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RAD51 homologous recombination repair gene haplotypes and risk of acute myeloid leukaemia☆

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Abstract

Homologous recombination (HR) is one of the main pathways for the repair of DNA double strand breaks (DSBs). To investigate whether inherited variants in genes encoding proteins that repair DSBs by HR modulate acute myeloid leukaemia (AML) risk, we have examined the frequency of two variants in the 5′ untranslated region (UTR) of *RAD51* (*RAD51 135 G>C* and the *RAD51 172 G>T*) in a large case–control study of acute myeloid leukaemia (AML). Inheritance of a *RAD51 135 C* allele was associated with a reduced risk of estimate for AML (odds ratio (OR) 0.56, 95% confidence intervals (CI), 0.38–0.83), while the *RAD51 172 T* allele was not associated with AML. The *RAD51 135* and *172* variants were in strong linkage disequilibrium, with three out of the four possible haplotypes being observed in the population. The protective effect associated with the *RAD51 135 C* allele was found to be associated with inheritance of the *RAD51 135–172 C–G* haplotype (cases 3.9% versus controls 6.5%, OR 0.61, 95% CI 0.42–0.90). These data suggest that variants in the *RAD51* HR gene may modulate genetic predisposition to AML.

Keywords: Acute myeloid leukaemia; DNA repair; Double strand break; Homologous recombination; Non-homologous end joining; Case–control study

1. Introduction

DNA damage in the haemopoietic precursor cell is thought to be an essential prerequisite for the development of acute myeloid leukaemia (AML). Double strand breaks (DSBs) are one of the most deleterious forms of DNA damage, representing a mechanism by which chromosomal translocations and other common molecular events in AML may occur. Due to the threat posed by DSBs, eukaryotic cells have evolved two main pathways for the repair of DSBs; non-homologous end joining (NHEJ) and homologous recombination (HR). Studies in mice and yeast have shown that the absence of either pathway leads to genomic instability [1,2], while cell lines defective in HR are known to have high rates of spontaneous chromosomal abnormalities [3]. In humans, inherited defects in HR pathways are known to predispose to AML, an example of this, Fanconi anemia (FA) [4] is characterized by spontaneous and mutagen-induced chromosome instability. Recently BRCA2, was identified as an FA protein, linking this pathway to HR through the interaction of BRCA2 with RAD51 [5].

HR effects DNA repair through the interaction of free DNA ends with a homologous DNA sequence that is used as a template for the high fidelity repair of the DSB. HR is

Abbreviations: AML, acute myeloid leukaemia; DSB, double strand break; HR, homologous recombination; NHEJ, non-homologous end joining; FA, Fanconi's anemia; OR, odds ratio; CI, confidence interval

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thought to be particularly important in DNA repair occurring during cellular replication [6–8]. RAD51 is one of the key proteins for HR, and functions by forming nucleoprotein filaments on single stranded DNA, mediating homologous pairing and strand exchange reactions between single and double stranded DNA during repair [9]. The central role of RAD51 in maintaining genomic stability is supported by the fact that null mice are embryo lethal [10], while in the chicken DT40 system disruption of the *RAD51* gene results in chromosome instability [11], a molecular feature commonly seen in AML. In contrast, over-expression results in increased resistance to apoptosis induced by ionizing radiation [9], a well defined cause of DNA DSBs. The emerging role of HR in myeloid precursor cells [12] combined with the phenotypic features of cells associated with over- and under-expression of RAD51 suggest that genetic variation within the gene may mediate risk of AML.

Two polymorphisms have been described in the 5′ UTR of *RAD51*, a G > C substitution at position +135 bp, and a G > T substitution at position +172 bp from the start of the cDNA sequence (NCBI accession number D14134). While alone neither polymorphism has been associated with risk of breast or ovarian cancer [13,14], nor an effect on breast cancer survival [15], the *RAD51 135 C* allele has been associated with an increased risk of breast cancer and a reduced risk of ovarian cancer amongst individuals that carry germline mutations in *BRCA1* or *BRCA2* [16,17]. To examine the effects of polymorphisms in *RAD51* on AML risk, we have examined the frequency of the *RAD51 135* and *RAD51 172* variants in a large case–control study of AML.

2. Methods

2.1. Study design

Full details of the Leukaemia Research Fund population-based AML case–control study are given elsewhere [18]. Briefly, acute leukaemia cases aged between 16 and 69 years were recruited from the UK, and diagnoses were pathologically confirmed. For every case that participated in the study, two controls of the same sex, race and year of birth were recruited from the general practice of the case. All subjects included in the study gave informed consent, and ethical approval was obtained for all study subjects. Briefly, the study group comprised 479 cases, of which 424 were classified as *de novo* AML, and 55 as secondary AML (defined as a history of previous malignancy or myelodysplasia (MDS)). The average age of the *de novo* cases was 47.6 years and 54% were male, while for the secondary cases the average age was 51.8 years, and 45.4% were male. The study group also comprised of 952 age sex matched controls, with an average age of 48.1, with 53.5% being male. As the frequency of alleles has been shown to vary with race [19], the genotyping and subsequent analysis was restricted to Caucasian subjects.

2.2. Genotyping and assay validation

Genomic DNA was extracted from whole frozen blood using a proteinase K treatment, followed by a series of phenol:chloroform extractions and ethanol precipitation [20]. Quality checks, using both DNA negative and known genotype control samples, were run with each set of subject DNA for both assays.

Inheritance of the *RAD51 135* and *172* polymorphisms was determined using Taqman™ Allelic Discrimination. Primer and probe sequences were as follows (variant base in lower case), *RAD51 135* forward primer 5′-TCTGGGTTGTGCGCAGA-3′, reverse primer 5′-CCGCGCTCCGACTTCA-3′, G allele probe 5′-FAM-AGCGTAAGCCAgGGGCGTTGG-3′, C allele probe 5′-VIC-GCGTAAGCCAcGGGCGTTGG-3′. *RAD51 172* forward primer 5′-CGAGTAGAGAAGTGGAGCGTAAGC-3′, reverse primer 5′-CCGCGCTCCGACTTCA-3′, T allele probe 5′-FAM-CGTGCCACtCCCGCGGG-3′, G allele probe 5′-VIC-CGTGCCACgCCCGCGGG-3′. All reactions were carried out in 15 μl volumes using 900 pmol of each primer and 100 nmol of each probe, using the 2× Applied Biosystems (ABI) (Foster City, CA) universal master mix. Amplification was carried out on an ABI 9700 using the following amplification conditions for both assays; 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 61 °C for 1 min. Data capture and analysis were carried out using an ABI PRISM 7700 sequence detector and the Sequence Detection Systems software (ABI).

Genotyping results were verified by sequencing 60 randomly chosen samples. A 178 bp fragment of the *RAD51* sequence surrounding the two variants was amplified using the following primers and reaction conditions; 10 pmol each primer (forward 5′-ACCGAGCCCTAAGGAGAGTG-3′, reverse 5′-CCGCGCTCCGACTTCA-3′), 10 ng of DNA, 1× Amplitaq Gold buffer, 200 μmol dNTPs, 1.5 mM $MgCl_2$, 1 unit Amplitaq Gold DNA polymerase, in a final reaction volume of 20 μl. Amplification conditions were as follows; 10 min at 95 °C, followed by 35 cycles of 95 °C 1 min, 56 °C 1 min, and 72 °C 1 min. Products were run out on 1% agarose gels and purified using the QiaQuick system from Qiagen (West Sussex, UK). Samples were sequenced using ABI PRISM® BigDye™ Terminator cycle sequencing, the sequencing products being visualized on an ABI 377 sequencer.

To verify the linkage disequilibrium predicted using statistical analysis between the *RAD51 172* and *RAD51 135* variants, five samples identified as dual *RAD51 172 GT* and *RAD51 135 GC* heterozygotes, were blunt-end cloned into the pT7Blue-3 Blunt vector following the manufactures instructions (Novagen, Germany). Plasmids that contained insert were then amplified using the R-20mer primer (Novogen) in combination with the reverse primer previously used for sequencing, and the products were then gel purified and sequenced as described previously.

Out of the 120 genotypes checked by sequencing, all genotypes agreed for the *RAD51 135*, while 1 genotype (*RAD51*

Table 1
Number of AML cases (*de novo* and secondary) and controls, odds ratios (OR) and 95% confidence intervals (CI) for *RAD51 135* and *172* polymorphisms, generated using conditional logistic regression

	Total				*De novo*				Secondary			
	Controls *n* (%) (*n* = 952)	Cases *n* (%) (*n* = 479)	OR	95% CI	Controls *n* (%) (*n* = 730)	Cases *n* (%) (*n* = 424)	OR	95% CI	Controls *n* (%) (*n* = 97)	Cases *n* (%) (*n* = 55)	OR	95% CI
RAD51 135												
GG	817 (87.3)	431 (92.5)	1		636 (87.6)	381 (92.3)	1		82 (85.4)	50 (94.3)	1	
GC	115 (12.3)	34 (7.3)	0.55	0.37–0.83	87 (12.0)	31 (7.5)	0.58	0.38–0.90	14 (14.6)	3 (5.7)	0.36	0.10–1.28
CC	4 (0.4)	1 (0.2)	0.55	0.06–5.39	3 (0.4)	1 (0.2)	0.55	0.06–5.39	–	–	–	–
GC + CC	119 (12.7)	35 (7.5)	0.56	0.38–0.83	90 (12.4)	32 (7.7)	0.58	0.38–0.89	14 (14.6)	3 (5.7)	0.36	0.10–1.28
SNA	16	13			4	11			1	2		
RAD51 172												
GG	331 (35.2)	144 (30.7)	1		256 (35.3)	131 (31.5)	1		35 (36.5)	13 (24.5)	1	
GT	445 (47.3)	225 (48.0)	1.11	0.86–1.45	350 (48.2)	200 (48.1)	1.09	0.82–1.43	42 (43.8)	25 (47.2)	1.37	0.56–3.38
TT	164 (17.4)	100 (21.3)	1.40	1.02–1.94	120 (16.5)	85 (20.4)	1.33	0.94–1.89	19 (19.8)	15 (28.3)	1.99	0.79–5.01
GT + TT	609 (64.7)	325 (69.3)	1.19	0.93–1.52	470 (64.7)	285 (68.5)	1.15	0.89–1.49	61 (63.6)	40 (75.5)	1.63	0.73–3.61
SNA	12	10			4	8			1	2		

SNA, sample non-amplified; –, genotype not found.

172) differed between the sequencing and genotyping data (98.3% concordance).

2.3. Statistical analysis

All analyses were carried out using Stata [21], with Phase Version 2.1 used to reconstruct haplotypes from genotype data [22,23]. To estimate to the risk of AML associated with *RAD51* odds ratios and 95% confidence intervals were derived using conditional logistic regression adjusting for the matched variables age, sex and region [24].

The study had 90% power ($p = 0.05$) to detect an odds ratio of 0.5 when the exposure level was 10% in the control population. The power to detect associations for the secondary cases alone is obviously reduced due to the rarity of secondary AML. Subgroup analyses were conducted for *de novo* and secondary AML. AML cases were considered to be secondary if prior to their current diagnosis, they were treated with chemotherapy and/or radiotherapy, or if they had been diagnosed with myelodysplastic syndrome, chronic myeloid leukemia, or a chronic myeloproliferative disorder; otherwise, their diagnosis of AML was designated as *de novo*. Cytogenetic abnormalities among AML cases were hierarchically classified into one of the following groups; normal, reciprocal translocations associated with good prognosis (*t*(15; 17), *t*(8; 21) or inv (16)), partial or complete deletion of chromosomes 5 or 7, or trisomy 8.

3. Results

The genotype distributions of the *RAD51 135* and *172* polymorphisms found in the Caucasian control group are in agreement with those previously published [13–15] (Table 1). Furthermore, both the *RAD51 135* and *172* polymorphisms are in Hardy–Weinberg equilibrium for both the case and control groups. No association was found with either polymorphism when stratified by age at diagnosis or sex (data not shown).

There were fewer individuals either heterozygous or homozygous for the *RAD51 135 C* allele amongst the cases compared to the controls (7.5% versus 12.7%; OR 0.56, 95% CI 0.38–0.83), with a similar distribution observed in the *de novo* and secondary AML cases (Table 1). An increased frequency of the *RAD51 172* homozygous *TT* genotype was seen in the cases compared to the controls (21.3% versus 17.4%; OR 1.40, 95% CI 1.02–1.94). While a similar frequency was observed for the *TT* genotype in *de novo* AML cases (20.4%), an increased frequency was observed for secondary AML (28.3%; OR 1.99, 95% CI 0.79–5.01). However, when a case–case analysis of *de novo* versus secondary AML was performed to control for the association of this polymorphism with *de novo* AML, the results suggest no association with secondary AML. Sub-classifying AML by recurrent cytogenetic abnormality (translocations, 5q/7q−, or trisomy 8) did not demonstrate any differences in genotype distribu-

Table 2
RAD51 135–172 haplotype frequency of AML cases and controls expressed as a percentage, associated odds ratios (OR) and 95% confidence intervals (95% CI) calculated for each haplotype, generated using conditional logistic regression

135 locus	*172* locus	Controls	All Cases	OR	95% CI	*De novo* cases	OR	95% CI	Secondary cases	OR	95% CI
G	G	52.4	50.8	1.0	–	51.5	1.0	–	45.2	1.0	–
G	T	41.1	45.3	1.13	0.97–1.33	44.5	1.1	0.95–1.35	51.9	1.40	0.86–2.29
C	G	6.5	3.9	0.61	0.42–0.90	4.0	0.62	0.43–0.99	2.9	0.44	0.12–1.63
C	T	0	0	–	–	0	–	–	0	–	–

Haplotype numbers estimated using PHASE, and OR and 95% CI calculated using Stata. (–) Haplotype too rare to estimate risk accurately.

tions for either the *RAD51 135* or *172* polymorphisms (data not shown).

The distribution of combined *RAD51 135* and *172* genotypes indicated strong linkage disequilibrium between the two polymorphisms. Statistical analysis supported a non-random association between the variants ($D' = 0.999$, $\chi^2 = 78$, $p < 0.0001$), which was confirmed by direct experimental analysis. Five combined *RAD51 135* (*GC*) and *172* (*GT*) heterozygotes were cloned and sequenced, and it was shown that the *RAD51 135 G>C 172 G>T* base substitutions were in *cis* (on opposite strands). Therefore, subjects genotyped as double heterozygotes possess the two *RAD51* haplotypes *135–172 C–G* and *135–172 G–T*. The *RAD51 135–172* haplotype frequencies (Table 2) suggest that the *RAD51 172 G>T* substitution occurred first, resulting in the common *135–172 G–T* haplotype, followed by a second event, the *135 G>C* substitution, producing the rarer *135–172 C–G* haplotype. Alternatively the *RAD51 135–172 C–G* haplotype may be seen at a low frequency due to evolutionary selection against this haplotype. While the estimated frequency for the *RAD51 135–172 C–T* haplotype in our study was too low to be reported, a frequency of 0.03% has been previously reported for the *RAD51 135–172 C–T* haplotype [14]. The rarity of the *RAD51 135–172 C–T* haplotype may result either from limited recombination owing to the close spatial proximity of the two variants (37 bp), or from the *RAD51 135* substitution occurring independently of the *RAD51 172* substitution.

Significant differences in haplotype frequencies were observed between the case and control populations for AML, suggesting that the *RAD51 135–172 C–G* haplotype confers a decreased risk of developing AML (cases 3.9% versus controls 6.5%; OR 0.61, 95% CI 0.42–0.90). Similar haplotype frequencies were observed for both the *de novo* AML and secondary case populations (Table 2). The frequency of the *RAD51 135–172 G–T* haplotype was not significantly different between the cases and controls for either *de novo* or secondary AML.

4. Discussion

Homologous recombination is one of the major DNA DSB repair mechanisms, and RAD51 a key protein in this process, promoting homologous pairing and strand exchange. Our results suggest a protective association for the *RAD51 135/172 C–G* haplotype for AML (OR 0.61, 95% CI 0.42–0.90).

The variants are situated in the 5′ UTR of the RAD51 mRNA, which may affect the regulation of transcription, translation or mRNA stability. The functional consequences of these variants are at present unknown, however data from breast cancer patients suggests that the *135 C* variant may increase RAD51 mRNA levels [25]. The exact mechanism by which these variants modulate AML risk is unknown, however changes in RAD51 expression associated with *RAD51 135/172 C–G* haplotype status may modulate the fidelity and activity of DNA repair within the target cells.

The linkage within the human genome has been well documented; as such there exists a possibility that the effect seen with this haplotypes is the result of a wider degree of linkage within this genomic area. The genes immediately adjacent to *RAD51* on chromosome 5 (within 10,000 kb) the confirmed transcripts NM_018145 and Q9BZ58 are both annotated as having unknown or ambiguous function. As a result it is difficult to make any conclusion on the possible effect haplotypes blocks containing these genes might confer, however the involvement of these transcripts in leukaemogenesis cannot be discounted, and warrants further investigation.

Previous studies have linked the *RAD51 135 C* allele with altered susceptibility to both breast and ovarian cancer. In breast cancer, although no association was seen for the variant [14], Wang et al. [17], report an increased risk of breast cancer and a lower risk of ovarian cancer amongst cases also possessing a *BRCA2* mutation, however, no association was seen for individuals known to have a *BRCA1* mutation. Apparently conflicting results have been reported by Jakubowska et al. [16] amongst Polish *BRCA1* mutation positive breast cancer cases, with a protective association for the *RAD51 135 C* allele being seen. These differences in associated risk among for *BRCA1* mutation carriers may be due to chance, but also could be explained by the nature of the *BRCA1* mutations reported in the two studies. The most common mutation seen in the Jakubowska study was the 5382insC, which results in a truncated protein but which retains an intact RAD51 binding site [16]. The primary mutation reported in the Wang study was the 185delAG, which also results in a truncated protein but abolishes the BRCA1 RAD51 binding site. This suggests that for a protective effect to be seen in *BRCA1* mutation carriers, the RAD51 interaction site must be present, enabling

the *RAD51 135 C* allele to enhance the activity of mutant BRCA1 [16].

An increased risk of breast cancer is associated with the *RAD51 135 C* allele when *BRCA2* mutations are present [17]. BRCA2 is required for the orderly assembly of RAD51 on single stranded DNA ends. In the absence of BRCA2, initiation of accurate HR is impaired and repair errors will rapidly accumulate [26]. The increased risk associated with the *RAD51 135 C* allele suggests an increase in repair errors. The biological explanation for this is uncertain but may reflect the use of an alternative pathway such as NHEJ [27], or may be a result of error prone HR [28].

This study highlights the importance of the link between RAD51, BRCA1, BRCA2 and a risk for AML. An increased risk for AML has been noted in patients previously diagnosed with breast cancer [29]. A positive association has also been reported between a family history of breast cancer and an increase in leukaemia incidence [30,31], exposure to known leukaemogens further strengthened the associations seen [32], suggesting genetic factors common to both diseases. Further evidence linking these molecules with variation in AML risk comes from the study of Fanconi anemia. BRCA2 has recently been identified as FANC-D1 [33], one of the eight FA gene products that function in an integrated DNA damage response pathway [34,35]. Aberrant DNA repair by both the NHEJ and HR pathways have been described in FA cells [36–38], supporting the hypothesis that FA cells have a defect in the regulation of the pathways that repair DNA DSBs. Both NHEJ and HR repair similar types of DNA damage, however the repair pathway utilized by the cell is important. NHEJ can be an imprecise pathway, however, it is less likely to generate chromosome translocations than HR [39]. While HR is less error prone than NHEJ, the presence of repetitive DNA sequences throughout the genome results in a higher probability of oncogenic recombination events occurring during repair. The increased risk for AML in FA carriers suggests that the HR pathway is important for maintaining cellular integrity in the myeloid progenitor cell. Impairment of HR may lead to the use of an error prone pathway that could increase the risk of transformation events. The protective effect we observe for the *RAD51 135–172 C–G* haplotype suggests that it may be associated with increased *RAD51* expression, modulating HR and protecting the cells against aberrant DNA repair events, thus reducing the risk of AML.

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