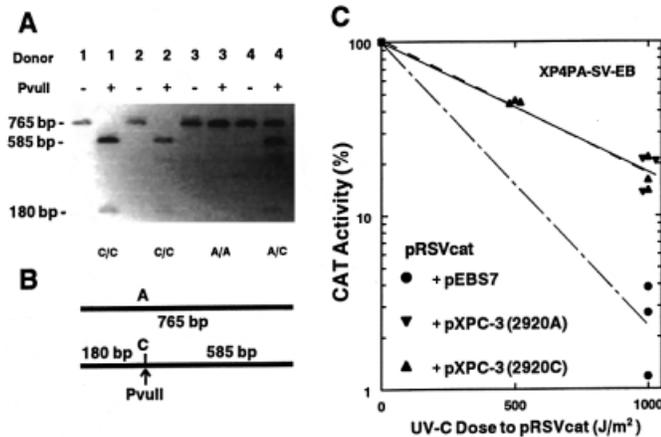


Fig. 2. XPC exon 15 A2920C polymorphism detection by PCR and host cell reactivation assay of DNA repair function. (A) DNA extracted from buccal cells from four donors was used in a PCR employing primers that flank the polymorphic region of exon 15 in the XPC gene. The product was subjected to electrophoresis on a 2% agarose gel without (-) or with (+) digestion with *PvuII*. (B) The undigested PCR product is 765 bp while *PvuII* digestion converts the C-containing product to two fragments of 585 and 180 bp. The product containing the A at position 2920 is resistant to *PvuII* digestion. Thus donors of genotype C/C have two bands (at 585 and 180 bp), A/A donors have a single band at 765 bp and C/A donors have all three bands. (C) Plasmids containing XPC cDNA with either an A [pXPC-3(2920A)] (▼) or a C [pXPC-3(2920C)] (▲) at position 2920 were constructed in the expression vector pEBS7 (●). These plasmids were co-transfected with a UV-treated plasmid (pRSVcat) expressing the CAT gene into an XPC cell line (XP4PA-SV-EB). CAT activity was measured after 2 days of repair as explained in Materials and methods. Co-transfection with the empty vector pEBS7 resulted in the typical low CAT activity indicating defective repair of the UV damage in pRSVcat by XPC cells (13). In contrast, co-transfection with plasmids containing XPC cDNA with either A or C at position 2920 showed an equal increase in CAT expression. This indicates that both XPC cDNAs were equally functional in correcting the XPC DNA repair defect.

Table II. Linkage disequilibrium between XPC intron 9 and XPC exon 15 polymorphisms in 98 NIH donors

XPC intron 9 PAT genotype	XPC exon 15 A2920C genotype (no. of donors with indicated genotype)		
	C/C	C/A	A/A
PAT+/+	19	3	0
PAT+/-	0	34	2
PAT-/-	0	0	40

have defective DNA nucleotide excision repair and a >1000-fold increase in cancers in sunlight-exposed portions of the body (3). Mutations have been identified in the genes involved in XP that result in large reductions in the DNA repair activity

of the encoded proteins and a severe clinical phenotype in XP complementation groups A (15–19), B (20–22), C (4,13,23), D (16), E (24), F (25,26) and G (27–29).

Polymorphisms in DNA repair genes

About 90% of sequence variants in humans are differences in single bases called single nucleotide polymorphisms. Single nucleotide polymorphisms have been identified in the XPA, XPC, XPD, XPF and XPG genes (27,30–36). Many of these polymorphisms did not change the amino acid sequence. However, a study of non-conservative amino acid substitutions in DNA repair genes found that the frequency of nine different variant alleles ranged from 0.04 to 0.45 in a group of 12 healthy donors (34,35). In the present study we describe a new insertion/deletion polymorphism (PAT) in intron 9 of the XPC gene (Figure 1). The PAT+ allele was originally detected in DNA from an XP patient (XP22BE) (13) who also had a splice donor mutation in exon 9 that resulted in several inactive alternatively spliced forms of XPC mRNA, including species containing exon 9a. Cells from normal donors with the PAT+ allele did not have these same alternatively spliced XPC isoforms. Here we report that this XPC PAT+ allele was common, with occurrence at a frequency of 36–44% in DNA from >600 donors.

There is an indication that normal individuals carrying specific polymorphic mutations in DNA repair genes may be at increased risk for certain cancers (34). Thus among 40 basal cell carcinoma patients and 40 controls, donors carrying two A alleles in XPD exon 23 (Lys751) (30,31) had a 4.3-fold higher (but not statistically significant) risk of basal cell carcinoma of the skin than donors with two C alleles (Gln751) ($P = 0.075$, 95% CI 0.79–23.57) (33). Rare microsatellite polymorphisms in the DNA repair genes XRCC1 and XRCC3 were reported to be associated with breast and internal cancers in a study of 34 controls and 19 cancer patients (37). Loss of heterozygosity of the XPG/ERCC5 gene was found in primary prostate cancers and metastases (38). The XPC PAT polymorphism may be a useful molecular marker for similar epidemiological studies.

Insertion/deletion polymorphism

Tandem repeats of a few to many nucleotides in introns of several genes have been associated with human disease (39). For example, Friedreich's ataxia is caused by a GAA triplet repeat expansion in intron 1 (40). However, variations in trinucleotide repeat expansion mutations is not a feature of XP (41). Variable numbers of an 86 bp tandem repeat in intron 2 of the human IL-1 receptor antagonist gene have been described as being associated with increased severity of alopecia areata, ulcerative colitis and systemic lupus erythematosus (42–45). A 799 bp deletion in intron 2 of the retinoblastoma gene was found in 2.2% of 185 patients without cancer but in 18% (four of 22) of patients with glioma ($P = 0.027$) (46).

In the present study the 83 bp insertion/5 bp deletion poly(AT) allele polymorphism in XPC intron 9 was not a tandem repeat and appeared to be stable, in that none of >600 donors showed intermediate bands on electrophoresis of PCR products (Figure 1). Insertion of the 83 bp in intron 9 results in a run of 103 nt that includes only A and T, possibly resulting in unusual DNA structures such as bends that are found in sequences that contain only A and T (47). Analysis of the PAT+ intron sequence with the RepeatMasker program (A.F.A.Smit and P.Green, RepeatMasker available at <http://ftp.genome.washington.edu/RM/RepeatMasker.html>) revealed that the 83 bp insertion is adjacent to a portion of a retrotransposon (48): a 0.7 kb inverse complement of a truncated portion of the 3'-end of a long interspersed nuclear element (LINE-1 or L1) of class L1MB3. Retrotransposons may play a role in exon shuffling (49) or abnormal splicing (50–52) and have been identified as active in the human genome (48). Previously, a large (4–6 kb) insertion/deletion polymorphism was described 5' to exon 1 of polymerase β (53), another gene involved in DNA repair. This polymorphism was present in ~20% of the human population (53). Polymerase β also demonstrates alternative splicing with deletion of exon II, inclusion of intron 9 or deletion of exon XI (54). In another gene, specific TGCATG repeats in the intron downstream have been implicated in alternative splicing of the fibronectin EIIIB exon (55). We have found low levels of alternatively spliced forms of the XPC mRNA containing exon 9a (13) in normal donors (S.G.Khan and K.H.Kraemer, unpublished results). We are presently exploring the possibility that the XPC PAT alleles may affect alternative splicing of the XPC DNA repair gene resulting in differences in DNA repair activity.

Single nucleotide polymorphism in XPC

Single nucleotide polymorphisms in the coding regions of genes or in the regulatory regions are more likely to cause functional differences than single nucleotide polymorphisms elsewhere in the gene. We found a single nucleotide polymorphism (A2920C) in exon 15 of the XPC gene that results in a change of the penultimate amino acid (Lys939) to Gln. We found this to be a relatively common polymorphism (38%) in the XPC gene (Table II) that was previously observed by others (23,32). We found that the allele containing C is in linkage disequilibrium with the PAT+ intron 9 polymorphism (Table II). From our sequencing studies we estimate that these sites are separated by ~10 kb in the XPC genomic DNA (S.G.Khan and K.H.Kraemer, unpublished observation). The non-radioactive PCR-based intron 9 PAT assay we have developed (see Materials and methods) is more rapid and economical than the exon 15 RFLP assay since it does not involve the additional step of restriction enzyme digestion and thus may be preferred for use in population studies.

XPC allele-specific complementation assay

We developed an allele-specific host cell reactivation complementation assay that can be used as a rapid laboratory test for assessing the function of single nucleotide polymorphisms in DNA repair genes in human cells. XPC cells are unable to repair UV-treated plasmids because of their defect in the XPC DNA repair gene (12). Introduction of the wild-type XPC gene in a plasmid expression vector can restore the normal activity (13). Site-directed mutagenesis can be used to create single nucleotide polymorphisms in the XPC gene in the expression vector. The separate alleles can be co-transfected with a UV-treated plasmid and their ability to restore normal

DNA repair is then measured. Using this assay we determined that a plasmid containing XPC cDNA with C at bp 2920 was fully as functional as a plasmid containing an A at that position in correcting the DNA repair defect in an XPC cell line (Figure 2). It is possible that additional functions exist for the XPC gene, such as a role in glycine metabolism (13), that might be altered by the change A2920→C, but these have not been reported. These polymorphisms can be used as a marker for finding other single nucleotide polymorphisms in the XPC gene or other nearby genes in linkage disequilibrium that may affect gene function. This allele-specific complementation assay may be used as a general test for function of polymorphisms in human DNA repair genes.

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