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

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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 the 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 0.22, 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 CT 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 (Ig) class switch recombination.

An effective immune response is dependent upon lymphocyte B cell and T cell antigen receptor diversity, generated by intrinsic gene rearrangement mechanisms including V(D)J recombination, somatic hypermutation, and Ig class switch recombination. Rare errors in these physiological processes can directly modify genomic DNA resulting in oncogene deregulation and subsequent malignant transformation.¹ A central feature of both physiological antigen receptor rearrangement and aberrant rearrangement leading to chromosomal translocation is the generation and repair of DNA DSBs. The repair of DNA DSBs is accomplished predominantly by NHEJ, with contribution from homologous recombination (HR).^{2,3} Studies in rodents have suggested that the NHEJ repair mechanism is an integral step in both V(D)J recombination and Ig class switching.^{4–6} Grawunder *et al*⁷ reported that targeted disruption of the *LIG4* gene, which functions in NHEJ, resulted in the abolition of V(D)J recombination in human precursor B cell lymphocytes.⁷ The mechanism of somatic hypermutation is likely to differ significantly from V(D)J recombination and switch recombination, with studies showing that NHEJ is not required.⁸

Components of the NHEJ pathway include the DNA-PK complex, consisting of a Ku70/Ku86 heterodimer and a large catalytic subunit called DNA-PK α . The Ku heterodimer has a high affinity for non-specific double stranded DNA ends. Following binding, Ku recruits and interacts with DNA-PK α , stimulating its kinase activity.⁹ The final step of NHEJ repair involves the ligation of the DNA ends by the XRCC4/DNA ligase IV complex.¹⁰ Mice deficient in any of the NHEJ genes show immunodeficiency and radiosensitivity. Recent studies have shown mouse embryonic fibroblasts lacking *Ku70*, *Ku86*, *DNA-PK α* , *Xrcc4*, or *Lig4* activity exhibit significant genomic instability.^{11–13} Furthermore, mice deficient in *DNA-PK α* , *Ku86*, or *Xrcc4*, plus the tumour suppressor gene *TP53*, were found to develop B cell lymphoma within months of birth, suggesting

that a p53 dependent pathway may prevent proliferation of genetically altered cells arising through defective NHEJ. The tumours resembled human lymphoid tumours characterised by chromosomal translocations into the IgH region, deregulating *c-myc*.^{14,15} An increased risk of lymphoma, typically involving translocations involving the antigen receptor loci,¹⁶ is also observed in ataxia-telangiectasia and Nijmegen breakage syndrome, two syndromes associated with aberrant responses to DNA DSBs. Additionally, a mutation in *LIG4* was detected in a leukaemia patient (patient 180BR) who dramatically over-responded to radiotherapy.¹⁷ Taken together, these studies suggest that aberrant processing of DNA DSBs and aberrant functioning of NHEJ in particular may be an important factor in the aetiology of lymphoid malignancy.

Recently, O'Driscoll *et al*¹⁸ identified further patients with mutations in *LIG4*. These patients, classified as LIGIV syndrome patients, displayed immunodeficiency and growth and developmental delay but, to date, tumours have not been found in the small number of patients reported. Cell lines derived from these patients exhibit radiosensitivity and defective DNA DSB repair, plus an impaired ability to carry out V(D)J recombination. The original leukaemia patient (patient 180BR) was developmentally normal with no overt immunodeficiency, but had the same homozygous germline mutation (R278H) as that found in one of the more severely affected LIGIV syndrome patients (patient 411BR). Patient 411BR was found, in addition to the R278H mutation, to be a double homozygote variant for two N-terminal polymorphisms, both C>T transitions, at nucleotide positions 8 and 26, resulting in the amino acid substitutions A3V and T9I, respectively. Although neither polymorphism is thought to alter significantly the structural conformation of the DNA ligase IV protein, collectively they are predicted to increase the hydrophobicity of that region.¹⁸ The NCBI accession number for *LIG4* is NM_002312, although the correct protein

Figure 1 Examples of DNA sequence data used to validate *LIG4* Taqman™ Allelic Discrimination assays. Electropherograms span both *LIG4* variant sites, showing alleles at mRNA nucleotide positions 281 and 299 correlating with polymorphisms A3V and T9I, respectively. Sequence A shows combined *LIG4* A3V CC (wild type) and T9I CT (heterozygote) genotype. Sequence B shows combined *LIG4* A3V CT (heterozygote) and T9I CT (heterozygote) genotype.

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,¹⁹ 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 categories²⁰: 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.²¹

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.²²

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'-TTGCTTACTAGTTAAACGAGAAGATTCA-3') and antisense (degenerative) (5'-AACGTGAGATGCAACA(GA)TTGTG-3') primers, 200 nmol/l of FAM labelled C allele probe (5'-ACCGCTTTGATGGCTGCCTCACA-3'), and 100 nmol/l of VIC labelled T allele probe (5'-ACCGCTTTGATGGCTGCTCACA-3'), with an annealing temperature of 63°C. The T9I assay was undertaken using 900 nmol/l of both sense (5'-GAGAAGATTATCACCCTTTGA-3') and antisense (5'-TTCGTTCTAAAGTTGAACACAAATCTG-3') primers, and 100 nmol/l of both FAM labelled C allele probe (5'-ACAAACTTCACAACTGTGCATCTCACGTT-3') and VIC labelled T allele probe (5'-ACAAACTTCACAAATGTTGCATCTCACGTT-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

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)

Ligase IV	Controls (n=220)	ALL (n=70)			Lymphoma (n=364)			Multiple myeloma (n=270)		
	No (%)	No (%)	OR*	95% CI	No (%)	OR*	95% CI	No. (%)	OR*	95% CI
A3V†										
CC	189 (85.9)	61 (87.1)	1	–	316 (86.8)	1	–	247 (91.5)	1	–
CT	31 (14.1)	9 (12.9)	0.90	0.41 to 2.00	48 (13.2)	0.93	0.57 to 1.51	20 (7.4)	0.49	0.27 to 0.89
TT	0 (0.0)	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.4)	–	–
SNA‡	–	–	–	–	–	–	–	2	–	–
T9I†										
CC	149 (67.7)	49 (70.0)	1	–	247 (67.9)	1	–	204 (75.6)	1	–
CT	58 (26.4)	21 (30.0)	1.10	0.61 to 2.00	108 (29.7)	1.12	0.77 to 1.64	61 (22.6)	0.77	0.51 to 1.17
TT	13 (5.9)	0 (0.0)	–	–	7 (1.9)	0.32	0.13 to 0.83	4 (1.5)	0.22	0.07 to 0.70
SNA‡	–	–	–	–	2	–	–	1	–	–

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

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 χ^2 analysis for *LIG4* genotype using the HWE software program (Linkage Utility Programs, Rockefeller University, USA). Haplotype frequency variation was assessed by χ^2 analysis using the Astatue for Excel package, plus the Yates correction for small numbers.

Linkage analysis

Investigation into possible linkage disequilibrium between *LIG4* 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 *LIG4* A3V-T9I haplotype frequency, followed by subsequent association analysis using 2LD, to obtain a linkage disequilibrium D' coefficient for the *LIG4* variant alleles. Five control subjects identified with the A3V T9I double heterozygote genotype were cloned using the pGEM®-T Easy Vector System (Promega), according to the manufacturer's instructions, and subsequently analysed by direct sequencing (BigDye Terminator Kit, Applied Biosystems).

RESULTS

The *LIG4* A3V and T9I allele frequencies in the control population were 0.07 and 0.19, respectively. The distribution of the A3V heterozygote genotype was similar in the control, ALL, and lymphoma case groups (table 1). A3V TT homozygous variants were not observed in either control or case series. The T9I heterozygote genotype distribution was also similar between case and control populations. However, the T9I TT genotype was found to be significantly under-represented within the ALL and lymphoma cases (table 1), suggesting that this genotype may protect against developing these disorders. This association was also seen following stratification of the lymphomas into FCL, tumours characterised by the t(14;18) abnormality²³ (OR 0.36, 95% CI 0.12 to 1.14), and DLBCL (OR

0.28, 95% CI 0.08 to 1.02) (data not shown). Strikingly, the distribution of both A3V and T9I genotypes differed significantly for the multiple myeloma case population compared to controls (table 1), whereby significantly fewer multiple myeloma cases were heterozygous for the A3V allele, and a gene dosage effect was observed for the T9I allele. Thus, inheritance of a single copy of either A3V or 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 1). Examination of combined A3V and T9I genotype showed no interaction between them, either within the ALL or lymphoma case series (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 *LIG4* 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 ($\chi^2=4.73$, $p=0.30$), 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 strong linkage disequilibrium between the *LIG4* 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 *LIG4* 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.

The combined *LIG4* 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 C>T substitution, on the same DNA strand producing the A3V-T9I TT haplotype. The rare A3V-T9I TC haplotype observed either resulted from limited recombination owing to the spatial proximity of the two alleles, or from an A3V C>T

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)

Ligase IV polymorphism		Controls (n=220)	ALL (n=70)			Lymphoma (n=364)			Multiple myeloma (n=270)		
A3V†	T9I†	No (%)	No (%)	OR*	95% CI	No (%)	OR*	95% CI	No (%)	OR*	95% CI
CC	CC	148 (67.3)	49 (70.0)	1	–	244 (67.0)	1	–	202 (74.8)	1	–
CC	CT	34 (15.5)	12 (17.1)	1.07	0.51 to 2.22	67 (18.4)	1.20	0.75 to 1.89	42 (15.6)	0.91	0.55 to 1.49
CC	TT	7 (3.2)	0 (0.0)	–	–	4 (1.1)	0.35	0.10 to 1.20	2 (0.7)	0.21	0.04 to 1.02
CT	CC	1 (0.5)	0 (0.0)	–	–	3 (0.8)	1.82	0.19 to 17.66	1 (0.4)	0.73	0.05 to 11.81
CT	CT	24 (10.9)	9 (12.9)	1.13	0.49 to 2.60	41 (11.3)	1.04	0.60 to 1.78	18 (6.7)	0.55	0.29 to 1.05
CT	TT	6 (2.7)	0 (0.0)	–	–	3 (0.8)	0.30	0.07 to 1.23	1 (0.4)	0.12	0.01 to 1.03
TT	CC	0 (0.0)	0 (0.0)	–	–	0 (0.0)	–	–	0 (0.0)	–	–
TT	CT	0 (0.0)	0 (0.0)	–	–	0 (0.0)	–	–	0 (0.0)	–	–
TT	TT	0 (0.0)	0 (0.0)	–	–	0 (0.0)	–	–	1 (0.4)	–	–
SNA‡		–	–			2			3		

*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

Ligase IV		Haplotype frequency*			
A3V locus	T9I locus	Controls	ALL	Lymphoma	Myeloma
C	C	0.81	0.85	0.83	0.87
C	T	0.12	0.09	0.11	0.09
T	C	0.00	0.00	0.00	0.00
T	T	0.07	0.06	0.06	0.04
$\chi^2=$, p=†			1.86, 0.60	1.20, 0.75	7.83, 0.05

*Haplotype frequency estimated by EH. †Haplotype frequency variation calculated by χ^2 analysis.

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 the observed linkage disequilibrium between the A3V and T9I alleles.

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.²⁴ 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.^{25–27} In particular, the FCL group is characterised by t(14;18), where *BCL2* is translocated from 18q32 into the J_H region of the Ig heavy chain (*IGH*) gene, with

subsequent deregulation of the *BCL2* gene.²³ In contrast, multiple myeloma is a malignant disorder of plasma cells characterised, in 60% of cases, by aberrant class switch recombination.²⁸ NHEJ and DNA ligation 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.²⁹ Therefore, although Ig class switching and somatic hypermutation share a number of features suggesting common machinery,³⁰ 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.^{4, 6, 8}

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

for other diseases in which defective DNA DSB repair is implicated as causative. It could be postulated that this observation of the *LIG4* A3V and T9I polymorphisms results from the ubiquitous nature of DNA ligase IV and the NHEJ pathway, and its involvement in both V(D)J recombination and Ig class switch recombination. *LIG4* syndrome patients show immunodeficiency through a reduced ability to undergo V(D)J 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 *LIG4* A3V and T9I in the absence of mutation such as R278H confer a reduced risk of chromosomal instability and subsequent malignancy as observed for multiple 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-catalytic regions of human DNA ligases contain unique regions that determine the protein-protein interactions, and therefore the differential cellular functions of these enzymes.³¹ Ligase IV forms a complex with XRCC4 via the non-catalytic 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 ligation.^{32,33} Recent structural analysis of XRCC4 suggests the protein forms a dumbbell-like tetramer, allowing multivalent ligase IV and DNA binding.³⁴ 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.¹⁹ 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 *LIG4* 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 ligation processes in which the enzyme is involved.

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ECHO

Unlinked genes influence severity of FAP within families

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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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