Genetic variants of NHEJ DNA ligase IV can affect the risk of developing multiple myeloma, a tumour characterised by aberrant class switch recombination

P L Roddam, S Rollinson, M O’Driscoll, P A Jeggo, A Jack, G J Morgan

The DNA double stranded break (DSB) repair mechanism, non-homologous end joining (NHEJ) represents an essential step in antigen receptor gene rearrangement mechanisms, processes believed to be intimately involved in the aetiology of lymphoproliferative disease. We investigated the potential impact that previously undescribed polymorphisms identified within NHEJ DNA ligase IV (LIG4) have upon predisposition to several lymphoproliferative disorders, including leukaemia, lymphoma, and multiple myeloma. Two LIG4 polymorphisms were examined, both C>T transitions, which result in the amino acid substitutions A3V and T9I. Inheritance of the LIG4 A3V CT genotype was found to be significantly associated with a two-fold reduction in risk of developing multiple myeloma (OR 0.49, 95% CI 0.27 to 0.89). Similarly, inheritance of the LIG4 T9I CT and T9I TT genotypes were found to associate with a 1.5-fold reduction (OR 0.77, 95% CI 0.51 to 1.17) and a four-fold reduction (OR 0.25, 95% CI 0.07 to 0.70) in risk of developing multiple myeloma respectively, suggesting a gene dosage effect for this polymorphism. The LIG4 A3V and T9I variant alleles are in linkage disequilibrium (D’=0.95, p<0.0001), and the protective effect associated with these polymorphisms was found to be the result of inheritance of the A3V-T9I and A3V-T9I TT haplotypes. These data suggest that genetic variants of NHEJ LIG4 may modulate predisposition to multiple myeloma, a tumour characterised by aberrant immunoglobulin (lg) class switch recombination.
coding sequence begins with an earlier ATG at position 274. This differs from that described in NM_002312; however, this region has been shown to be essential for DNA ligase IV activity, raising the possibility that the A3V and T9I polymorphisms might confer some impact on DNA ligase IV function. We therefore examined the impact of these genetic variants in the aetiology of lymphoproliferative disease using the REAL categories: adult acute lymphoblastic leukaemia (ALL), follicular lymphoma (FCL), diffuse large B cell lymphoma (DLBCL), and multiple myeloma, the pathogenesis of which involve different contributions of the various antigen receptor rearrangement mechanisms.

**METHODS**

**Study population**

Potential associations between LIG4 polymorphism and lymphoproliferative disease were investigated by Taqman™ Allelic Discrimination using a case-control approach. The study population was composed of archived tumour material, and of material collected by the Leukaemia Research Fund and the Myeloma VII population based case-control epidemiological studies. All case and controls were of European origin and for ALL, lymphoma, and multiple myeloma cases, all diagnoses were pathologically confirmed. Ethical approval was obtained for epidemiological study subjects. Control subjects used as the reference group were randomly chosen from a large population based lymphoma case-control study undertaken by the Leukaemia Research Fund. Genomic DNA was prepared for 220 controls, 364 lymphoma cases composed of 180 FCL cases and 184 DLBCL cases, 70 ALL cases, and 270 multiple myeloma cases using a standard laboratory protocol.

**LIG4 genotyping**

LIG4 A3V and T9I genotypes were determined using the ABI PRISM 7700 sequence detection system, with assay primers and probes designed by Primer Express™ v1.5. The A3V assay was undertaken using 900 nmol/l of both sense (5′-TTGCTTACTAGTTAACGAGAAGATCA-3′) and antisense (degenerative) (5′-AACGTGAGATGCAACAAG(A)TTTGTG-3′) primers, 200 nmol/l of FAM labelled C allele probe (5′-ACCGCTTTGATGGCTGCCTACA-3′), and 100 nmol/l of VIC labelled T allele probe (5′-ACCGCTTTGATGGCTGTCTACA-3′), with an annealing temperature of 63°C. The T9I assay was undertaken using 900 nmol/l of both sense (5′-GAGAAGATCATCAGCGCTTGAG-3′) and antisense (5′-TGGTCTAAAGTTGAAACCAAAATCTG-3′) primers, and 100 nmol/l of both FAM labelled C allele probe (5′-ACAAACCTCACAACTGTTGCATCTCACGT-3′) and VIC labelled T allele probe (5′-ACAATACTCACAAAATGTTGCATCTCACGT-3′), with an annealing temperature of 66°C. The T9I assay was undertaken using 900 nmol/l of both sense (5′-GAGAAGATCATCAGCGCTTGAG-3′) and antisense (5′-TGGTCTAAAGTTGAAACCAAAATCTG-3′) primers, and 100 nmol/l of both FAM labelled C allele probe (5′-ACAAACCTCACAACTGTTGCATCTCACGT-3′) and VIC labelled T allele probe (5′-ACAATACTCACAAAATGTTGCATCTCACGT-3′), with an annealing temperature of 66°C. PCR reactions were carried out using 20-50 ng of DNA and 1X Taqman™ Universal PCR Master Mix, in a total volume of 20 µl. LIG4 A3V and T9I wild type (CC), heterozygote (CT), and homozygote variant (TT) genotype controls, plus non-template controls, were included within each plate. As a quality control measure, 10% of the control population were
randomly selected and genotyped for the A3V and T9I polymorphisms a second time by direct sequencing (BigDye Terminator Kit, Applied Biosystems) (fig 1).

Statistical analysis
Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated using logistic regression, conducted by Stata (Intercooled Stata 6.0 for Windows NT, Stata Corporation, 1999). Each disease group was analysed as an independent case series using the control group as a reference population where, with the exception of ALL (a relatively rare disease), analysis had approximately 80% power to detect an OR of 0.5 with a 95% confidence interval as determined by Epi Info v5.01a. Hardy-Weinberg equilibrium was tested by $\chi^2$ analysis for Ligase IV genotype using the HW package program (Linkage Utility Programs, Rockefeller University, USA). Haplotype frequency variation was assessed by $\chi^2$ analysis using the Astute for Excel package, plus the Yates correction for small numbers.

Linkage analysis
Investigation into possible linkage disequilibrium between Ligase IV A3V and T9I polymorphisms took two forms: statistical prediction using linkage analysis software, EH and 2LD (Linkage Utility Programs, Rockefeller University, USA), and direct experimental analysis through A3V and T9I double heterozygote sequencing. EH was used to estimate Ligase IV A3V-T9I haplotype frequency, followed by subsequent association analysis using 2LD, to obtain a linkage disequilibrium $D'$ coefficient for the Ligase IV variant alleles. Five control subjects identified with the A3V T9I double heterozygote genotype were cloned and sequenced, supported this statistical prediction of linkage disequilibrium as all A3V T9I double heterozygotes possessed the two spatial proximity of the two alleles, or from an A3V C>T base substitution occurring first, producing the T9I variant allele. Alternatively, the absence of selective pressure observed for the A3V genotype may be the result of the small number of controls used in this study and the relative rarity of the A3V variant allele in comparison with the T9I variant allele. Alternatively, the protective effect of the A3V polymorphism may arise as a consequence of association with the actual protective allele, T9I.

The combined Ligase IV A3V T9I genotype distribution suggests that the T9I C>T base substitution occurred first, producing the A3V-T9I CT haplotype, followed by a second event, the A3V T9I allele confers a 1.5-fold reduction in risk of developing multiple myeloma, and inheritance of two copies of the T9I variant allele confers a four-fold reduction in risk of developing multiple myeloma (table 2). However, a significant interaction ($\chi^2=12.6, p<0.05$) was observed among multiple myeloma cases, with 50% fewer multiple myeloma cases genotyped as A3V T9I double heterozygotes compared to controls (table 2).

Distribution of the Ligase IV A3V and T9I polymorphisms within the control population were investigated for Hardy-Weinberg equilibrium. The T9I genotype distribution was found to deviate significantly from equilibrium ($\chi^2=4.73, p=0.03$), with positive selection bias upon the T9I TT genotype. In contrast, distribution of the A3V genotype did not significantly deviate from Hardy-Weinberg equilibrium. However, the distribution of combined A3V T9I genotypes indicated significant linkage disequilibrium between the Ligase IV polymorphisms. Statistical analysis using EH and 2LD predicted non-random association between the two variant alleles ($D'=0.95, \chi^2=128.8, p<0.0001$). Furthermore, direct experimental analysis, whereby five control subjects genotyped as double heterozygotes for A3V and T9I were cloned and sequenced, supported this statistical prediction of linkage disequilibrium as all A3V T9I double heterozygotes were found to possess the A3V and T9I C>T base substitution in cis, not trans (data not shown). Therefore, subjects genotyped as double heterozygotes possess the two Ligase IV haplotypes A3V-T9I CC and A3V-T9I TT. The apparent absence of selective pressure observed for the A3V genotype may be the result of the small number of controls used in this study and the relative rarity of the A3V variant allele in comparison with the T9I variant allele. Alternatively, the protective effect of the A3V polymorphism may arise as a consequence of association with the actual protective allele, T9I.

Table 1 Ligase IV A3V and T9I genotype distributions of controls and lymphoproliferative disorder cases, with odds ratios (OR) and 95% confidence intervals (95% CI)

<table>
<thead>
<tr>
<th>Ligase IV</th>
<th>Controls (n=220)</th>
<th>ALL (n=70)</th>
<th>Lymphoma (n=364)</th>
<th>Multiple myeloma (n=270)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No (%)</td>
<td>No (%)</td>
<td>OR* 95% CI</td>
<td>No (%)</td>
</tr>
<tr>
<td>A3V†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>189 (85.9)</td>
<td>61 (87.1)</td>
<td>1 –</td>
<td>316 (86.8)</td>
</tr>
<tr>
<td>CT</td>
<td>31 (14.1)</td>
<td>9 (12.9)</td>
<td>0.90 0.41 to 2.00</td>
<td>48 (13.2)</td>
</tr>
<tr>
<td>TT</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>– –</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>SNA‡</td>
<td>– –</td>
<td>– –</td>
<td>– –</td>
<td>2</td>
</tr>
<tr>
<td>T9I†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>149 (67.7)</td>
<td>49 (70.0)</td>
<td>1 –</td>
<td>247 (67.9)</td>
</tr>
<tr>
<td>CT</td>
<td>58 (26.4)</td>
<td>21 (30.0)</td>
<td>1.10 0.61 to 2.00</td>
<td>108 (29.7)</td>
</tr>
<tr>
<td>TT</td>
<td>13 (5.9)</td>
<td>2 (2.9)</td>
<td>– –</td>
<td>7 (1.9)</td>
</tr>
<tr>
<td>SNA‡</td>
<td>– –</td>
<td>– –</td>
<td>– –</td>
<td>2</td>
</tr>
</tbody>
</table>

*ORs estimated by logistic regression; †CC: wild type, CT: heterozygote, TT: homozygote variant; ‡SNA: sample non-amplifiable.
substitution occurring independently from the T9I mutation. No significant difference in haplotype frequencies was observed between the control population, and either the ALL or lymphoma case populations (table 3). However, LIG4 haplotype frequencies for the multiple myeloma cases differed significantly from those of the control population (table 3) ($\chi^2 = 7.83$, $p = 0.05$), suggesting that both the A3V-T9I CT and A3V-T9I TT haplotypes are associated with protection against the development of multiple myeloma. These data reflect the genotype analysis, whereby inheritance of a single copy of either the A3V or T9I variant alleles, independently or in combination, or inheritance of two copies of the T9I variant allele is associated with a significant reduction in risk of multiple myeloma. A similar association was not observed for inheritance of two copies of the A3V allele, possibly owing to the rarity of the A3V-T9I TC haplotype or variation in effect observed for the different lymphoproliferative disease groups.

**DISCUSSION**

Our data provide evidence to implicate germline variation within the NHEJ pathway in the risk of developing lymphoproliferative disease. We examined several lymphoproliferative disorder case series, ALL, FCL, DLBCL, and multiple myeloma, recognised by the REAL classification as distinct pathological entities, with potentially different aetiological mechanisms. ALL is a precursor cell disorder, originating from an early bone marrow progenitor which is in the process of V(D)J recombination. The germinal centre lymphomas analysed in this study, made up of both FCL and DLBCL cases, may have arisen through aberrant V(D)J recombination or receptor editing, possibly with contribution from somatic hypermutation and, in some instances, class switch recombination. In particular, the FCL group is characterised by t(14;18), where BCL2 is translocated from 18q32 into the $J_a$ region of the Ig heavy chain (IGH) gene, with subsequent deregulation of the $BCL2$ gene. In contrast, multiple myeloma is a malignant disorder of plasma cells characterised, in 60% of cases, by aberrant class switch recombination. The germinal centre lymphomas and DNA ligase IV by LIG4 is important in all of these mechanisms of antigen receptor rearrangement. Our data show a protective effect associated with inheritance of the LIG4 A3V-T9I CT and TT haplotypes, suggesting that functional variation of NHEJ as a consequence of LIG4 polymorphism, can modulate the risk of lymphoproliferative disease. The risk is, however, most significant for multiple myeloma, a tumour associated with abnormal class switch recombination as an early pathogenetic event. The variation in effect observed for LIG4 A3V and T9I for the different lymphoproliferative disease groups could be attributed to the different DNA DSB repair mechanisms, essential for effective immunological response, used by the respective target for transformation. The various antigen receptor rearrangement processes may involve different components of repair pathways, as has been recently shown by studies in rodents investigating the role of mismatch repair proteins in somatic hypermutation and class switch recombination. Therefore, although Ig class switching and somatic hypermutation share a number of features suggesting common machinery, they are distinct mechanisms with specific components, including those involved in DNA repair as shown by the requirement for functional NHEJ in class switch recombination, but not somatic hypermutation.

The LIG4 A3V and T9I polymorphisms were initially identified in a LIGIV syndrome patient (411BR), suggesting a deleterious impact of the polymorphisms upon DNA ligase IV function. Yet these data provide evidence to suggest the LIG4 A3V and T9I polymorphisms are protective in nature, conferring a reduced risk of developing multiple myeloma. Further support is provided by the apparent positive selection upon the T9I variant allele within the control population, suggesting that these LIG4 genetic variants may possess a protective role.

### Table 2 Combined ligase IV A3V and T9I genotype distribution of controls and lymphoproliferative disorder cases, with odds ratios (OR) and 95% confidence intervals (95% CI)

<table>
<thead>
<tr>
<th>Ligase IV polymorphism</th>
<th>Controls (n=220)</th>
<th>ALL (n=70)</th>
<th>Lymphoma (n=364)</th>
<th>Multiple myeloma (n=270)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A3V†</td>
<td>T9I†</td>
<td>A3V†</td>
<td>T9I†</td>
</tr>
<tr>
<td>CC</td>
<td>148 (67.3)</td>
<td>49 (70.0)</td>
<td>244 (67.0)</td>
<td>202 (74.8)</td>
</tr>
<tr>
<td>CT</td>
<td>34 (15.5)</td>
<td>12 (17.1)</td>
<td>67 (18.4)</td>
<td>42 (15.6)</td>
</tr>
<tr>
<td>TT</td>
<td>7 (3.2)</td>
<td>0 (0.0)</td>
<td>4 (1.1)</td>
<td>2 (0.7)</td>
</tr>
<tr>
<td>CC</td>
<td>1 (0.5)</td>
<td>0 (0.0)</td>
<td>3 (0.8)</td>
<td>1 (0.4)</td>
</tr>
<tr>
<td>CT</td>
<td>24 (10.9)</td>
<td>9 (12.9)</td>
<td>41 (11.3)</td>
<td>18 (6.7)</td>
</tr>
<tr>
<td>TT</td>
<td>248 (67.3)</td>
<td>49 (70.0)</td>
<td>244 (67.0)</td>
<td>202 (74.8)</td>
</tr>
<tr>
<td>A3V†</td>
<td>0.81</td>
<td>0.00</td>
<td>0.12</td>
<td>0.00</td>
</tr>
<tr>
<td>T9I†</td>
<td>0.08</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>A3V†</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>T9I†</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

*ORs estimated by logistic regression; †CC: wild type, CT: heterozygote, TT: homozygote variant; ‡SNA: sample non-amplifiable.

### Table 3 Ligase IV A3V-T9I haplotype frequency distribution of controls and lymphoproliferative disorder cases

<table>
<thead>
<tr>
<th>Ligase IV</th>
<th>Haplotype frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3V locus</td>
<td>T9I locus</td>
</tr>
<tr>
<td>Controls</td>
<td>ALL</td>
</tr>
<tr>
<td>C</td>
<td>0.81</td>
</tr>
<tr>
<td>C</td>
<td>0.12</td>
</tr>
<tr>
<td>T</td>
<td>0.00</td>
</tr>
<tr>
<td>T</td>
<td>0.07</td>
</tr>
</tbody>
</table>

$\chi^2$, p<0.05

*Haplotype frequency estimated by EH. †Haplotype frequency variation calculated by $\chi^2$ analysis.
for other diseases in which defective DNA DSBR is implicated as causative. It could be postulated that this observation of the LG4 A3V and T9I polymorphisms results from the ubiquitous nature of DNA ligase IV and the NHEJ pathway and its involvement in both V(DJ) recombination and Ig class switch recombination. LGIV syndrome patients show immunodeficiency through a reduced ability to undergo V(DJ) recombination and thus to produce viable lymphocytes, a preliminary process to Ig class switching. Multiple myeloma, in contrast, is characterised by aberrant class switch recombination; therefore, A3V and T9I variants may have a different impact upon each of these rearrangement mechanisms. Alternatively, it may be proposed that LG4 A3V and T9I in the absence of mutation such as R278H confer a reduced risk of chromosomal instability and subsequent malignancy as observed in a single myeloma in this study. However, when combined with other genetic defects, A3V and T9I enhance the effect of that mutation upon DNA ligase IV, producing a more severe clinical phenotype, such as that observed for patient 411BR.

It has been proposed that the non-catastrophic regions of human DNA ligases contain unique regions that determine the protein-protein interactions, and therefore the differential cellular functions of these enzymes. Ligase IV forms a complex with XRCC4 via the non-catastrophic C-terminus, which contains tandem BRCT motifs between which XRCC4 binds. XRCC4 is essential for ligase IV protein stabilisation and activity, stimulating adenylation of ligase IV, the initial step in ligase binding. Recent structural analysis of XRCC4 suggests the protein forms a dumbbell-like tetramer, allowing multivalent ligase IV and DNA binding. Although the N-terminus sequence of DNA ligase IV containing A3V and T9I is not highly conserved, this region has been shown to be essential for ligase IV activity. It has been shown that ligase IV stability is not affected by the A3V or T9I polymorphisms, but they are predicted to increase the hydrophobicity of the region. More detailed analysis using structural modelling programmes predicts an impact on the protein’s tertiary structure (P A Jeggo, unpublished data). It may be proposed that the protective effect of the LG4 variants seen in this study results from the A3V and T9I substitutions altering the protein-protein interactions of DNA ligase IV with components of the NHEJ pathway, such as XRCC4, or with other ligase IV processes in which the enzyme is involved.

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REFERENCES

Unlinked genes influence severity of FAP within families

A study of how familial adenomatous polyposis (FAP) varies within families supports the hypothesis that modifier genes affect the severity of the disease. This in turn may provide new insights into tumour formation in the gut.

Severity of FAP was significantly related to truncation mutations of the APC gene. Individual family members with severe disease, associated with a high polyp count, had mutations clustering in the MCR region, and those with moderate or mild disease had mutations clustering in other regions of the gene.

Polyp counts showed a higher, significant, correlation for close relatives within a family than more distant relatives. The correlation coefficient, corrected for germline APC mutation, in sibling pairs was 0.42, for parent-sibling pairs was 0.29, and for more distant relatives was even weaker. Segregation analysis showed that a mixed model of inheritance not linked to the APC gene best fitted the data.

One hundred and sixty six patients from 55 families were studied. They had classic FAP and had had a prophylactic colectomy; 128 had known APC gene mutation. Counts of polyps in the colectomy samples were grouped by APC mutation indicating the genotype-phenotype relation, and correlation coefficients were calculated to assess variation within families independently of APC mutation, on the premise that closer relatives have more shared modifier genes.

FAP shows characteristic phenotypic variation, only some of which is due to APC gene mutations. The rest might be explained by a modifier gene or genes, as occurs in a mouse model.

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