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Unusual Haplotypic Structure of *IL8,* a Susceptibility Locus for a Common Respiratory Virus

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Interleukin-8 (IL8) is believed to play a role in the pathogenesis of bronchiolitis, a common viral disease of infancy, and a recent U.K. family study identified an association between this disease and the IL8−251A allele. In the present study we report data, from a different set of families, which replicate this finding; combined analysis of 194 nuclear families through use of the transmission/disequilibrium test gives $P = .001$. To explore the underlying genetic cause, we identified nine single-nucleotide polymorphisms (SNPs) in a 7.6-kb segment spanning the *IL8* gene and its promoter region and used six of these SNPs to define the haplotypic structure of the *IL8* locus. The IL8−251A allele resides on two haplotypes, only one of which is associated with disease, suggesting that this may not be the functional allele. Europeans show an unusual haplotype genealogy that is dominated by two common haplotypes differing at multiple sites, whereas Africans have much greater haplotypic diversity. These marked haplotype-frequency differences give an F_{ST} of .25, and, in the European sample, both Tajima's D statistic ($D = 2.58, P = .007$) and the Hudson/Kreitman/Aguade test ($\chi^2 = 4.9, P = .03$) reject neutral equilibrium, suggesting that selective pressure may have acted on this locus.

Introduction

Interleukin 8 (IL8 [MIM 146930]) is one of a family of 13 human CXC chemokines. These small basic heparin-binding proteins are proinflammatory and primarily mediate the activation and migration of neutrophils into tissue from peripheral blood. Radiation-hybrid mapping has indicated that 11 of the 13 genes for these proteins reside on the long arm of chromosome 4, spanning a region of ~2.75 Mb (Modi and Chen 1998). IL8 has been implicated in the pathogenesis of the viral lower respiratory tract–infection bronchiolitis caused by the respiratory syncytial virus (RSV). This disease is responsible for major epidemics each year, with many thousands of infants requiring hospital treatment. High levels of IL8 have been found in nasal secretions (Noah et al. 1995) and tracheal aspirates (Abu-Harb et al. 1999) of infants with RSV bronchiolitis, and the level of IL8 appears to be correlated with disease severity (Smyth et al. 2000). RSV infection of airway cells in culture induces rapid expression of *IL8* (Fiedler et al. 1996), providing a mechanism by which IL8 could result in airway inflammation. However, it is also possible that the IL8 production in RSV infection is a consequence of airway damage, rather than being the cause.

We have attempted to address this issue through investigation of functional variability at the *IL8* locus. Resequencing of the promoter region of *IL8* identified a common single-nucleotide polymorphism (SNP) at position −251 (Hull et al. 2000). Stimulation assays in whole blood through use of lipopolysaccharide (LPS) showed that the IL8−251A allele tends to result in greater expression of *IL8*. In 117 case-parent triad families, we have shown that the IL8−251A allele is associated with susceptibility to severe disease after infection with RSV (transmission/disequilibrium test [TDT] $P = .001$), raising the possibility that the susceptibility to bronchiolitis is a consequence of increased IL8 production after infection with RSV (Hull et al. 2000). One mechanism by which this could occur is if the IL8−251A allele is itself a functional variant that directly affects IL8 production—for example, by influencing binding of transcription factors in the promoter region of the gene. Alternatively, it may be in linkage disequilibrium with a functional variant elsewhere in *IL8* or in a neighboring gene.

Two key questions arise from the above observations: First, is this a true disease association? The use of a family-based design removes the most common source of artifact, that of population structure, but it is important to know whether the association can be repli-

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Table 1

ARMS-PCR Primers Used in Genotyping Reactions

Primer Name	Sequence (5′→3′)	PCR Temperature (A, B)[a] (°C)
−1722delT	GTAAAATACAGTGATGAGTGTTACGATAC	68, 61
−1722insT	GTAAAATACAGTGATGAGTGTTACAATAA	68, 61
−1722 consensus	GTTGTGTCCATATGAGAATGTGTC	
−251A	CCACAATTTGGTGAATTATCAAT	68, 61
−251T	CCACAATTTGGTGAATTATCAAA	68, 61
−251 consensus	TGCCCCTTCACTCTGTTAAC	
396G	TTTACGTTAAATATATGCATGTTACC	67, 60
396T	TTTACGTTAAATATATGCATGCTACA	67, 60
396 consensus	AACATGACTTCCAAGCTGGC	
781C	TCATAACTGACAACATTGAACG	70, 64
781T	AGTCATAACTGACAACATTGAACA	70, 64
781 consensus	TGAGTTGAGCAAGGTAACTCAG	
1633C	TATGTATGGTCTTTCTGGTCGTG	67, 60
1633T	AACTATGTATGGTCTTTCTGGTCGTA	67, 60
1633 consensus	GGACTTAGACTTTATGCCTGACTTAAG	
2767A	CCCAGTTAAATTTTCATTTCAGATAT	67, 60
2767T	CCCAGTTAAATTTTCATTTCAGATAA	70, 64
2767 consensus	GACAAACACTTGATTACTTTGACAACA	
Control primer 1	GGTAGGACACTTTTGGCAACAC	
Control primer 2	CCCATATTAGTACATAACTCTTCCATCA	

NOTE—Primers are identified by the position of the SNP. Allele specificity is determined by the 3′ base of the primer, and additional mismatches were introduced in four of the allele-specific primers (396G, 1633C and 1633T, and −1722delT).

[a] See text for thermocycling conditions.

cated in an independent sample. Second, if it is assumed that this is a true disease association, how can the DNA variant that is functionally involved in the disease process be identified? To dissect the effect of the IL8−251A allele from that of any neighboring linked polymorphisms, a detailed understanding of the pattern of genetic diversity at the *IL8* locus is required.

The present study represents the first step toward addressing these complex issues. To examine the replicability of the association with IL8−251A, we have collected DNA samples from a further 77 affected nuclear families, in a new prospective study. We have extended our search for SNPs in the *IL8* region and have identified a further eight novel polymorphisms spanning a distance of 7.6 kb. We have characterized the haplotypic structure of this locus in both a European and an African population and have identified a disease-susceptibility haplotype among those bearing the IL8−251A allele.

Subjects and Methods

Subjects

Infants with RSV-positive bronchiolitis were identified from five hospitals in the Oxfordshire region. To be included in the study, patients had to be aged <12 mo and had to have RSV-positive bronchiolitis characterized by tachypnea, retractions, and bilateral crackles (wheeze alone was not accepted). The infants were included only if their disease was considered sufficiently severe to require gavage feeding, intravenous fluids, or oxygen. DNA was collected from 77 affected children and from both parents. DNA samples from 36 blood donors recruited at the John Radcliffe Hospital were used for SNP discovery.

To assess the haplotypic diversity in the general population, DNA was extracted from 95 cord blood samples randomly drawn from infants born at the John Radcliffe Hospital, Oxford. For the generation of the African haplotypes, *IL8* SNPs were typed through use of DNA from 61 Gambian nuclear families recruited in Banjul, The Gambia. The study was approved by the Multi-Centre Ethics Committee, United Kingdom, and by The Gambia Government/MRC joint ethical committee. Primate DNA from a chimpanzee (*Pan troglodytes*) was used to determine the ancestral haplotype.

DNA Collection

For the RSV family archive, DNA was collected by use of a mouth-swab method. It was subsequently extracted, quantified, and pre-amplified prior to storage, as described elsewhere (Hull et al. 2000).

Identification of Novel SNPs

A total of 7,618 bp of sequence around *IL8* (−3,908 to 3,711), including 2,428 bp of novel sequence (−1,480 to 3,908), was screened for SNPs by means of denaturing high-pressure liquid chromatography (dHPLC), in 36

Table 2

DNA Sequence Polymorphisms Identified at the *IL8* Locus

SNP[a]	Position	Base Variation	Frequency[b]
−3121	Upstream region	G/C	.39
−2901	Upstream region	C/T	.01
−1722	Upstream region	insT	.01
−251	Upstream region	A/T	.50
396	Intron 1	G/T	.50
781	Intron 1	C/T	.39
1238	Intron 2	insA	.50
1633	Intron 3	T/C	.39
2767	Downstream region	A/T	.39

[a] SNPs are identified by their position relative to the *IL8* gene transcriptional start site.

[b] Frequencies are estimates based on dHPLC data from 72 European chromosomes.

European blood donors. The upstream sequence was obtained by sequencing (ABI 377 automated sequencer, PE Applied Biosystems) of gel-purified PCR products generated by the Genome Walker kit (Clontech Laboratories UK Ltd). DNA variants detected by dHPLC were confirmed and characterized by sequencing.

Typing of SNPs

SNPs were typed through use of the amplification refractory mutation system, as described elsewhere (Hull et al. 2000). The primers used in these reactions are shown in table 1. Also shown in the table are the annealing temperatures used in the PCR. The basic PCR conditions were as follows (see table 1 for values of temperatures A and B): 96°C for 60 s, 96°C for 35 s, temperature A for 45 s, 72°C for 35 s (4 cycles), 96°C for 25 s, temperature B for 50 s, 72°C for 40 s (20 cycles), 96°C for 35 s, 58°C for 60 s, and 72°C for 90 s (8 cycles). Control primers were used for all reactions. To obtain the best differentiation between the different alleles at each locus, additional mismatches were introduced in four of the allele-specific primers (396G, 1633C and 1633T, and −1722delT). DNA samples of known genotype were included as controls in each experiment.

Analysis

Association of alleles and haplotypes with disease within families was analyzed by the TDT (Spielman et al. 1993). *IL8* haplotypes from the unrelated European samples were assigned by the computer program PHASE (Stephens et al. 2001; Mathematical Genetics Group Web site). PHASE is an implementation of the Stephens-Donnelly method of haplotype reconstruction, which uses a Bayesian approach incorporating a priori expectations of haplotypic structure from population genetic and coalescent theory. Haplotypes from the Gambian samples were assigned manually by inspection of the family data. In cases where there was ambiguity, PHASE was used to construct the parental haplotypes. Haplotype genealogies were constructed by inspection, assuming a minimum number of mutational events. Recombination was favored over recurrent mutation to explain homoplastic sites. Identical genealogies were derived by Network2.0c (Shareware Phylogenetic Network Software Web site). Genetree (Griffiths and Tavare 1994; R. C. Griffiths and M. Bahlo, unpublished data; Monash University FTP Archives Web site) was used to estimate the likelihood that the observed European haplotypic distribution could arise through neutral evolution under a simple out-of-Africa model. Two tests of neutrality were applied to data from the 36 European blood donors who were screened for SNPs over 7.6 kb by dHPLC. Tajima's *D* statistic (Tajima 1989) compares two estimates of θ, the population mutation parameter: one derived from the average number of pairwise differences between sequences, and one based on the number of segregating sites. Under neutrality and equilibrium, these two measures have equal expectation, and Tajima's *D* will be ~0. Tajima's *D* analyses were performed using Arlequin software (Arlequin's Home on the Web). The Hudson/Kreitman/Aguade (HKA) test of neutrality (Hudson et al. 1987) is based on the expectation that the amount of intraspecies polymorphism at a locus is proportional to the amount of interspecies divergence, since both are dependent on the same underlying rate of neutral mutation. For this test, 820 bp of sequence data from chimpanzee *IL8* intronic regions were used to estimate interspecies divergence at this locus, and the lipoprotein lipase gene (*LPL* [MIM 238600]; Clark et al. 1998) was used as a reference locus. HKA analyses were performed using DnaSP software (DnaSP Software Web site).

Results

Genetic Association between IL8−251A and RSV Bronchiolitis

We tested the association between the IL8−251A allele and RSV bronchiolitis, using DNA from the 77 families identified in the 1998–1999 bronchiolitis season, by

Table 3

Frequencies of Six SNPs in Two Populations

	Frequency in[a]	
SNP	Europeans	Africans
−1722insT	.01	.26
−251A	.47	.89
396G	.46	.52
781T	.43	.08
1633T	.44	.08
2767T	.42	.08

[a] Frequencies are estimated from a sample of 190 European chromosomes and 244 African chromosomes.

Table 4

Frequencies of the 12 Different Haplotypes Observed in Two Populations

Haplotype Identifier	Haplotype[a]	Frequency in[b]	
		Europeans	Africans
1	delTTTCCA	.52	.10
2	delTTGCCA	...	.004
3	delTTGTTT	.005	...
4	delTATCCA	.01	.19
5	insTATCCA	.005	.18
6	delTAGCCA	.03	.36
7	insTAGCCA	...	.08
8	delTAGCTT	.005	...
9	delTAGTTA	.01	.004
10	delTATTTA	.005	...
11	delTATTTT	...	.004
12	delTAGTTT	.41	.07

[a] The SNPs comprising these haplotypes are those listed in table 3: −1722insT, −251A, 396G, 781T, 1633T, 2767T.

[b] Frequencies are estimated from a sample of 190 European chromosomes and 244 African chromosomes. ... = not found.

use of the TDT statistic. When all informative families were considered, the proportion of occasions in which the IL8−251A allele was transmitted to affected infants was 62% (95% CI 51%–73%), significantly more frequently than expected ($P = .036$). This is very close to the proportion transmitted in our previous DNA collection from 117 families (62%; 95% CI 53%–71%, $P = .014$) (Hull et al. 2000). When the two data sets are combined, giving a total of 194 families, the proportion transmitted is 62% (95% CI 55%–69%) with a probability of $P = .001$.

Nucleotide Diversity

Screening of 7.6 kb of DNA from 36 European blood donors was performed. Eight novel SNPs—in addition to IL8−251, which has been reported previously—were identified. Three of the novel SNPs are upstream of the transcription start site, four are in intronic regions, and one is downstream of the final exon. The nucleotide diversity for the 7,618 bp screened was .0005 ± .0003. Approximate frequencies of each SNP, based on the dHPLC data from 72 chromosomes, are shown in table 2. Three SNPs were not suitable for typing by the amplification refractory mutation system (ARMS)–PCR technique; −3121G/C and −2901C/T lie within an *Alu* repeat, and 1238insA is within a run of six adenine bases. ARMS reactions were successfully developed for the remaining six SNPs.

Haplotypic Analysis of the IL8−251A Association with RSV Bronchiolitis

To dissect the IL8−251A association, we constructed haplotypes by typing this SNP and five adjacent SNPs in 95 European umbilical cord blood samples, using PHASE (Stephens et al. 2001). All phase assignments were made with >90% certainty. The frequencies of each of the six SNPs are shown in table 3. Nine different haplotypes were identified; their relative frequencies are given in table 4, and a minimum-mutation network is shown in figure 1 (*top*). Remarkably, the picture is dominated by two common haplotypes that differ at five of the six positions tested (both haplotypes are represented in sequences held in GenBank: accession numbers M28139 and NT_006216). As might be expected from this haplotype distribution, linkage disequilibrium between segregating sites is high, with $D' > .95$ for all pairwise comparisons.

In the European population, the haplotype structure allows only limited refinement of the IL8−251A bronchiolitis association. The most useful additional marker is the 781 polymorphism: 90% of IL8−251A alleles occur together with 781T, and 10% occur together with 781C. Complete data for both SNPs were available in

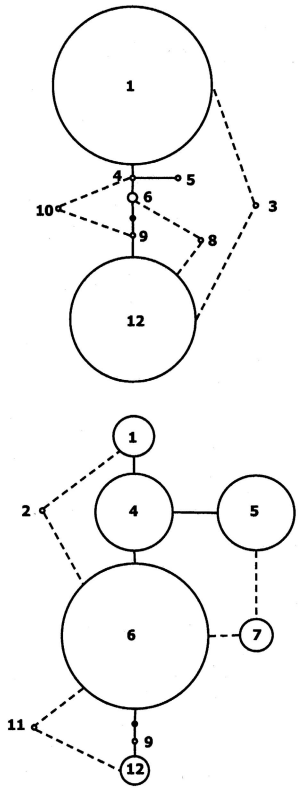

Figure 1 Suggested minimum-mutation network of the haplotypes in European (*top*) and African (*bottom*) populations. Each haplotype is represented by a circle, the radius of which is proportional to its allele frequency. The solid branches (—) represent mutation events, and the dashed branches (- - -) represent recombination events. In each genealogy, the haplotype numbers refer to haplotypes shown in table 4. Sequence data from the chimpanzee suggest that the ancestral root is haplotype 4.

Table 5

Results of the TDT

Haplotype	No. of Haplotypes		% of Haplotypes Transmitted (95% CI)	*P*
	Transmitted	Nontransmitted		
−251A/781T	103	60	63 (56–71)	.0008
−251A/781C	12	18	40 (22–58)	.3

Note—The transmission of haplotypes were tested for departure from the expected rate of 50% transmission, through use of the TDT. The transmitted:nontransmitted ratio is significantly different between the two haplotypes ($P = .02$).

184 families from the combined RSV family archive (table 5). The −251A/781T haplotype showed a significant association with susceptibility to bronchiolitis (63% transmitted, $P = .0008$). In contrast, the −251A/781C haplotype showed a nonsignificant trend in the direction of protection against bronchiolitis (40% transmitted, $P = .3$). The −251A/781T haplotype is significantly more likely to be transmitted than the −251A/781C haplotype, when the transmission ratios are compared through use of a proportions test ($P = .02$; table 5).

Haplotype Frequency Distribution

A striking feature of the European data set was the unusual haplotype distribution, which shows a marked predominance of two haplotypes that are separated by five mutational events and shows a low frequency of intermediate haplotypes. To determine whether the observed haplotype distribution was consistent with the expectations of a neutral equilibrium model, we applied two tests of neutrality: Tajima's *D* and the HKA test. Using dHPLC and sequencing data from 36 European blood donors over a 7.6-kb region spanning the *IL8* locus, we calculated Tajima's $D = 2.58, P = .007$ (table 6). These findings reject a model of neutrality and population equilibrium. The HKA test was applied to the *IL8* sequence data from the 36 European blood donors and one chimpanzee, with published data from the *LPL* gene serving as a reference locus (Clark et al. 1998). Nine segregating sites were found in 5,979 bp of noncoding sequence from 72 European chromosomes. There were nine fixed differences between the human and chimpanzee, over 820 bp of *IL8* intronic sequence. These data were compared with the published data at the LPL locus (Clarke et al. 1998), in which 61 segregating sites were found in 8,736 bp of sequence from 94 European chromosomes and in which 108 fixed differences were found between the human and chimpanzee, over 7,261 bp of sequence. The HKA test rejected the hypothesis that both genes have evolved under the same neutral parameters ($\chi^2 = 4.9, P = .03$).

These observations raised the question of whether a similar haplotype structure exists in other populations. To address this question we typed 183 African individuals from 61 three-member families. African parental haplotypes were constructed on the basis of pedigree information, and any remaining phase-unknown sites were assigned by PHASE (Stephens et al. 2001). Of the 732 phase assignments, 720 were made with >90% certainty. SNP and haplotype frequencies are shown in tables 3 and 4, respectively, and minimum-mutation networks of the haplotypes are shown in figure 1 (*bottom*). On the basis of sequence data from the chimpanzee, the root of the haplotype tree appears to be haplotype 4. In the African population, the distribution of haplotypes is spread much more evenly than in Europeans, with six different haplotypes seen at frequencies of >7%. Linkage disequilibrium between most segregating sites in the African population is also high, with values of $D' > .90$ for all pairwise comparisons, with the exception of that between loci 1 and 3, where $D' = .40$. Gene diversity, the probability that two haplotypes randomly chosen from the sample will be different, is higher in the African population (.7823 [SD .0146]) than in the European population (.5619 [SD .0188]) (Nei 1987). The haplotypes that are most common in the European population are not the most common in the African population. The two populations appear quite different in terms of haplotype frequencies, and the F_{ST} (Wright 1931) is .25, which is considerably higher than that typically observed (Cavalli-Sforza et al. 1994).

Discussion

In the present study, we have shown that the genetic association between severe RSV bronchiolitis and the *IL8* gene region is reproducible in a separate family archive. Previous data (Hull et al. 2000) suggest that whole blood from individuals carrying the −251A allele pro-

Table 6

Tajima's *D* Test of Selective Neutrality

Population	No. of Sites	π[a]	θ[b]	Tajima's *D*	*P*
European	9	3.680 ± 2.087	1.857 ± .763	2.579	.007

[a] Average number of pairwise differences between sequences.
[b] Estimated from the number of segregating sites.

duces a greater IL8 response when stimulated with LPS in vitro. This provides evidence that the genetic association we have observed is a consequence of functional variability in *IL8* gene expression. We have not demonstrated that the −251A allele is itself functional. By determining the haplotype structure across the *IL8* region, we will be able to map the observed genetic association more finely and be able to focus functional studies on the region that shows the strongest genetic association.

In the present study, we have described nine novel SNPs at the *IL8* locus, spanning 7.6 kb (−3908 to 3771). We have typed six of these SNPs in two populations and have shown the haplotypes derived from these data. The distribution of the haplotypes in the European population shows that 52% of chromosomes carry one haplotype, and 41% carry a different haplotype separated by five mutation events. The division of the European SNPs into two major haplotypes limits the resolution with which we can map the disease association. Nevertheless, the 781T allele dissects the −251A-bearing haplotype into two groups: 90% of haplotypes carrying −251A also carry 781T, and 10% carry 781C. When the −251A/781T haplotype was analyzed by use of the TDT, a significant association with severe RSV bronchiolitis was revealed (63% transmitted, $P = .0008$). In contrast, the −251A/781C haplotype showed a nonsignificant trend in the direction of protection against bronchiolitis (40% transmitted, $P = .3$). The −251A/781C haplotype is relatively rare, and, as a consequence, the two-marker haplotype does not markedly increase the strength of the association compared with −251A alone. When the proportion of transmissions of the −251A/781T haplotype was compared with that of the −251A/781C haplotype, the −251A/781T haplotype was transmitted significantly more often. This suggests that the functional allele may lie on chromosomes of the −251A/781T haplotype and that −251A may not be functional. The greater haplotypic diversity of the African population will allow fine mapping of the disease association at a higher resolution than in the European population and illustrates the importance of establishing DNA archives of relevant diseases in these populations.

Results from the Tajima's *D* and HKA tests suggest that the *IL8* locus has evolved under conditions that are inconsistent with a selectively neutral model, an equilibrium population model, or both. The F_{ST} at the *IL8* locus between African and European populations is high (.25). High F_{ST} values have been interpreted as evidence for positive natural selection (Hamblin and Di Rienzo 2000). Previous applications of the Tajima's *D* statistic (Tajima 1989; Hamblin and Di Rienzo 2000) rejected neutrality because of a negative value of *D;* that is, the number of segregating sites was high, but the average pairwise sequence differences was low. This can result from the star-shaped genealogy that follows a selective sweep. Interestingly, the *IL8* locus, in Europeans, gives a strong positive value of *D,* resulting from a relatively high estimate of average pairwise sequence differences given the number of segregating sites observed. This results from a genealogy dominated by two common haplotypes that are deeply divergent, separated by five mutational events (fig. 1, *top*).

Although selection is one explanation for the observed haplotypic distribution in the European population, other possibilities should be considered. It is conceivable that a reduction in population size (e.g., during migration out of Africa) would cause chromosomes to be lost because of genetic drift, leaving two predominant haplotypes. On inspection, it seems improbable that the European haplotype distribution could be obtained by sampling from the African population. Coalescent modeling of this process through use of Genetree (data not shown) shows that the two most common European haplotypes are among the least likely to become common after migration. This suggests that migration and drift alone are unlikely explanations for the reduction in European haplotypic diversity. This analysis required the assumption that the current African sample is representative of an ancestral African population, so the result should be interpreted with caution. Nevertheless, this observation, in combination with the high value of F_{ST} and the significant results of Tajima's *D* and the HKA test, raises the intriguing possibility that one of the European haplotypes may have increased in frequency through selection of a favorable functional element.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Arlequin's Home on the Web, http://lgb.unige.ch/arlequin/ (for Arlequin version 1.1)

DnaSP Software, http://www.bio.ub.es/~julio/DnaSP.html (for DnaSP version 3.5)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/index.html (for *IL8* [accession numbers M28139 and NT_006216])

Mathematical Genetics Group: Software, http://www.stats.ox.ac.uk/mathgen/software.html (for PHASE version 0.0)

Monash University FTP Archives, http://ftp.monash.edu.au/pub/gtree/ (for Genetree version 8.3)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for IL8 [MIM 146930] and LPL [MIM 238600])

Shareware Phylogenetic Network Software, http://www.fluxus-engineering.com/sharenet.htm (for Network2.0c)

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