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ORIGINAL ARTICLE

The severity of *FIP1L1–PDGFRA*-positive chronic eosinophilic leukaemia is associated with polymorphic variation at the *IL5RA* locus

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We have investigated the hypothesis that constitutional genetic variation in IL-5 signalling may be associated with the development or severity of *FIP1L1–PDGFRA*-positive chronic eosinophilic leukaemia (CEL) in humans. We genotyped six single-nucleotide polymorphisms (SNP) within or close to the *IL5RA* or *IL5* genes in 82 patients with *FIP1L1–PDGFRA*-positive CEL plus, as controls, healthy individuals ($n=100$), patients with *FIP1L1–PDGFRA*-negative eosinophilia ($n=100$) or patients with chronic myeloid leukaemia (CML) ($n=100$). We found no association between SNP allele frequency between *FIP1L1–PDGFRA*-positive and control cases. However, for *FIP1L1–PDGFRA* cases, we found an association between the genotype at rs4054760, an SNP in the 5′-UTR of *IL5RA* and peripheral blood eosinophil count ($P=0.026$) as well as the presence or absence of tissue infiltration ($P=0.032$). Although these associations fell below the level of significance once corrected for multiple testing, no such association was seen in *FIP1L1–PDGFRA*-negative cases and no difference in allele frequencies for rs4054760 was seen in control populations across Europe. Furthermore, in an analysis of 112 patients with CML, *IL5RA* expression was strongly related to rs4054760 genotype ($P<0.001$). These data suggest that the variations in *IL5RA* expression are linked to constitutional *IL5RA* genotype and severity of *FIP1L1–PDGFRA* disease.
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Introduction

The World Health Organization (WHO) defines hypereosinophilic syndrome (HES) as unexplained eosinophilia of $\geqslant 1.5\times10^9$ per litre for at least 6 months associated with signs of organ involvement and dysfunction.[1] While the pathogenesis of HES is often idiopathic, excess eosinophil proliferation in many cases is believed to be a primary consequence of an underlying myeloproliferative disorder or a secondary consequence of a covert T-cell lymphoproliferative disorder. In the latter scenario, aberrant T cells overproduce IL-5, the principal cytokine, that drives eosinophil production via a heterodimeric receptor comprised of a specific α subunit encoded by the gene *IL5RA*, and the common β subunit shared with the receptors for IL-3 and granulocyte-macrophage colony-stimulating factor.

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Understanding of the myeloproliferative subtype of HES was greatly advanced by the finding of the *FIP1L1–PDGFRA* fusion, formed by a cytogenetically cryptic 800 kb interstitial deletion at chromosome band 4q12.[2] Although initially described in more than 50% of cases with idiopathic HES, subsequent studies estimated the prevalence to be lower at 3–17%.[3–5] Detection of *FIP1L1–PDGFRA* by reverse transcription-PCR or fluorescent *in situ* hybridization analysis is important for patient management since positive cases are exquisitely sensitive to imatinib, with most achieving rapid haematologic and molecular remission.[5] According to the WHO classification, HES patients who are positive for *FIP1L1–PDGFRA* should be reclassified as having chronic eosinophilic leukaemia (CEL).

FIP1L1–PDGFRα is a constitutively active tyrosine kinase that can transform Ba/F3 cells to be independent of growth factor and induce myeloproliferative disease in mice.[2,6] Using a standard Balb/c mouse strain, *FIP1L1–PDGFRA* induced a chronic myeloid leukaemia (CML)-like disease with relatively modest eosinophilia but without evidence of one of the hallmarks of HES/CEL, eosinophilic tissue infiltration.[6] However, when expressed in mice that had been engineered to overexpress IL-5, the absolute and relative eosinophil counts increased and tissue infiltration was observed.[7] These observations indicated that *FIP1L1–PDGFRA* alone is insufficient to induce true HES/CEL in mice, and that a secondary event associated with IL-5 overexpression is required for the full phenotype. Whether constitutional or acquired changes in IL-5 signalling are relevant to the human disease is unknown.

Polymorphic variation of the *IL5* and/or *IL5RA* loci has been associated with a range of human diseases including asthma, coeliac disease, atopic dermatitis and susceptibility to helminthic or viral infections.[8–12] Such associations are postulated to be a consequence of natural variation in the expression levels of IL-5 or IL5RA and hence the strength of signalling through this pathway. In this study, we have investigated the hypothesis that genetic variation in IL-5 signalling may be associated with the development or severity of *FIP1L1–PDGFRA*-positive CEL in humans.

Materials and methods

Patient samples

We studied DNA extracted from peripheral blood or bone marrow leucocytes from 82 patients with HES or persistent unexplained eosinophilia who tested positive for *FIP1L1–PDGFRA* by reverse transcription-PCR or fluorescent *in situ* hybridization using established procedures.[2] Patients were

identified in the United Kingdom ($n=26$), Germany ($n=30$), France ($n=23$) and Italy ($n=3$). To ensure sufficient material for analysis, some samples were amplified first by multiple-displacement whole genome amplification using the Illustra GenomiPhi DNA Amplification kit according to the manufacturer's instructions (GE Healthcare UK Limited, Little Chalfont, UK). To ensure adequate quality, amplified samples were considered acceptable only if they produced clear bands following a multiplex PCR designed to assess DNA quality.[13] As controls, we used DNA from (i) 100 age-matched healthy individuals (first-degree relatives of individuals ascertained for a variety of genetic conditions) from the United Kingdom, (ii) 100 individuals with unexplained eosinophilia who tested negative for *FIP1L1–PDGFRA* from the United Kingdom and (iii) 100 patients with CML from Germany. An additional 62 Greek *FIP1L1–PDGFRA*-negative HES cases were also studied for rs4054760, plus 107 healthy controls from Greece and 95 healthy controls from France. The study was approved by the South Wiltshire Regional Ethics Committee and informed consent was provided in accordance with the Declaration of Helsinki Principles.

Genetic analysis

Single-nucleotide polymorphisms (SNPs) within or close to *IL5RA* or *IL5* were selected on the basis of published data, indicating positive associations with one or more human diseases or as tagged SNPs with minor allele frequencies (maf) >0.2 from the International HapMap Project (release 21; www.hapmap.org). Five markers were analysed for *IL5RA*: rs334792, rs4054760, rs9814648, rs17882248 (also known as IL5RA385) and rs2290608 (IL5RA5091). For *IL5*, only a single informative SNP has been identified; rs2069812 (also known as IL5 746 or var929). For each SNP, we designed a pyrosequencing assay as described[14] using the primers and dispensation order detailed in Table 1. Markers were quantified using the Allele Frequency Quantification function in the SNP Software (Biotage AB, Uppsala, Sweden) and called as homozygous when one allele gave reading of $>90\%$ and heterozygous when both alleles read 40–60%.

Quantitative reverse transcription-PCR analysis

RNA extraction was performed using the Qiagen RNeasy system (Qiagen, West Sussex, UK) and reverse transcription was performed as described.[15] We measured *IL5RA* and, as a control for cDNA quality and quantity, *GUSB* levels by real-time quantitative reverse transcription-PCR using a Corbett Rotor Gene 6000 (Corbett Life Science, Sydney, Australia). Probes and primers used for *IL5RA* were sense primer 5′-CCCAGAGA TAAGGTTCGTTCT-3′, antisense primer 5′-CAACCCCAGCATC TAGCATAG-3′ and probe 5′-FAM-CGATGAGTCTCAATGCC CTGCCGCTGCTTCTCATCG-DABCYL-3′. Probes and primers for *GUSB* were as described by the Europe against Cancer group.[16] *IL5RA* levels were normalized to *GUSB* using the ΔC_t method.[17]

Statistical analysis

Genotype frequencies were compared between patient groups by χ^2 analysis using Minitab (State College, PA, USA). Clinical information was only available for approximately half of the *FIP1L1–PDGFRA*-positive cases. The Hardy–Weinberg equilibrium was also assessed by χ^2 analysis. Expression levels ($2^{-\Delta C_t}$)[17] were compared to genotype by Kruskal–Wallis analysis.

Results

To investigate the possibility that constitutional genetic variation at the *IL5* or *IL5RA* loci may be associated with the development

Table 1 Primers and dispensation orders for SNPs analysis by pyrosequencing

SNP	*Dispensation order*	*Primer*
rs2069812	Gt**C**TAGATC	→ F1: TTAAACAATCAGAAAAGCACAGC ←● R1: CTTTCCCATTGAGGTCTCAAGAT ← S1: GCTCATGAACAGAATACATA
rs2290608	C**G**aGTTACAT	→ F1: ATTTGCTTAGAGAAAACGTGTTGC ←● R1: TTTAACTTAGAGCGGTTCTTCA → S1: CGAAGCTGCCTGAGA
rs17882248	GCA**C**tCGAG	●→ F1: ACTTTTGACTACCTAGGGTCTCCT ← R1: GCAGTGAAGGGAAACTTGGTAT ← S1: GGTGCGAGTTAAATTCA
rs334792	GAc**T**CAGAG	→ F1: TCACAGTTTACCACTGAGGGTTCT ←● R1: TTTGGTCAGAAGATCAGACTAACA → S1: AGCTGACTAATACACCTACC
rs4054760	Gt**C**GTGCGC	→ F2: CGCCTCTAAGTTAAAAAGTGCACC ←● R8: GTGAAGCATTTGATTCCCACTTAC → S7: GGTTCGTTCTCAATGC
rs9814648	A**C**tAGCTGC	●→ F1: ACAAGCACGCATCAAGCATCT ← R1: TTTGGGCTTCGTCCATTTGACT ← S1: CTTCGTCCATTTGACTC

Abbreviations: F, forward primer; R, reverse primer; S, sequencing primer; SNP, single-nucleotide polymorphisms; ●, 5′-biotin.
SNP rs2060812 corresponds to the *IL-5* gene; all others correspond to *IL5RA*. In the dispensation order, the bases in bold and lower cases correspond to the major and minor alleles, respectively.

Table 2 Genotypes for *FIP1L1–PDGFRA*-positive cases and initial control groups

	FIP1L1–PDGFRA			*Normals*			*Other eosinophilia*			*CML*		
	a/a	*a/b*	*b/b*	*a/a*	*a/b*	*b/b*	*a/a*	*a/b*	*b/b*	*a/a*	*a/b*	*b/b*
rs2069812	36	31	15	49	43	8	53	34	13	48	41	11
rs2290608	37	39	6	56	34	10	47	44	9	45	47	7
rs17882248	37	37	7	43	39	18	37	46	17	42	43	12
rs4054760	49	31	2	62	28	10	60	34	6	50	44	6
rs9814648	43	33	6	52	39	9	50	43	7	52	45	3

Abbreviation: CML, chronic myeloid leukaemia.

Table 3 Rare allele frequencies (*f*) and *P*-values (*P*) for *FIP1L1–PDGFRA*-positive patients compared to the presence or absence of splenomegaly, peripheral blood eosinophil count and whether tissue infiltration was clinically apparent

Marker	*Splenomegaly*			*Eosinophils*			*Tissue infiltration*		
	f_{yes}	f_{no}	P	$f_{<median}$	$f_{>median}$	P	f_{yes}	f_{no}	P
rs2069812	0.42	0.36	0.65	0.35	0.40	0.67	0.38	0.43	0.67
rs2290608	0.35	0.23	0.27	0.27	0.31	0.65	0.25	0.40	0.16
rs17882248	0.37	0.27	0.41	0.33	0.31	0.83	0.38	0.30	0.44
rs334792	0.47	0.50	0.80	0.50	0.48	0.84	0.44	0.40	0.17
rs4054760	0.23	0.18	0.47	***0.13***	***0.31***	***0.026***	***0.31***	***0.10***	***0.032***
rs9814648	0.34	0.23	0.33	0.27	0.33	0.51	0.38	0.23	0.16

Significant differences are indicated in bold italics.

of *FIP1L1–PDGFRA*-positive CEL, we genotyped six SNPs in patient and control populations. None of the markers deviated significantly from the Hardy–Weinberg equilibrium; however, significant distortions in allele ratios in patient and control populations were seen for rs334792, which was therefore not considered further. The genotyping results for the other five markers are summarized in Table 2. There was no significant difference between the allele frequencies seen for patients with *FIP1L1–PDGFRA* and the three control groups (healthy individuals from the United Kingdom; patients with *FIP1L1–PDGFRA*-negative persistent eosinophilia from the United Kingdom and patients with CML from Germany) either considered separately or as a single group. Furthermore, there was no difference between any other pairwise combination, including the control groups, or other comparisons such as patients with eosinophilia (both *FIP1L1–PDGFRA*-positive and -negative cases) compared to healthy individuals or CML controls.

To determine if variation at the *IL5* or *IL5RA* loci might be associated with the severity of *FIP1L1–PDGFRA*-positive CEL, we compared SNP allele frequencies with peripheral blood eosinophil count prior to treatment ($n=48$; greater or less than the median value of 5.8×10^9 per litre), the presence or absence of splenomegaly ($n=42$) and the presence or absence of eosinophilic infiltration into the heart, skin, lung, liver, lymph node, nervous system or any other tissue ($n=41$). No associations were found with splenomegaly, but we found that the minor allele at rs4054760 was more common in *FIP1L1–PDGFRA*-positive cases with high eosinophil counts ($P=0.026$) and evidence of clinically significant tissue infiltration ($P=0.032$; Table 3). Although these associations fell below statistical significance once corrected for multiple testing, we considered them sufficiently suggestive to warrant further investigation.

Single-nucleotide polymorphism allele frequencies often vary between different ethnic groups and we considered the possibility that the association between rs4054760 and disease severity might reflect simply the fact that our *FIP1L1–PDGFRA* patients were drawn from several different countries. To address this possibility, we genotyped rs4054760 in two additional control populations. No significant differences between United Kingdom (maf = 0.24; $n=100$), German (maf = 0.28; $n=100$), French (maf = 0.27; $n=95$) and Greek (maf = 0.22; $n=107$) controls were observed.

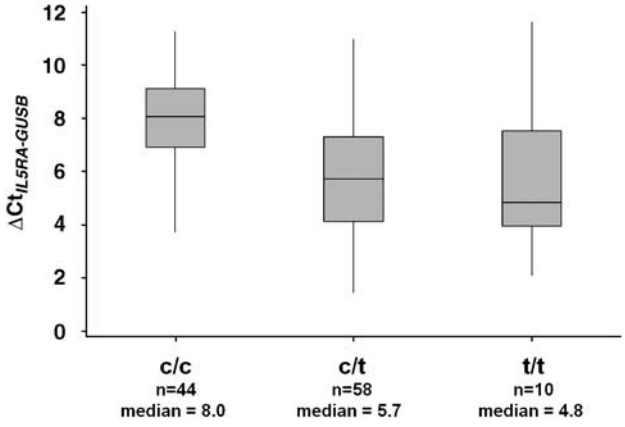

Figure 1 *IL5RA* expression normalized to *GUSB* is significantly related to rs4054760 genotype in 112 patients with chronic myeloid leukaemia ($P=<0.001$; Kruskall–Wallis test).

To determine if this SNP is generally associated with the severity of eosinophilic disease, we genotyped additional *FIP1L1–PDGFRA*-negative HES cases for whom baseline clinical data were available. No association was seen with eosinophil count ($n=97$; greater or less than the median value of 2.3×10^9 per litre; $f_{<\text{median}}=0.19$ versus $f_{>\text{median}}=0.26$; $P=0.27$), the presence or absence of splenomegaly ($n=66$; $f_{\text{no}}=0.20$ versus $f_{\text{yes}}=0.26$; $P=0.37$) or the presence/absence of tissue infiltration ($n=79$; $f_{\text{no}}=0.24$ versus $f_{\text{yes}}=0.17$; $P=0.25$).

Next, we sought to determine if rs4054760 genotype is associated with the expression level of *IL5RA*. Since *FIP1L1–PDGFRA* is a deregulated tyrosine kinase, we considered that the most appropriate control group would be patients with CML. As shown in Figure 1, we found that normalized *IL5RA*

expression levels of *IL5RA* were significantly different ($P<0.001$) when compared to rs4054760 genotype.

Discussion

Our study was motivated by the finding that a secondary event associated with IL-5 overexpression is required for the full HES phenotype in mice engineered to express *FIP1L1–PDGFRA*.[7] If elevated IL-5 signalling is also important for human *FIP1L1–PDGFRA*-positive CEL, it is possible that constitutional differences in the expression or function of the IL-5 system may be associated with the development or severity of disease. Alternatively, abnormalities of this system may be acquired before or after the acquisition of the fusion gene. We aimed to test the former hypothesis by analysing the constitutional genotype at *IL5* and *IL5RA* in *FIP1L1–PDGFRA*-positive patients and controls. Although we did not find any differences between *FIP1L1–PDGFRA*-positive and control cases, we did find an association between the genotype at rs4054760 and (i) peripheral blood eosinophil count and (ii) the presence or absence of tissue infiltration in *FIP1L1–PDGFRA*-positive disease. Furthermore, we found that rs4054760 was significantly related to the expression levels of *IL5RA*. It is possible that rs4054760 may itself be causal, or alternatively it may be in linkage disequilibrium with a true (unknown) functional variant that is responsible for the observed differences in expression levels.

At least three problems arise in a study that tries to link inherited polymorphisms with a rare clonal haematological disorder. First, the number of subjects available for analysis is small for genetic association analysis, despite the fact that our study group was relatively large for a rare disease and indeed constitutes the largest group of *FIP1L1–PDGFRA*-positive cases studied to date. Second, to accumulate reasonable numbers of cases, we had to recruit patients from different European countries and, because this may introduce additional genetic variation, it was important to include relevant control groups. Finally, we analysed peripheral blood leucocyte DNA and there is a possibility that allele ratios may be distorted in clonal populations due to karyotypic evolution. Although conventional chromosome analysis does not usually highlight any abnormalities in *FIP1L1–PDGFRA*-positive cases, we cannot exclude the possibility that submicroscopic copy number abnormalities at 3p26 (*IL5RA*) and 5q31 (*IL5*) may be present, or the possibility of other changes that could distort allele frequencies, for example, acquired uniparental disomy. To mitigate against this possibility, we quantified the proportion of each allele by Pyrosequencing and called SNPs as homozygous only when one allele gave reading of $>90\%$ and heterozygous when both alleles read 40–60%. In positive cases, fluorescent *in situ* hybridization typically detects the *CHIC2* deletion (a surrogate for *FIP1L1–PDGFRA*) in only a minority of total peripheral blood or bone marrow leucocytes[3] and therefore any distortions within the clone would be expected to yield allele ratios outside the ranges above. In fact, the SNPs we studied consistently gave readings within these ranges except for rs334792. Since these distortions were seen in both patient and control samples, this marker may not be a simple biallelic polymorphism.

Inspection of Hapmap data (release 21a, accessed March 2007) indicates low linkage disequilibrium at *IL5RA*, with 22 tagged SNPs being required to capture allelic variation with an $r^2>0.8$ in the Caucasians, meaning that we have only sampled part of the natural genetic variation at this locus. Nevertheless, our study suggests that *IL5RA* genotype may be associated with the severity of *FIP1L1–PDGFRA* disease. Clearly, this finding will need to be confirmed and explored further in a larger study group, the assembly of which will require a concerted international effort.

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