



BRIEF COMMUNICATION

Allele associations reveal four prominent haplotypes at the human interleukin-6 (IL-6) locus

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We have examined four polymorphic elements in the human interleukin-6 (IL-6) locus and described their allele distribution in 73 unrelated, healthy individuals from the West-of-Scotland. These comprised three single nucleotide polymorphisms (SNP) in the 5' promoter region of the gene and one VNTR in the 3' region of IL-6. A statistical consideration of the relationship between alleles at each locus was carried out. Of a total of 12 possible haplotypes observed in the population, the analysis suggested that four were prominent. These accounted for 41.1%, 28.1%, 14.4% and 3.4% respectively; in total, 87% of the haplotypes present. Frequently, these proposed haplotypes were supported by homozygosity across all four loci within individuals. We propose that these haplotypes be identified as IL6.0103, IL6.0204, IL6.0207 and IL6.0307, in recognition of their frequency in this population and the alleles that they contain. *Genes and Immunity* (2000) 1, 451–455.

Keywords: cytokines; immunogenetics; IL-6; haplotypes; autoimmunity

Cytokines are a group of multi-functional proteins used predominantly, but not exclusively, as signalling molecules between cells of the immune system. Recently, much interest has begun to focus on the contribution of cytokines to the genetic variation known to occur between the immune responses of different individuals.¹ While the majority of work in cytokine genetics has been carried out on the TNF locus,^{2–7} other cytokines have also featured, notably IL-1^{8–10} and IL-10.^{10–13}

A major point of interest as to whether these cytokine markers can be related to human disease status^{1,2,4–13} and studies in malignant² and autoimmune^{4–7} disease have suggested that the TNF locus can contribute to disease susceptibility. Indeed, similar studies have demonstrated that IL-1 and IL-10 are also associated with the presence or severity of autoimmune diseases across a variety of ethnic backgrounds.^{9–13} This work has been complemented by studies which demonstrate a relationship between certain markers and some aspect of gene function, for example, cytokine secretion.^{7,11}

Interleukin-6 (IL-6) was originally defined as a B cell growth factor,¹⁴ but was rapidly identified as underpinning the induction of the acute-phase response¹⁵ and being heavily involved in inflammatory responses generally.¹⁶ More recently, the role of IL-6 in human malignancy has become a topic of interest. For example, it was recently shown that levels of serum IL-6 correlate mark-

edly with disease status in gastric cancer¹⁷ and that IL-6 is an autocrine growth factor for some gastric cancer cell-lines.¹⁸

We examined the distribution of four polymorphic elements in the human IL-6 gene; three point mutations in the 5' promoter region and one VNTR in the 3' region of the gene. The location of these markers is shown in Figure 1. Two of the point mutations have been previously described in Caucasians: the G/C element defined by the enzyme Nla-III at position –172 by Olomolaiye *et al*¹⁹ and the G/A element defined by the enzyme Fok-I by Faulds *et al*²⁰ at position –594. The third is a G/C at position –570 which has recently been independently characterised by Nakajima *et al*²¹ in Japanese subjects and Osiri *et al*²² in an African-American population. The nucleotide position numbers quoted in the present study correspond to the numbering from the sequence in Accession no. Y00081.¹⁴ The 3' minisatellite was originally described by Bowcock *et al*.²³ Since polymorphic alleles at loci so close together are likely to be transmitted together, it was therefore of interest to define these relationships for the human IL-6 gene. In order to do this, we examined the distribution of polymorphic alleles in our West-of-Scotland control population and determined the relationship of these alleles to one another.

The distribution of alleles at individual polymorphic sites is shown in Table 1. The distribution observed in our West-of-Scotland population at the –172 (Nla-III) polymorphism is similar to that recently described for Caucasians by Fishman *et al*²⁴ (–172C, 44.5% vs 40.3% and –172G, 55.5% vs 59.7%, this study and Fishman *et al*,²⁴ respectively). A similar distribution was observed at the –592 (Fok-I) polymorphism, previously described by Faulds *et al*²⁰ (–594A, 43.2% and –594G, 56.8%). The SNP at position –570, defined by the enzyme Fnu4HI has not

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Joyce Eskdale was supported in part by the Breast Cancer Campaign.

Received 20 April 2000; revised and accepted 6 June 2000

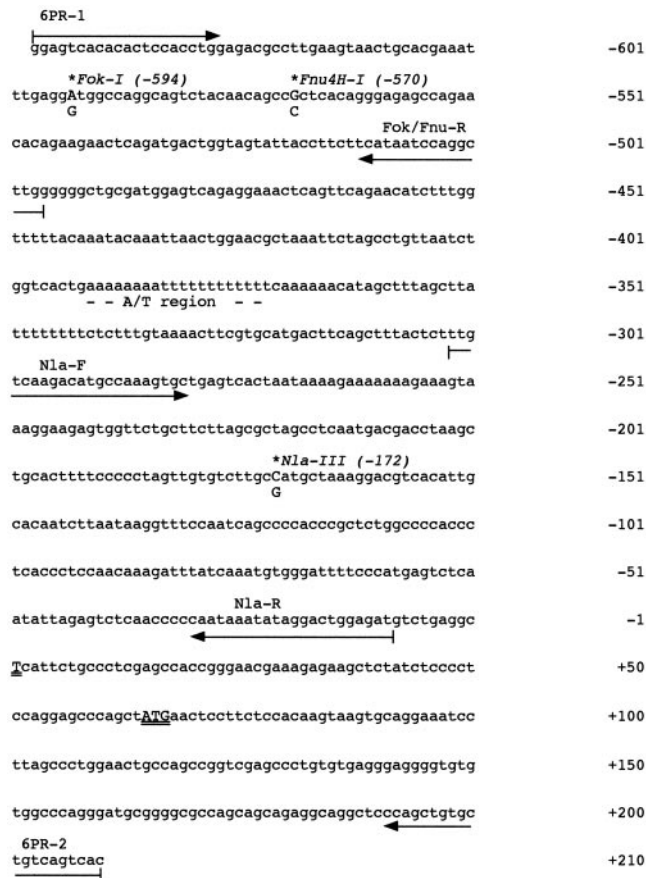


Figure 1 Representation of the human IL-6 promoter. The human IL-6 promoter, as enumerated in the original description of the IL-6 gene (ref 14 and Genbank/EMBL Accession number Y00081) is shown. The three polymorphic SNP loci are indicated in capitals, with the alternative bases shown under the main sequence. The polymorphic A(n)T(m) region is indicated as are the codon for the initiating methionine (ATG, double-underlined) and the transcription start site (T; double-underlined). Genotypes were obtained by a nested PCR technique. In the first round, primers '6PR-1' and '6PR-2' were used to amplify an 858 bp fragment of genomic DNA from test individuals. This fragment then served as a template for further amplification and genotyping-amplification with the primers 'Nla-F' and 'Nla-R' allowed genotyping of the -172G/C locus following digestion of the product with the enzyme Nla-III (New England Biolabs); amplification with '6PR-1' and 'Fok/Fnu-R' allowed genotyping of the -594A/G following digestion with enzyme Fok-I (New England Biolabs) or alternatively the -570G/C locus could be genotyped if a separate aliquot of this PCR product was digested with the enzyme Fnu4H (New England Biolabs).

previously been described in Caucasians. In this study, we observed that the -570C allele was sparsely distributed in our population at 4.1%, with the -570G allele being much the more common, at 95.9%. However, at 4.1%, it is probably only barely polymorphic enough to be of interest in its own right, although it was critical in haplotype definition (below); this marker may be more frequent in populations of different ethnic origin.²¹

The 3'VNTR of the IL-6 gene was originally described by Bowcock *et al*,²³ following amplification by PCR and separation on agarose gels. In that paper they described four alleles but questioned whether others may be present. When we examined this locus on agarose gels, we too observed four alleles, each of which occasionally

Table 1 Allele distribution at four polymorphic loci in the human interleukin-6 gene

Locus	Enzyme used	Allele	No. (%) observed
-594	Fok-I	A	63 (43.2)
		G	83 (56.8)
-570	Fnu4H-I	G	140 (95.9)
		C	6 (4.1)
-172	Nla-III	C	65 (44.5)
		G	81 (55.5)
3'VNTR	—	3	64 (43.8)
		4	45 (30.8)
		6	5 (3.4)
		7	27 (18.5)
		8	4 (2.7)
		9	1 (0.7)
		13	1 (0.7)

appeared to vary slightly in size or appear to be double bands. We therefore adopted denaturing 3.5% acrylamide sequencing gels, to give a higher resolution separation. Using this system, we have seen a total of 13, irregularly-spaced, alleles in a variety of Caucasian control and patient groups from the West-of-Scotland (Figure 2). In the control panel, seven of these were represented: allele 3 (610 bp), allele 4 (616 bp), allele 6 (639 bp), allele 7 (648 bp), allele 8 (658 bp), allele 9 (672 bp), and allele 13 (773 bp). As shown in Table 1, alleles 3 and 4 were most common (43.8% and 30.8%, respectively) with allele 7 also frequently represented (18.5%); other alleles were rare in this population. Four genotypes constituted the majority; 3*4 (27.4%), followed by 3*3 (19.2%), 3*7 (17.8%) and 4*4 (12.3%). The observation of 13 alleles using a 3.5% denaturing acrylamide, contrasts with the observations of Ralston *et al*²⁵ who observed only two dominant alleles from a total of six observed in their Caucasian control population. It should be noted that the irregular nature of this locus may well mean that additional alleles exist and these could be size and/or sequence variants; further work will be required to define this fully.

The IL-6 gene spans some 6 kb and the 5' region studies here is just under 600 bases long. It is therefore to be expected that alleles within the various polymorphic elements present would be related to one another, as has recently been described in this population for the TNF locus³ and the IL-10 locus.²⁶ As in our previous reports, associations between loci were estimated by constructing a series of 2 x 2 tables, which were then analysed by the Chi-square test (using Minitab software). The probability (P) obtained was corrected (Bonferoni) for multiple comparisons (pc) according to the number of allele combinations observed. Associations between alleles were considered as 'strong' where pc < 0.05.

The associations described between the multi-allelic IL-6 3'VNTR and the point mutations in the 5' region are summarised in Table 2. Associations were observed between the 3' IL-6 minisatellite alleles 3, 4 and 7, and the three 5' SNPs. There was a strong association between the 3' minisatellite allele 3 and both the -172C (Chi-square = 57.718, pc < 0.00013) and -594A alleles (Chi-square = 55.223, pc < 0.00013). No association was observed with either of the -570 alleles (P = 0.0527).

Similarly, IL-6 3' minisatellite allele 4 was strongly

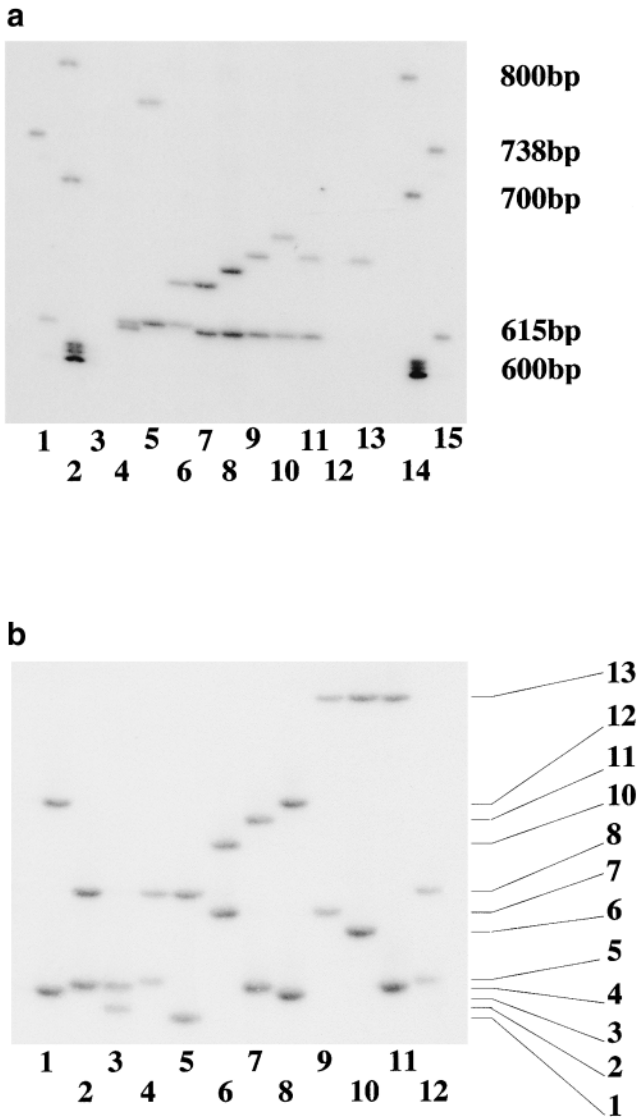


Figure 2 Demonstration of multiple alleles at the IL-6 3'VNTR locus. The imperfect VNTR in the 3' region of the human IL-6 locus was amplified by PCR using the primers and method previously described by Bowcock *et al.*²³ PCR products were internally-labelled with [32]-P dCTP and separated on 3.5% denaturing polyacrylamide sequencing gels (acrylamide: bis-acrylamide, 19:1). Size ladders were end-labelled with [32]-P dATP. Following electrophoresis, gels were dried onto filter paper and allowed to expose X-ray film. In panel (a), a range of alleles is shown in comparison to size markers from the West-of-Scotland population. Lane 1, 123 bp ladder; Lane 2, 100 bp ladder; Lane 3 water blank; Lane 4, 3/4; Lane 5, 4/13; Lane 6, 4/6; Lane 7, 3/6; Lane 8, 3/7; Lane 9, 3/8; Lane 10, 3/9; Lane 11, 3/8; Lane 12, 8/8; Lane 13, blank; Lane 14, 100 bp ladder; Lane 15, 123 bp ladder. In panel (b), the overall range of alleles, as seen in a variety of patient groups, is illustrated (note, allele 9 not represented). Lane 1, 3/12; Lane 2, 4/8; Lane 3, 2/4; Lane 4, 5/8; Lane 5, 1/8; Lane 6, 7/10; Lane 7, 5/11; Lane 8, 4/12; Lane 9, 7/13; Lane 10, 6/13; Lane 11, 4/13; Lane 12, 5/8.

associated with the complimentary -172 allele, -172G (Chi-square = 14.765, *pc* = 0.00013) and additionally with the -594G allele (Chi-Square = 12.534, *pc* = 0.0052), but not with either of the -570 alleles. Finally, there were strong associations between the IL-6 3' minisatellite allele 7 and the -172G and -594G alleles (Chi-square = 11.214, *pc* = 0.0104; Chi-square = 14.015, *pc* = 0.0026); and a weak

Table 2 Associations between common 3'VNTR and 5'SNP alleles

VNTR allele	-594	-570	-172
3	A <i>P</i> < 0.00001	G <i>P</i> = 0.05271	C <i>P</i> < 0.00001
4	G <i>P</i> = 0.0004	G <i>P</i> = 0.65552	G <i>P</i> = 0.0001
6	G <i>P</i> = 0.0313	G <i>P</i> = 0.33962	G <i>P</i> = 0.0254
7	G <i>P</i> = 0.00022	C <i>P</i> = 0.00412	G <i>P</i> = 0.0008

association with -570C which was not significant following Bonferroni correction (Chi-square = 8.242, *P* = 0.0041, *pc* = 0.0533).

These associations between the 5' and 3' locus alleles were complimented by associations within the 5' locus alleles themselves. In particular, a clear pattern emerged whereby the -594A allele was in strong linkage disequilibrium with the -172C allele and the -594G allele with the corresponding -172G allele. In general, both these combinations were found with the common -570G allele. However, it was notable that the rare -570C allele was found only with the -594G and -172G alleles, suggesting that it had arisen against this background (data not shown).

Using these associations, we present four extended associations in our population which integrate alleles within the IL-6 locus:

- 594A; -570G; -172C, IL-6 3'VNTR.3 (nb: contains the Fnu4H-I.1(G) allele by default)
- 594G; -570G; -172G; IL-6 3'VNTR.4 (nb: contains the Fnu4H-I.1(G) allele by default)
- 594G; -570G; -172G; IL-6 3'VNTR.7 (nb: contains the Fnu4H-I.1(G) allele by default)
- 594G; -570C; -172G; IL-6 3'VNTR.7

The associations within these groups are such that we propose that they will behave as haplotypes and that they be termed IL6.0103, IL6.0204, IL6.0207 and IL6.0307 respectively, where the first part of the terminology is from their respective frequencies in our population and the latter part makes use of the allele present in the 3' minisatellite to further differentiate one haplotype from another. In support of this, we observed 13 individuals who were homozygous for the proposed IL6.0103 haplotype and a further eight who were homozygous for the proposed IL6.0204 haplotype, from our test population of 73. One individual was homozygous IL6.0207. In addition, 18 individuals had genotypes consistent with their being heterozygous for the IL6.0103 and IL6.0204 haplotypes and a further nine were likely to be heterozygous for the IL6.0103 and IL6.0207 haplotypes. In Table 3, we have examined the distribution of these proposed haplotypes in our test population. It will be seen that collectively they potentially account for 87% of the genotypes observed. Subtraction of these haplotypes⁶ from the population revealed the remainder of the proposed haplotypes given in Table 3.

Considering these haplotypes from their 5' components only, it is interesting to compare them with the 5'

Table 3 Putative haplotypes observed in the West of Scotland population

Haplotype	Alleles				No. (%) observed
	-594	-570	-172	3'VNTR	
IL-6.0103	A	G	C	3	60 (41.1)
IL-6.0104	A	G	C	4	3 (2.1)
IL-6.0203	G	G	G	3	3 (2.1)
IL-6.0204	G	G	G	4	41 (28.1)
IL-6.0206	G	G	G	6	5 (3.4)
IL-6.0207	G	G	G	7	21 (14.4)
IL-6.0208	G	G	G	8	4 (2.7)
IL-6.0209	G	G	G	9	1 (0.7)
IL-6.0304	G	C	G	4	1 (0.7)
IL-6.0307	G	C	G	7	5 (3.4)
IL-6.0403	G	G	C	3	1 (0.7)
IL-6.0407	G	G	C	7	1 (0.7)

haplotypes recently reported by Osiri *et al.*²² Our most common 5' haplotype was G-G-G (51.4%), as it was in the study of Osiri *et al* although at a higher incidence (84.1%). Their next most frequent haplotype was G-C-G (9.5%), which was more poorly represented in our population (4.1%). In contrast, a minor component of their population carried the haplotype A-G-C (2.4%), which was in fact very common in this study (43.2%). Thus, although the same haplotypes were present between these two populations, their distribution was markedly different and this probably reflects the ethnic disparity of the two populations, African-American in the case of the Osiri study and the northern European Caucasians reported here.

Having defined these four common IL-6 locus haplotypes, we examined the 5' combinations in the other individuals to establish how the remainder of the variation in the IL-6 3'VNTR was distributed. In only one individual, was any variation found on the IL-6.0103 haplotype and this person was homozygous for the genotype:

-594A; -570G; -172C; IL6 3'VNTR.4 (ie IL-6.0104)

Interestingly, none of the IL-6 3' minisatellite alleles 6, 7, 8 or 9 appeared on this background of -594A; -570G; -172C, suggesting that the proposed IL6.0103 haplotype is either recent or contains (as yet undefined) elements within it which inhibit or prevent its change, or is under selection pressure to remain stable; this appears analogous with the strongly-conserved B8-DR3 MHC haplotype and its associated TNF locus alleles in Caucasians.³

A recent study by Fishman *et al.*²⁴ described the polymorphic nature of the [A]n[T]m stretch which lies between the -570G/C site and the -172G/C site in the IL-6 promoter. This study described considerable variation within both the 'A' and the 'T' runs, such that the number of 'A' and 'T' residues were varying independently of one another. Interestingly, only one of 18 individuals sequenced showed variation in this 'AT' element was found where the -172C allele was present and all other variants were found in association with the -172G allele. These data are wholly consistent with those presented in this report and emphasise the stable nature of the IL6.0103 haplotype. Genotyping this locus directly using

a recently-described method²⁷ should formally resolve this question.

Fishman *et al.*²⁴ also demonstrated that the -172C allele was significantly less frequent in a population of systemic-onset Juvenile Chronic Arthritis patients and that promoter constructs containing this allele were less transcriptionally active following LPS stimulation,²⁴ (although others have failed to observe differential IL-6 secretion in relation to this allele²⁸). This *in vitro* functional association was mirrored in the lower serum IL-6 levels observed in normal individuals who were homozygous for this allele, compared with those who carried the more common -172G allele. Our demonstration here that this marker is in strong linkage disequilibrium with two other 5' alleles and one 3' allele allows for the association with IL-6 secretion observed by Fishman *et al.*²⁴ to be contributed to by one or more of these additional alleles. Similarly, Linker-Israeli *et al.*²⁹ have demonstrated an association between the 3'VNTR region of the IL-6 gene and SLE, although in this instance, additional, SLE-related alleles may also be involved in dysregulation of IL-6 secretion.²⁹

In conclusion, we have examined the distribution of alleles at four polymorphic elements in the human IL-6 gene locus and demonstrated that these alleles come together to form four dominant haplotypes in the West-of-Scotland population. We propose that these be termed IL6.0103, IL6.0204, IL6.0207 and IL6.0307. Recently, much attention has focussed on the detection of genetic markers within cytokine genes and using them as indicators of disease susceptibility and/or progression.^{1,2,4,5-13,17,21,24-26,29} The results reported here may assist this process by demonstrating that markers cannot necessarily be considered independent of one another across human cytokine genes and that where markers fall in, or close to, functional elements such as transcription factor binding sites, it should be remembered that other polymorphic elements may be contributing to the observed effect.

Acknowledgements

We are grateful to Dr Lou Bridges and Dr Janet McNicholl for informing us of the -570A/G polymorphism pre-publication.

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