Polymorphisms in Genes Involved in Homologous Recombination Repair Interact to Increase the Risk of Developing Acute Myeloid Leukemia

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ABSTRACT

Purpose: Double-strand break repair via homologous recombination is essential in maintaining genetic integrity. RAD51 and XRCC3 are involved in the repair of DNA by HR and polymorphisms have been identified in both the RAD51 (RAD51-G135C) and XRCC3 (XRCC3-Thr241Met) genes. The object of this study was to examine whether these polymorphisms may modulate susceptibility to the development of acute myeloid leukemia (AML), a disease that is characterized by genetic instability.

Experimental Design: We studied the distribution of polymorphisms in RAD51 and XRCC3 in 216 cases of de novo AML, 51 cases of therapy-related AML (t-AML), and 186 control subjects using PCR followed by restriction enzyme digestion. The polymorphic deletion of the detoxification gene glutathione S-transferase M1 (GSTM1) was also examined by PCR.

Results: The risk of the development of AML was found to be significantly increased when both variant RAD51-135C and XRCC3-241Met alleles are present [odds ratio (OR), 3.77; 95% confidence interval (CI), 1.39–10.24], whereas the risk of t-AML development is even higher (OR, 15.26; 95% CI, 2.22–96.8), presumably because of the large genotoxic insult these patients receive after their exposure to radiotherapy or chemotherapy. If we further divide the AML group into patients in which the burden of DNA damage is increased, because of the deletion of the GSTM1 gene, the risk of development of AML is further increased (OR, 15.26; 95% CI, 1.83–127.27).

Conclusions: These results strongly suggest that DNA double-strand breaks and their repair are important in the pathogenesis of both de novo and t-AML.

INTRODUCTION

Acute myeloid leukemia (AML) is a clonal hemopoietic disorder that is frequently associated with genetic instability characterized by a diversity of chromosomal and molecular changes. Most cases of AML arise de novo; however, ~10–20% of all cases of AML arise after exposure to chemotherapy or radiotherapy after the treatment of a primary malignancy [therapy-related AML (t-AML); reviewed by Pedersen-Bjergaard et al. (1)].

DNA is at constant risk from damage from both endogenous and exogenous sources. A large number of highly complex mechanisms have evolved to protect DNA from damage including DNA repair pathways and systems that protect against oxidative stress and other damaging agents. These pathways play a vital role in maintaining genetic integrity. The ability of an individual to prevent and repair damage is genetically determined and is the result of combinations of multiple genes that may display subtle differences in their activity (2–4).

Genetic polymorphisms have now been identified in a number of DNA repair genes and damage-detoxification genes. Polymorphisms can affect protein function, promoter activity, mRNA stability, and splice variants and, hence, can result in a change in the cellular ability to cope with DNA damage, which contributes to an altered disease susceptibility. The genotype distributions of a number of these polymorphic genes have been shown to be associated with AML and/or t-AML including the base excision repair gene XRCC1 (5) and the detoxification genes NAD(P)H:quinone oxidoreductase (6, 7) and glutathione S-transferases (8, 9).

Double-strand breaks (DSBs) in DNA are arguably the most important class of DNA damage because they may lead to either cell death or loss of genetic material resulting in chromosomal aberrations. The balance of DSB repair activity appears to be critical to the genetic stability of cells. Too little repair leads to the acquisition and persistence of mutations, whereas elevated levels of repair can inhibit the apoptotic pathway and can enable a cell with badly damaged DNA to attempt repair, potentially mis-repair, and survive. DSBs are predominantly repaired by either homologous recombination (HR) repair or nonhomologous end-joining pathways in mammalian cells. Polymorphisms have now been identified in a number of genes involved in protecting from, and repairing, DSBs and we have chosen to study the distribution of several of these, in genes belonging to the HR DNA DSB repair pathway.

HR uses the second, intact, copy of a chromosome as a template to copy the information lost at the DSB site on the first chromosome, and, hence, HR repair is a high-fidelity process.
A Role for HR Repair in AML

One of the central proteins in the HR repair pathway is RAD51. RAD51 binds to DNA and promotes ATP-dependent homologous pairing and strand transfer reactions [reviewed in Baumann and West (10)]. The RAD51 protein is essential to the viability of a cell and gene knock-out models in mice are embryonically lethal (11, 12). Cells lacking RAD51 are characterized by an accumulation of chromosomal breaks before cell death (13). Hence the role of RAD51 is vital in maintaining genetic stability within a cell.

A G/C polymorphism at position –135 in the 5' untranslated region of the RAD51 gene has been identified (RAD51-G135C; Ref. 14). Because of the interaction of RAD51 with the breast/ovarian cancer susceptibility gene products BRCA1 and BRCA2 (15–17), this polymorphism has been the focus of a number of studies in breast and ovarian cancer (14, 18–20). The polymorphism has been shown to be associated with breast cancer risk in BRCA2 mutation carriers (18). However, because of the importance of the HR DSB repair pathway and the fact that AML is often characterized by a high degree of genetic instability, we have decided to look at the frequency of this polymorphism in AML.

The XRCC3 protein also functions in the HR DNA DSB repair pathway and directly interacts with and stabilizes RAD51 and the closely related RAD51C (21–25). XRCC3 is a paralog of RAD51 and, similar to RAD51, it is essential for genetic stability (26–30). A polymorphism at codon 241 in the XRCC3 gene results in a Thr-to-Met amino acid substitution (31). Positive associations between XRCC3-241Met and malignancies have been made by several groups, including studies on bladder cancer (32) and melanoma skin cancer (33). The variant XRCC3-241Met allele has also been associated with a higher level of DNA adducts compared with those individuals with the wild-type gene (34). However, we have previously reported that there is no association between XRCC3-241Met and AML or t-AML (5).

A malignant phenotype is likely to result from the accumulation of many minor genotypes, and it is probable that associations exist between polymorphisms in DNA repair genes and polymorphisms in genes encoding proteins involved in minimizing the effects of carcinogens. The glutathione S-transferases (GSTs) are a multigene family that detoxify reactive electrophiles via conjugation to glutathione and, hence, prevent damage to DNA. A deletion polymorphism in one isozyme of this family [glutathione S-transferase M1(GSTM1)] occurs in ~50% of Caucasians (35) resulting in the absence of functional GSTM1 protein. GSTM1 detoxifies a variety of genotoxic agents (reviewed in Hayes and Pulford; Ref. 36) and a reduction in GSTM1 protein activity, due to the deletion polymorphism, has previously been shown to be associated with significantly elevated levels of DNA adducts in WBCs (37) and with higher levels of sister-chromatid exchange (38) compared with individuals with wild-type GSTM1 protein levels.

We hypothesized that polymorphisms in the RAD51 and XRCC3 HR DNA DSB repair genes may lead to aberrations within the pathway and contribute to genetic instability and subsequent AML. In addition, we wanted to establish whether an increase in DNA damage caused by a deletion polymorphism in GSTM1 would further contribute to the pathogenesis of AML.

MATERIALS AND METHODS

Study Subjects. Blood or bone marrow samples were obtained at AML diagnosis after informed consent, according to the declaration of Helsinki, from 267 Caucasian patients, including 51 with t-AML. The diagnosis of AML was made using the French-American-British criteria after conventional cytochemical and surface marker analysis. The median ages of the AML patients were as follows: de novo AML, 64 years (range, 11–96 years); and t-AML, 59 years (range, 27–88 years). Control peripheral blood samples were obtained from an equivalent number (n = 186) of white Caucasian individuals, with no known malignancies, from the same small geographic area in the United Kingdom. The median age of the controls was 50 years (range, 15–97 years). Genomic DNA was extracted from cells using QIAamp blood DNA isolation kits (Qiagen, Crawley, United Kingdom) according to the manufacturer’s protocol.

Studies on the majority of these patients and controls have been described previously (5).

PCR RFLP Analysis. PCR followed by either direct analysis (GSTM1) or enzymatic digestion of the PCR products (RAD51 and XRCC3) was used for the genotyping of the three polymorphisms. Approximately 50 ng of genomic DNA was used as template in each of the PCR amplifications. The 50-μl reaction also consisted of each dNTP (at 150 μM; Amersham Biosciences United Kingdom Ltd, Little Chalfont, United Kingdom), each primer (at 1 μM), 1.5–2.0 mM MgCl2, and 2 units of AmpliTaq Gold (PE Applied Biosystems, Warrington, United Kingdom) in the manufacturer’s buffer. After an initial heat activation step at 95°C for 10 min, amplification was performed in a PTC-100TM Programmable Thermal Controller (MJ Research, Inc, Watertown, MA) using the following conditions: XRCC3-Thr241Met, denaturation at 95°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min for a total of 35 cycles ending with a final extension at 72°C for 10 min. The conditions for the RAD51 PCR were as described except an annealing temperature of 52°C was used; an annealing temperature of 57°C was used for the GSTM1 amplification. PCR products were purified using a QIAquick PCR purification kit (Qiagen) before digestion and/or sequencing.

XRCC3-Thr241Met. XRCC3-Thr241Met was amplified in a 415-bp product using primers described previously (31) with 2 mM MgCl2. The PCR products were digested at 37°C overnight with 10 units of NlaIII [New England Biolabs (United Kingdom) Ltd, Hitchin, United Kingdom] in 1× buffer supplied with the enzyme and supplemented with 100 ng/μl BSA. All XRCC3 PCR products contain an internal NlaIII site, and, in addition, the presence of the Met polymorphism also generates an additional NlaIII site resulting in 104-bp, 141-bp, and 170-bp products for the polymorphic allele and 141-bp and 274-bp products for the wild-type threonine allele.

The digested products were resolved on 3% agarose gels (Helena Biosciences, Sunderland, United Kingdom), stained with ethidium bromide and analyzed under UV light.

RAD51-G135C. The RAD51-G135C polymorphism was amplified in a 157-bp fragment using primers described previously (18) with 1 mM MgCl2. The PCR products were digested with 10 units of MvaI (Helena Biosciences) in the manufactur-
er’s buffer. The polymorphic C allele eliminates the MvaI restriction site, and, therefore, the digestion of polymorphic samples resulted in a single band at 157 bp, whereas the wild-type allele resulted in two bands at 71 bp and 87 bp. All of the polymorphic samples were sequenced.

Two reviewers (C.S., E.D-G.) independently scored all of the genotypes, and samples that could not be scored were sequenced. Sequencing was also carried out on a 10% random sample population of control and AML PCR products.

**GSTM1.** The GSTM1 gene was amplified using previously described primers (39), β-globin was also amplified as an internal control using 2.5 mm MgCl$_2$. The GSTM1 null genotype was indicated by the presence of the 268-bp β-globin gene and lack of the 215-bp GSTM1 gene.

**Sequencing Reactions.** Sequencing reactions were set up with ~200 ng of purified PCR product and 10 pmol primer using a Thermo Sequenase II dye terminator cycle sequencing premix kit according to the manufacturer’s instructions (Amersham Biosciences United Kingdom Ltd). The primers used for sequencing were the same as those used for the PCR amplifications. The reactions were electrophoresed using an ABI 377 automated DNA sequencer (PE Applied Biosystems) as recommended by the manufacturer.

**Statistical Analysis.** The observed genotype frequencies of the XRCC3-Thr241Met and RAD51-G135C polymorphisms in the control cohorts were compared with those calculated by the Hardy-Weinberg equilibrium ($p^2 + q^2 + 2pq = 1$; where $p$ is the variant allele frequency). The distribution of genotypes in AML populations compared with the control population were tested for significance using odds ratios (ORs) and their 95% confidence intervals (95% CIs) calculated by logistic regression analysis and adjusted for the effect of age. Values of $P \leq 0.05$ were considered significant.

All of the analyses were performed by the statistical package SPSS for Windows, Version 9 (SPSS Inc, Chicago, Illinois).

## RESULTS

**RAD51-G135C, XRCC3-Thr241Met, and GSTM1 Polymorphisms.** We have examined the frequency of three polymorphisms in 216 cases of de novo AML and 186 controls. We also analyzed a subgroup of 51 patients with t-AML. Because of an inadequate amount of DNA in some cases, several samples did not generate complete information on all three polymorphisms. There was very little discrepancy in genotype scores between the two reviewers; the PCR was repeated on ambiguous samples. In all of the cases in which sequencing was performed, the results confirmed the RFLP findings. Among the controls, the variant allele frequencies were: RAD51-G135C, 0.06; and XRCC3-241Met, 0.29; 44.1% of the controls lacked both GSTM1 alleles. The RAD51-G135C and XRCC3-Thr241Met genotype frequencies among the control population were consistent with those expected from the Hardy-Weinberg equilibrium; RAD51-G135C, $\chi^2 = 0.576$, $P = 0.750$; XRCC3 Thr241Met, $\chi^2 = 1.030$, $P = 0.957$.

The distributions of the three genotypes in control individuals and AML and t-AML patients and the adjusted ORs for each genotype are shown in Table 1. The results for the RAD51-G135C polymorphism demonstrate that the proportion of de novo AML and t-AML patients possessing at least one polymorphic C allele is higher than in the control group; this results in a significant 2.66-fold increase in the risk of developing AML in the t-AML group. Neither the XRCC3-Thr241Met nor the GSTM1 genotype distributions demonstrated statistical differences in either de novo AML or t-AML compared with the control group. The XRCC3-Thr241Met genotype results on a section of this population have previously been reported (5).

**Combined Analysis of Polymorphisms.** Table 2 shows the combined analysis of the two polymorphisms present in genes that encode proteins involved in the HR DSB DNA repair pathway in both AML and t-AML. Interactions between the two polymorphisms can be seen; this is associated with a significant

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (n)</th>
<th>AML</th>
<th>t-AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAD51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g/g</td>
<td>186</td>
<td>206</td>
<td>51</td>
</tr>
<tr>
<td>g/c</td>
<td>166</td>
<td>171</td>
<td>39</td>
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<tr>
<td>c/c</td>
<td>18</td>
<td>32</td>
<td>1.0</td>
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<tr>
<td>g/c+c/c</td>
<td>2</td>
<td>3</td>
<td>1.60</td>
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<td>XRCC3-241</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Thr/Thr</td>
<td>175</td>
<td>216</td>
<td>44</td>
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<td>Thr/Met</td>
<td>64</td>
<td>87</td>
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<td>19</td>
<td>30</td>
<td>1.56</td>
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<tr>
<td>Thr/Met + Met/Met</td>
<td>83</td>
<td>117</td>
<td>1.38</td>
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<tr>
<td>GSTM1</td>
<td></td>
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<tr>
<td>Present</td>
<td>177</td>
<td>200</td>
<td>42</td>
</tr>
<tr>
<td>Null</td>
<td>78</td>
<td>101</td>
<td>1.28</td>
</tr>
</tbody>
</table>

* OR, odds ratio; CI, confidence interval; t-AML, therapy-related AML; Ref, used as reference.

* Adjusted for age.

* Carriers of at least one intact allele are used as reference.
A Role for HR Repair in AML

The efficient repair of DSBs is essential for genetic stability. The knock-out model of the HR repair gene RAD51 is embryonically lethal (11, 12) and XRCC3 is also essential for genetic stability (26–30). Mutations in these genes are rare, demonstrating the absolute requirement of the encoded proteins. However subtle differences in protein levels or protein activity resulting from polymorphisms are likely to be tolerated by cells, although these differences would be expected to have an effect when a high level of DNA damage is present. We have demonstrated that this may indeed be the case in AML. The presence of variant RAD51-135C and XRCC3-241Met is associated with an increase in the risk of developing t-AML. We have demonstrated significant ORs in AML and t-AML associated with the presence of polymorphisms in two HR genes, namely RAD51-135C and XRCC3-241Met. In addition, when a deletion polymorphism of the detoxification gene GSTM1 is taken into consideration, there is a greater than 15-fold increase in the risk of development of AML when the three variant genotypes are present.

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<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>AML</th>
<th>OR* (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All WT</td>
<td>38</td>
<td>29</td>
<td>1 (Ref)</td>
<td></td>
</tr>
<tr>
<td>One variant</td>
<td>79</td>
<td>86</td>
<td>1.55 (0.85–2.82)</td>
<td>0.16</td>
</tr>
<tr>
<td>Two variants</td>
<td>41</td>
<td>48</td>
<td>1.57 (0.80–3.07)</td>
<td>0.19</td>
</tr>
<tr>
<td>Three variants</td>
<td>1</td>
<td>12</td>
<td>15.26 (1.83–127.27)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Table 2  Combined logistic regression analysis to study the association between DNA double-strand break repair genotypes and acute myeloid leukemia (AML) and therapy-related AML (t-AML) risk

Table 3  Combined logistic regression analysis to study the association between DNA double-strand break repair genotypes, RAD51-135C and XRCC3-241Met, and GSTM1 genotype and acute myeloid leukemia (AML) risk

increase in the risk of development of both AML (OR, 3.77; 95% CI, 1.39–10.24) and t-AML (OR, 8.11; 95% CI, 2.22–29.68) in individuals with at least one variant RAD51-135C and at least one variant XRCC3-241Met allele. In addition to the analysis on the combined effects of the HR repair genes, the control and AML groups have also been analyzed with respect to the presence or absence of the detoxification GSTM1 gene. These results are shown in Table 3. The combination of a null GSTM1 genotype with the double HR gene variants results in a large increase in the risk of AML development (OR, 15.26; 95% CI, 1.83–127.27).

**DISCUSSION**

Our findings suggest that the HR DSB DNA repair pathway may be important in the pathogenesis of both de novo and t-AML. We have demonstrated significant ORs in AML and t-AML associated with the presence of polymorphisms in two HR genes, namely RAD51-135C and XRCC3-241Met. In addition, when a deletion polymorphism of the detoxification gene GSTM1 is taken into consideration, there is a greater than 15-fold increase in the risk of development of AML when the three variant genotypes are present.

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Table 2  Combined logistic regression analysis to study the association between DNA double-strand break repair genotypes and acute myeloid leukemia (AML)/therapy-related AML (t-AML) risk

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<th>XRCC3-241</th>
<th>Control</th>
<th>n</th>
<th>OR* (95% CI)</th>
<th>P</th>
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<tr>
<td>WT</td>
<td>WT</td>
<td>78</td>
<td>74</td>
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<tr>
<td>V</td>
<td>WT</td>
<td>10</td>
<td>14</td>
<td>1.31 (0.53–3.24)</td>
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</tr>
<tr>
<td>WT</td>
<td>V</td>
<td>74</td>
<td>83</td>
<td>1.19 (0.74–1.90)</td>
<td>0.48</td>
</tr>
<tr>
<td>V</td>
<td>V</td>
<td>6</td>
<td>21</td>
<td>3.77 (1.39–10.24)</td>
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*OR, odds ratio; CI, confidence interval; WT, wild-type genotype; Ref, used as reference group; V, the presence of a variant allele (either heterozygous or homozygous).

* Adjusted for age.
A deletion polymorphism also occurs at the GSTT1 locus (40), and the distribution of the GSTT1 genotype was studied in our patient and control cohorts (data not shown). Although a similar trend to that described among the GSTM1, RAD51, and XRCC3 polymorphisms was also evident among GSTT1, RAD51 and XRCC3, because of the lower frequency of the GSTT1 deletion polymorphism (20% frequency of GSTT1 null compared with 50% for GSTM1 null), the numbers of individuals required for statistical analysis are much greater than those available to us, and we were unable to draw any firm conclusions from this work. However, our results encourage the establishment of large population analyses to study the combined effects of polymorphisms in DNA repair and detoxification genes.

Work is still required to determine the exact functional consequences of the DNA repair gene polymorphisms. Little is known about the effect of the RAD51-135C variant, although recent data suggest that this polymorphism may increase the levels of RAD51 mRNA in patients with breast cancer (41). If this also results in an increase in RAD51 protein expression, then it is possible that the fine balance of HR protein levels will be disrupted. Overexpression of RAD51 or RAD52 have previously been demonstrated to have a dominant-negative effect on HR initiation (42). In addition, it is possible that an increase in the cellular levels of RAD51 protein may inhibit the stimulation of the apoptotic pathway and allow a cell with damaged DNA to survive, acquire mutations and chromosomal aberrations, and subsequently convey the damage to daughter cells. This is a plausible explanation as to why we found a significant increase in the risk of the development of t-AML when a variant RAD51-135C allele was present (irrespective of other genotypes). We suggest that the variant level of RAD51-135C alone is sufficient to confer a significant increase in the risk of development of t-AML (OR, 2.66; 95% CI, 1.17–6.02) because of the huge genotoxic insult that precedes the disease.

Recent evidence indicates that the XRCC3-241Met variant is functional in HR repair of DSBs (43). The variant protein was shown to be able to complement the HR defect in a XRCC3-deficient cell line; however, the investigators (Araujo et al.; Ref. 43) note that the possibility of a genetic interaction between variant XRCC3 and polymorphic alleles of other DNA repair genes that may lead to a HR defect could not be ruled out. This is supported by our results: we did not see an effect for the variant XRCC3-241Met gene alone in either AML or t-AML, confirming our previous results (5); however, when combining the variant XRCC3-241Met genotype with a variant RAD51-135C genotype a significant risk in the development of AML and t-AML was observed (AML: OR, 3.77; 95% CI, 1.39–10.24; t-AML: OR, 8.11; 95% CI, 2.22–29.68).

We did not find a difference between proportions of patients with AML or t-AML with the GSTM1 gene deletion compared with control individuals. This is in keeping with other studies (44–46), although Allan et al. (9) reported a slight increase in risk in de novo AML (OR, 1.28; 95% CI, 1.02–1.61). Although we have demonstrated a significant and large effect of combining this genotype with the variant HR genotypes in AML, we cannot determine whether this trend is also seen in t-AML because of the smaller number of patients in this group. However, it would not be surprising if such a large effect were observed in t-AML, because exposure to chemotherapy or radiotherapy and the resulting high levels of DNA damage may mask the requirement of the GSTM1 protein.

Mismatches repair and base excision repair pathways have already been demonstrated to contribute to the pathogenesis of AML and t-AML (5, 47–49); however, double-strand break repair has been, thus far, largely unstudied in AML. We have shown that the efficient functioning of this pathway is significantly important in protecting individuals from both de novo AML and t-AML. In addition, minimizing damage to DNA by harboring functional GSTM1 protein is also a vital requirement for protecting against de novo AML. AML is characterized by genetic instability, and it is likely that defects in the HR repair pathway will contribute to the chromosomal aberrations seen in the majority of AML cases. Larger studies are now required to confirm these findings and to also further divide the patients into cytogenetic groups to establish whether the genotype described here results in a particular pattern of chromosomal abnormality. Studies involving greater numbers of individuals are also required to demonstrate that none of our findings were achieved by chance because of the large number of statistical analyses that we performed for the work reported here.

This report illustrates the importance of studying the combined effects of genotypes, particularly those from more than one gene in a specific pathway. Our work demonstrates a positive association between polymorphisms in genes involved in protecting the cells from DSBs and an increased risk of de novo and t-AML susceptibility.

ACKNOWLEDGMENTS

We thank Stephen Langabeer and Steve Chatters and the Kay Kendall Leukemia Fund for t-AML DNA samples from the DNA/RNA banking facilities at University College Hospital, London, and the Medical Research Council Acute Leukemia Working Party for access to samples from patients with AML entered into Medical Research Council trials.

REFERENCES

Polymorphisms in Genes Involved in Homologous Recombination Repair Interact to Increase the Risk of Developing Acute Myeloid Leukemia

Claire Seedhouse, Rowena Faulkner, Nadia Ashraf, et al.