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ORIGINAL ARTICLE

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Linkage disequilibrium and haplotype analysis among four novel single-nucleotide polymorphisms in the human leukemia inhibitory factor (*LIF*) gene

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Abstract Leukemia inhibitory factor (LIF) is a pleiotropic cytokine implicated in various pathological conditions, such as rheumatoid arthritis and osteoporosis. Despite the possible importance of LIF as a therapeutic target, little is known about the bioregulation of the human *LIF* gene. We here sequenced the entire structure of the *LIF* gene of 48 alleles in the Japanese population. These experiments identified four single-nucleotide polymorphisms (SNPs) and determined their allelic frequencies from a 48-allele sequence in the Japanese population. All four SNPs found in the *LIF* gene were located within exon 3, that is, a C/T at nucleotide (nt) position 3951, a C/G at nt position 4376, an A/C at nt position 4442, and a G/A at nt position 5961 (nucleotide numbering starts from the ATG start codon). Based on the genotypic data, we constructed four major haplotypes in the tested population. Two-way comparisons of SNPs revealed complete linkage disequilibrium between SNPs at positions 3951, 4376, and 4442. These results may prove to be useful as genetic markers for population-based disease-association studies in osteoporosis.

Key words Leukemia inhibitory factor (LIF) · Single-nucleotide polymorphism · Japanese population · Direct sequence · Haplotype frequency · Linkage disequilibrium

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Introduction

Leukemia inhibitory factor (LIF) was initially described based on its ability to induce differentiation in the murine myeloid leukemia cell line M1. Later, LIF turned out to be a synonym for at least nine different factors defined on the basis of their effects on a variety of cell types, including lymphomas, liver cells, embryonic stem cells, carcinoma cells, neurons, melanomas, and osteoclasts (Van Vlasselaer 1992). We sequenced the entire genomic DNA sequence from 24 Japanese individuals (48 alleles), identified four single-nucleotide polymorphisms (SNPs) at the *LIF* locus, and analyzed haplotypes and allelic frequencies. We also looked for linkage disequilibrium among the SNPs in the hope of explaining the genetic diversity observed at this locus in our test population.

Subjects and methods

DNA extraction

Blood samples were obtained, with informed consent, from 24 healthy Japanese individuals. Genomic DNA was prepared from each sample according to procedures described previously (Ikegawa et al. 1999).

Polymerase chain reaction (PCR) condition and primer design

PCR and sequencing primers were determined by analyzing the *LIF* genomic sequence with DNASIS 3.0 software (Hitachi Software Engineering, Yokohama, Japan). The sequence numbering was adjusted to the RNA transcription-start site (A at the top of GenBank U20816.1 is now +1). Eleven pairs of PCR primers were chosen at approximately 500-bp intervals, with approximately 100-bp overlaps. PCR was performed as described previously (Tsukamoto et al. 1998). PCR amplification was performed under the follow-

ing conditions: 94°C for 3 min, 72°C for 3 min, then 5 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 1 min, and 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min.

DNA sequencing

Following purification by affinity membrane, PCR products were subjected to cycle sequencing with the indicated primer as described previously (Tsukamoto and Emi 1998). The differences among sequences in 24 samples were regarded as SNPs.

Determination of haplotype frequencies and tests of linkage disequilibrium

Haplotype frequencies among the 48 alleles investigated were calculated by Arlequin software (Genetics and Biometry Laboratory, Geneva, Switzerland). We investigated linkage disequilibrium for all possible two-way comparisons of the novel SNPs using several widely used methods (D, D′, and r^2; Lewontin 1988; Miller et al. 2000). Significance levels were determined by χ-squared statistics for the corresponding 2×2 table.

Results and discussion

A total of 24 Japanese individuals were genotyped for sequence variations of all three exons and surrounding regions of the *LIF* structural gene. A total of four SNPs were found in the gene by this method. The locations of these SNPs are shown in Fig. 1 in relation to the genomic structure of the *LIF* gene. All four of the SNPs found in the *LIF* gene were located within exon 3, that is, a C/T at nucleotide (nt) position 3951, a G/C at nt position 4376, an A/C at nt position 4442, and a G/A at nt position 5961. Table 1 summarizes the SNPs and their allelic frequencies detected in our sample of 48 Japanese alleles. None of the SNPs we found were reported in the IMS-JST SNPs database (http://snp.ims.u-tokyo.ac.jp/index.html), nor in the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html).

Haplotype frequencies among the 48 alleles investigated were calculated by Arlequin software (Genetics and Biometry Laboratory). The results are summarized in Table 2. The SNPs at nt position 5961 were excluded from this analysis because their minor alleles were too rare. Nevertheless, we identified four distinct haplotypes among the 24 Japanese subjects; one of them (haplotype 1, Table 2) accounted for 56% of all haplotypes. Although we measured the frequencies of four major haplotypes in the Japanese population, our analysis of a modest sample size is not enough to provide solid data for haplotype frequencies in this population. Future analysis of a larger sample size would be indispensable for accurate determination of these haplotype frequencies.

We investigated linkage disequilibrium for all possible two-way comparisons of the novel SNPs using several widely used methods (D, D′, and r^2; Lewontin 1988; Miller et al. 2000). SNPs with rare alleles (at nt position 5961) were excluded from the analysis. Results are shown in Table 3. Complete linkage disequilibrium was identified between the SNPs at nt positions 3951 and 4376, as well as between the SNPs at nt positions 3951 and 4442. "C" variation at nt position 3951 probably occurred in the background haplotype of nt positions 4376 (C)–4442 (A).

LIF is a pleiotropic cytokine implicated in various pathological conditions, such as rheumatoid arthritis and osteoporosis (Van Vlasselaer 1992). Despite the possible

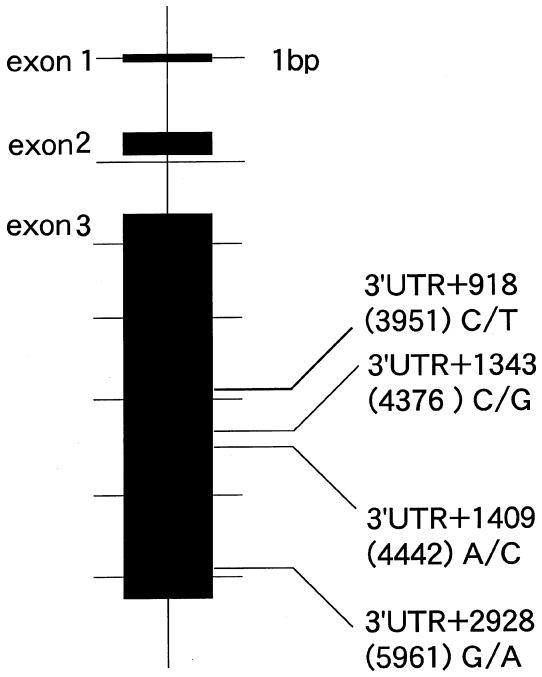

Fig. 1. Relationship between genomic structure and location of single-nucleotide polymorphisms in the human *LIF* gene. *UTR*, Untranslated region

Table 2. Frequencies of haplotypes constructed from four single-nucleotide polymorphisms

No.	Frequency	3′UTR + 918	3′UTR + 1343	3′UTR + 1409
1	0.5625	C	C	A
2	0.3125	T	G	C
3	0.1042	T	C	C
4	0.0208	T	G	A

Table 1. Novel polymorphisms in the human *LIF* gene in the Japanese population

Gene region	Position[a]	Nomenclature	Frequency
Exon three	3951	3′UTR + 918 C/T	C (0.56), T (0.44)
Exon three	4376	3′UTR + 1343 G/C	C (0.67), G (0.33)
Exon three	4442	3′UTR + 1409 A/C	A (0.58), C (0.42)
Exon three	5961	3′UTR + 2928 G/A	G (0.94), A (0.06)

UTR, untranslated region
[a]Nucleotide numbering starts from ATG start codon

Table 3. Analysis of linkage disequilibrium for all possible two-way comparisons among four single-nucleotide polymorphisms

	3′UTR + 1343 (4376) (C/G)	3′UTR + 1409 (4442) (A/C)
3′UTR + 918 (3951) (C/T)	$D' = 1.0000$ $r^2 = 0.6435$ $\chi^2 = 30.887$ $P = 2.7 \times 10^{-7}$	$D' = 1.0000$ $r^2 = 0.9178$ $\chi^2 = 44.053$ $P = 3.2 \times 10^{-11}$
3′UTR + 1343 (4376) (C/G)		$D' = 0.8922$ $r^2 = 0.5581$ $\chi^2 = 26.787$ $P = 2.3 \times 10^{-7}$

importance of human LIF as a therapeutic target, little is known about its biological activity. It is known that osteoclast and osteoblast activity is stimulated or suppressed by LIF, depending on the developmental stage of the respective cells (Bamberger et al. 1997). These data suggest that *LIF* is a candidate gene involved in the regulation of differentiation and activity of osteoblasts and osteoclasts, and thus in the regulation of bone mineral density in human physiology and pathology.

In conclusion, these ten polymorphisms, their haplotypes, and the linkage disequilibrium data will be useful for investigating a possible relationship between genetic variation at the human *LIF* locus and human diseases, especially in association studies between specific polymorphisms and susceptibility to inflammatory diseases and osteoporosis.

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