The \textbf{XRCC1} \textit{−77T→C} variant: haplotypes, breast cancer risk, response to radiotherapy and the cellular response to DNA damage

\textbf{Introduction}

The X-ray repair cross-complementing 1 (XRCC1) protein is required for DNA single-strand break repair in human cells (1). These lesions arise either directly following exposure to certain DNA damaging agents such as ionising radiation (IR) or indirectly as intermediates in the base-excision repair (BER) pathway. Left unrepaired, single-strand breaks (SSBs) are a major threat to genetic stability and cell survival, accelerating mutation rates and increasing levels of chromosomal aberrations (2–5). \textit{XRCC1} deficiency in mice results in embryonic lethality and mutant mouse or Chinese hamster ovary cells lacking a functional \textit{XRCC1} (6–8) or human cells in which \textit{XRCC1} protein levels have been depleted by RNAi (1,9) are hypersensitive to a broad range of DNA damage induced by alkylating agents, reactive oxygen species or IR.

Over the last few years, associations between genetic variants in the \textit{XRCC1} gene and the risk of developing certain cancers (10,12) and clinical prognostic factors or adverse reactions to radiotherapy have been reported (13–16). The most extensively studied variants in the \textit{XRCC1} gene are Arg194Trp, Arg280His and Arg399Gln. The \textit{−77T→C} variant alone showed no significant association with BC risk or therapeutic radiation sensitivity. The H5 haplotype (variant allele codon 280, wild-type allele other positions) was associated with increased BC risk [odds ratio (OR), 1.90; 95\% confidence interval (CI), 1.12–3.23] and the H3 haplotype (wild-type allele all four positions) was inversely associated with therapeutic radiation sensitivity compared with the reference group (H1 haplotype, −77C, wild-type allele codons 194, 280, 399) (OR, 0.39; 95\% CI, 0.16–0.92). However given that the global tests for association were not significant these results should be interpreted carefully. Lymphoblastoid cell lines heterozygous for the H1/H3 haplotypes had a significantly higher cell survival (\textit{P} = 0.04) after exposure to ionising radiation (IR) than those with the H1/H1 haplotypes, in agreement with the association study. However no haplotype-specific differences in \textit{XRCC1} expression or cell cycle progression were noted in the 24 h following IR exposure. These results suggest that the \textit{−77T→C} genotype or another variant in linkage disequilibrium influences the cellular response to DNA damage, although the underlying molecular mechanisms remain to be established.

\textbf{Materials and methods}

\textbf{Study population and genotyping for XRCC1 \textit{−77T→C}}

The BC cases and controls investigated in this study have been described previously (20). Briefly, BC cases were recruited over the period of February 1996 to April 2002 among women treated in the Radiotherapy Department at Lyon-Sud Hospital (Pierre-Bénite, France). The average treatment dose
was 50 Gy, in dose fractions of 2.5 Gy over a 6-week period, followed by a 10 Gy boost to the tumour bed. For all cases a blood sample was collected after written informed consent had been obtained, on the occasion of a routine clinical visit to the radiotherapy clinic. DNA samples from a total of 247 BC cases over the age of 35 were available. Of the 247 BC cases, 66 were from women who had developed an adverse reaction to their radiotherapy (RS+ group) in the 2-year period following treatment as classified and graded by the European Organisation for Research and Treatment of Cancer (21). A total of 13 women developed an early reaction (4, grade I; 5, grade II; and 4, grade III); 42 a late reaction (18, grade I; 15, grade II; and 9, grade III) and 11 showed early and late effects. Of the late reactions being at least grade II. The remaining 181 samples were from BC patients who had not developed any adverse reactions to their radiotherapy over a 2-year period following treatment (RS– group).

In addition DNA samples from 380 female blood donors over the age of 35 in the catching area of the hospital through community based collections of the Regional Blood transfusion service were genotyped. The polymorphism in the 5′-UTR was genotyped using a restriction enzyme digestion of a PCR product with the Mhi1 enzyme (Fermentas). The PCR primers (F sense or forward; R antisense or reverse) are as follows: XRCCI –77F, 5′-gccttgaggaaacgcttc-3′ and XRCCI –77R 5′-ttccagcagagactaagag-3′. The PCR reaction was carried out in a total volume of 25 µl containing 50 ng of genomic DNA, 1× Hot-Start Tag buffer, 100 µM deoxyribonucleotide triphosphates, 0.4 µM of each primer, 1 U of Hot-Start Taq polymerase (Qiagen) and 1× solution Q (Qiagen). The cycling conditions were 15 min at 95°C, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 5 min. Aliquots of 10 µl of this PCR product were digested overnight with 20 U of EcoRI in 1× TaqMan Buffer (Fermentas). The homozygous C allele produced a 173 and 46 bp fragment and the homozygous T allele three fragments of 116, 57 and 46 bp, which were resolved on a 3% agarose gel. DNA samples known to be carrying the variant allele were included in each analysis and two investigators read each gel. In addition 10 samples were sequenced for this polymorphism and the results were identical to that of the initial analysis by restriction fragment length polymorphism (RFLP).

Cell survival

As part of a previous study (20), LCLs were established by Epstein–Barr virus (EBV) infection from some of the BC patients analysed here. They were routinely grown in RPMI 1640 Glutamax-1 medium (Life Technologies) supplemented with 10% heat-inactivated FCS (Integro) and 1% pencillin/streptomycin (Biochrom KG) at 37°C under an atmosphere of 5% CO₂. The survival of the LCLs following exposure to IR was assessed by comparing their relative growth 72 h post-treatment (22). The cells were seeded at 2 x 10⁵ cells/ml the day before treatment and survival was assessed 3 days later by counting the number of living cells assessed by trypan blue exclusion. The number of viable cells in the non-treated cultures was used as 100% survival. Cells were exposed to a 1/3CEF (i.e. the required time to give a dose of 1, 2 or 4 Gy). Experiments were performed at least in duplicate for each cell line.

Cell-cycle analysis

Following treatment with IR as described above, cells were processed at the indicated time points using the Cycle Test PLUS DNA reagent kit (Becton Dickinson) or using BrdU/propidium iodide as described previously (23). Analysis was carried out using a Facscalibur (Becton Dickinson), cell-cycle distribution was assessed using the Modfit LT 2.0 software (Verity Software House).

XRCCI expression

Lymphoblastoid cells were plated at a concentration of 0.5 x 10⁵ cells/ml the night prior to RNA or protein extraction or IR exposure. To assess differential XRCCI expression after IR, cells were exposed to defined doses and then returned to the incubator before harvesting at fixed time points. A quantitative RT-PCR approach was used to measure the constitutive and post-treatment levels of XRCCI mRNA in cell lines with different haplotypes. Total RNA was extracted from a minimum of 5 x 10⁶ cells using Trizol (Invitrogen) according to the manufacturer’s protocol at timepoints up to 24 h following treatment and cDNA was synthesised with 1µg total RNA using the iScript cDNA synthesis kit (Biorad). Real-time PCR was run in a Stratagene MX 3000P system and consisted of a 10min initial denaturation at 95°C, followed by 40 cycles of 30s at 95°C and 60s at 60°C. The reactions were prepared with TaqMan® Master Mix (Applied Biosystems). Two replicates of each cDNA were analysed on each plate and at least two cDNAs from independent RNA isolations were analysed. The primers and probes used to quantify the transcripts of XRCCI, p21 (WAF1/Cip1) and the reference gene GAPDH were Taqman gene expression assays (Applied Biosystems). To assess variation between experiments a standard RNA was included in each batch of samples analysed. The threshold cycle (CT), defined as the cycle where the amplification of the PCR product enters the exponential phase was determined for each gene and the relative expression of the XRCCI and p21 (WAF1/Cip1) genes in the different cell lines was then estimated by calculating the dCT value, defined as the difference in the CT value for the target gene (XRCCI or p21) and the reference gene (GAPDH). The dCT is inversely related to the mRNA level, with a high dCT value corresponding to a lower mRNA level.

Statistical analysis

Associations between cancer risk or radiation sensitivity and SNPs or haplotypes were carried out using SAS version 8.1. Hardy–Weinberg equilibrium and linkage disequilibrium (LD) were tested using PROC ALLELE. Haplotypes were calculated using an expectation–substitution approach to assign haplotype probabilities based on the unphased genotype data and to account for uncertainty in assignment (24,25). Odds Ratios (ORs) were calculated using PROC LOGISTIC. The global test for association between phenotype (BC or radiation sensitivity) and haplotypes was calculated comparing the log likelihood of the logistic regression model including all haplotypes to the log likelihood of the logistic regression model without haplotypes.

A variant of anova (ANOVA) was used to test the effect of genotype and radiation dose on the proportion of surviving cells. The ANOVA model included main effects for genotype and dose and factor ‘patient’ as a block effect, to allow for repeated measures on the same patient. Stata version 8 (Stata Corporation) was used for this statistical analysis.

Results

SNP frequencies and associations with risk

The frequencies of the alleles at position −77 in the controls in this study were 0.401 for the C allele and 0.599 for the T allele. The genotype distributions were in Hardy-Weinberg equilibrium in the controls and the BC cases. The association between this SNP and BC and radiation sensitivity are given in Table I. All risk estimates are controlled for age. The −77C variant allele was neither significantly associated with BC risk nor the risk of developing adverse reactions to radiotherapy in BC patients.

Extensive LD exists at the XRCCI locus. The minimum D’ between any pair of SNPs analysed in this study was 0.82. The XRCCI haplotypes carried in this study population based on the genotypes at codons 194, 280 and 399 have been previously determined (19). In order to investigate whether the inclusion of the −77T→C genotype modified the risk associations, the haplotypes were reconstructed based on the four variants. The most frequently found haplotype (H1) contained the C variant at position −77 together with the wild-type alleles at codons 194, 280 and 399, whilst the haplotype (H3) based on the wild-type alleles at all four positions was only present in 11.8% of the controls. An inverse association was observed between the haplotype H3 and therapeutic radiation sensitivity [OR, 0.53; 95% confidence interval (CI), 0.13–0.83]. In terms of BC risk, the haplotype H5 containing the wild-type alleles at −77 (T), codon 194 and 399 and the variant allele at codon 280 was associated with increased risk (OR, 1.90; 95% CI, 1.12–3.23). However, given that the global tests for association between haplotypes and these phenotypes were not significant, these results should be carefully interpreted (global P for BC = 0.07, global P for RS = 0.14) (Table II).

Cell survival and cell-cycle progression after DNA damage

As the H3 haplotype was inversely associated with radiation sensitivity, we next investigated if the presence of the −77T→C variant had any effect on the cellular phenotype. To do this, we measured cell survival and cell-cycle
progression after exposure to IR in two sets of LCLs that were either homozygous for the reference haplotype (H1) or carrying the H3 haplotype in combination with a H1 haplotype. After exposure to IR, ANOVA analysis showed that the lines with the H1/H3 haplotypes had a significantly higher cell survival than those with the H1/H1 haplotypes, $P = 0.04$.

The cell-cycle progression was investigated in the same two sets of cell lines (Figure 1). A trend towards fewer cells in S phase and a higher number of cells in G2 was noted 8 h after exposure to IR for all the cell lines studied, and by 24 h after treatment only between 3 and 14% of all cells were found in S phase while the number of cells in G2 had further increased. However, between the two sets of cell lines studied there were no apparent differences in the cell-cycle progression after exposure to IR suggesting that the XRCC1 genotype did not influence the cell-cycle progression.

Expression of XRCC1

In order to investigate whether the differences in cell survival were related to changes in the level of expression of XRCC1, mRNA and protein levels were measured (by RT–PCR and western blotting, respectively) in the same LCLs and also in three lines homozygous for the H2 haplotype and three lines carrying the H1/H5 haplotypes. Whilst minor variations in the constitutive XRCC1 mRNA and protein levels were noted between these four sets of lines, no significant correlation was seen between mRNA or protein levels and XRCC1 haplotype (data not shown). In order to investigate whether XRCC1 mRNA levels varied after the formation of DNA damage, two lines carrying the H1/H1 haplotypes and two lines carrying the H1/H3 haplotypes were treated with 2 or 4 Gy of IR and RNA was extracted at various time points afterwards. Figure 2 shows the results of a representative experiment where relative expression levels for XRCC1 and GAPDH, respectively, are presented as dCT, which shows an inverse correlation with absolute mRNA levels. It should be noted that over the time course of the experiment the relative level of the GAPDH reference transcript remained constant (data not presented). As was expected an induction in XRCC1 and p21 (WAF1/Cip1), which served as a treatment control, were measured 4, 8 and 24 h post-treatment. Levels are presented as dCT, which shows an inverse correlation with absolute mRNA levels. It should be noted that over the time course of the experiment the relative level of the GAPDH reference transcript remained constant (data not presented). As was expected an induction in p21 (WAF1/Cip1) mRNA levels, seen as a decrease in the dCT value, was clearly detected in all the cell lines studied after exposure to IR showing that these cells were clearly capable of responding to DNA damage. However in the same cells the level of XRCC1 mRNA remained fairly constant with slight fluctuations in levels but no systematic induction being noted and there were no differences based on the XRCC1 genotype.

**Discussion**

The XRCC1 protein plays a critical role in SSB repair and BER in human cells and many molecular epidemiological studies have investigated possible associations between the common XRCC1 polymorphisms and cancer risk, with several studies having shown gene–environment interactions between XRCC1 genotypes and exposure (10,11). However, many of the studies, which have investigated individual SNPs, produced apparently conflicting results. This can be due to small sample sizes (giving rise to chance results) and to large ethnic differences in variant allele frequencies. For instance, the frequency of the two alleles at position

### Table I. Association between the −77T→C XRCC1 SNP and risk of BC and adverse reaction to radiotherapy

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Cases (%)</th>
<th>Controls (%)</th>
<th>OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T</td>
<td>90 (36.4)</td>
<td>134 (35.3)</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td>C/T</td>
<td>107 (43.3)</td>
<td>187 (49.2)</td>
<td>0.83 (0.57–1.19)</td>
</tr>
<tr>
<td>C/C</td>
<td>50 (20.2)</td>
<td>59 (15.5)</td>
<td>1.23 (0.71–1.97)</td>
</tr>
</tbody>
</table>

### Table II. Association between haplotypes and therapeutic radiation sensitivity risk

<table>
<thead>
<tr>
<th>Haplotype*</th>
<th>RS+ (%)</th>
<th>RS− (%)</th>
<th>OR (95% CI)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1...C-C-G-G</td>
<td>205 (40.4)</td>
<td>311 (39.7)</td>
<td>1.00 (Ref.)</td>
</tr>
<tr>
<td>H2...C-T-G-A-G</td>
<td>169 (33.3)</td>
<td>284 (36.2)</td>
<td>0.90 (0.67–1.17)</td>
</tr>
<tr>
<td>H3...C-T-G-G</td>
<td>51 (10.0)</td>
<td>92 (11.8)</td>
<td>0.79 (0.52–1.20)</td>
</tr>
<tr>
<td>H4...T-C-G-G</td>
<td>34 (6.7)</td>
<td>51 (6.9)</td>
<td>1.01 (0.55–1.49)</td>
</tr>
<tr>
<td>H5...T-C-A-G</td>
<td>40 (7.9)</td>
<td>39 (5.0)</td>
<td>1.90 (1.12–3.23)</td>
</tr>
</tbody>
</table>

### Table III. Genotype-specific differences in cell survival

<table>
<thead>
<tr>
<th>Haplotype*cell line</th>
<th>Relative cell survival (mean ± SD)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1/H1</td>
<td></td>
</tr>
<tr>
<td>BC26 (2)</td>
<td>1 0.38 ± 0.01 0.17 ± 0.02 0.06 ± 0.03</td>
</tr>
<tr>
<td>BC229 (2)</td>
<td>1 0.57 ± 0.04 0.19 ± 0.03 0.08 ± 0.04</td>
</tr>
<tr>
<td>BC157 (2)</td>
<td>1 0.49 ± 0.03 0.15 ± 0.03 0.05 ± 0.01</td>
</tr>
<tr>
<td>C/C wt wt</td>
<td>1 0.48 ± 0.09 0.17 ± 0.02 0.06 ± 0.02</td>
</tr>
<tr>
<td>H1/H3</td>
<td></td>
</tr>
<tr>
<td>BC19 (3)</td>
<td>1 0.65 ± 0.14 0.28 ± 0.09 0.08 ± 0.04</td>
</tr>
<tr>
<td>BC43 (2)</td>
<td>1 0.56 ± 0.16 0.32 ± 0.17 0.11 ± 0.05</td>
</tr>
<tr>
<td>BC52 (2)</td>
<td>1 0.41 ± 0.11 0.18 ± 0.01 0.07 ± 0.01</td>
</tr>
<tr>
<td>C/T wt wt</td>
<td>1 0.54 ± 0.12 0.26 ± 0.07 0.09 ± 0.02</td>
</tr>
</tbody>
</table>

Figures in parentheses represent number of independent experiments.

*Haplotypes as defined in Table II.

**Mean values ± SD for each cell line based on the results of at least two independent experiments. ANOVA analysis showed that the lines with the H1/H3 had a significantly higher cell survival than those with the H1/H1 haplotypes, $P = 0.04$.**
77 shows considerable variation. In the present study, the C allele was found at a frequency of 0.401 and the T allele at 0.599, whilst in Chinese subjects the allele frequencies were 0.10/0.11 for the C allele and 0.90/0.89 for the T allele (17,18).

Only a few studies have examined multiple SNPs in the XRCC1 gene and assessed associations based on the presence of inferred haplotypes. In this study we identified a haplotype (H5) carrying the variant allele at codon 280 which was associated with increased BC risk. In the previous analysis of

---

Fig. 1. Cell-cycle progression of cells with different XRCC1 genotypes. Cells were treated with 2 or 4 Gy IR and the cell-cycle distribution was assessed 8 and 24 h after treatment by flow cytometry. Values shown are for one typical experiment. Haplotypes for each cell line are given in table III.

Fig. 2. Variation in XRCC1 and p21 (WAF1/Cip1) mRNA levels in cells after DNA damage. Cells were treated with 2 or 4 Gy IR, RNA was extracted 2, 4, 8 and 24 h later and mRNA levels were determined by quantitative RT–PCR. An induction of p21 (WAF1/Cip1) mRNA was seen in all cell lines after treatment—the dCT is inversely proportional to the mRNA level with a lower dCT value corresponding to a higher mRNA level.
this data set (19) a haplotype carrying only this variant was also found to be associated with increased BC risk (OR, 1.80; 95% CI, 1.06–3.08). Increased risk of BC for a haplotype containing the codon 280 His allele has been observed in one other study (26) in which it was also found solely on a chromosomal background containing the wild-type alleles at codons 194 and codon 399. However no association with BC risk was reported in four other studies (27–30). One important observation in this respect was reported by Takehami et al. (31), who introduced human XRCC1 variant proteins into XRCC1 mutant CHO cells and found that in contrast to the wild-type allele, XRCC1 carrying 280 His failed to rescue the SSB repair defect of the mutant cells. The haplotype-specific differences in BC risk seen in the cohort studied might reflect the involvement of XRCC1 in several DNA repair pathways. SSBs and base modifications which arise spontaneously or are induced by a number of DNA damaging agents are repaired via the BER pathway, in which XRCC1 plays a key role. A certain number of DNA double-strand breaks (DSBs) will also be formed after an SSB-induced replication fork collapse. There is evidence that XRCC1 may also contribute to backup pathways of DSB repair (32,33) and a functional link between XRCC1 and the heterodimeric complex DNA–PK has been recently demonstrated (34). Thus if the XRCC1 variant proteins show differences in affinity for protein partners, haplotype and exposure-specific differences in cancer risk might be expected.

In addition, we identified a haplotype inversely associated with therapeutic radiation sensitivity. This haplotype carried the common alleles at the four positions investigated and was not identified in our previous study. In this population although the T wild-type allele is found at a higher allele frequency, the most frequently found haplotype contains the C allele at position −77 in combination with the wild-type alleles at codons 194, 280 and 399. Thus the inclusion of the −77T→C genotype into the haplotypes allows a subdivision of the carriers of the wild-type alleles which would have otherwise all been included in the reference group.

In an attempt to investigate the underlying causes of this modified risk of developing an adverse response to radiotherapy the cellular response to DNA damage produced by IR were examined in lymphoblastoid cell lines heterozygous or homozygous for the variant allele at position −77 and wild-type for the three other common XRCC1 SNPs (haplotypes H1/H3 compared to H1/H1). The set of lines with the H1/H3 haplotypes had a higher cell survival compared with those with the H1/H1 haplotypes after exposure to IR which is in agreement with the findings that the H3 haplotype is inversely associated with therapeutic radiation sensitivity. However these differences did not appear to be related to XRCC1 expression as significant variations based on the XRCC1 genotype were found neither in the constitutive mRNA or protein levels nor in levels over the 24-h period after treatment of the cells with IR. These expression results are in contrast to those recently published by Hao et al. (35) who reported that the −77T→C variant was within the binding motif of the Sp1 transcription factor and that the T→C substitution enhanced its binding. Using transient transfection of a luciferase reporter gene, they reported that the Sp1-high-affinity-C-allelic XRCC1 promoter was associated with a reduced transcriptional activity relative to the T-containing construct. The reasons for the differences in the expression in their and our analysis are unclear. It may be cell type specific and that in the rapidly growing lymphoblastoid cell lines haplotype-specific differences in the constitutive level of XRCC1 transcription are masked. As the construct used in the luciferase assays contained only the 797 bp XRCC1 promoter region, it is also possible that alone this variant might influence expression but that because of the high LD at the XRCC1 locus, other yet unidentified variants in the same haplotype or linkage to another gene might modify expression.

In conclusion whilst this study has several limitations, not least the small numbers of cases and controls, which means that the haplotype risk associations can only be considered as preliminary, the results do suggest that association studies in which individual XRCC1 SNPs are only considered might lead to errors in risk estimations and perhaps explain some of the apparently conflicting results published. The inclusion of the −77T→C variant in larger association studies is clearly warranted as are further mechanistic studies.

Acknowledgements

We gratefully acknowledge the financial support of EDF. Reto Brem carried out this work during the tenure of an IARC Special Training Award.

Conflict of Interest Statement: None declared

References

12. Chang-Claude,J., Popanda,O., Tan,X.L. et al. (2005) Association between polymorphisms in the DNA repair genes, XRCC1, APE1, and XPD and


Received April 20, 2006; revised June 1, 2006; accepted June 20, 2006