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Identification of *cis*-regulatory variations in the *IL6R* gene through the inheritance assessment of allelic transcription

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ABSTRACT

Background: The level of circulating interleukin-6 receptor in human blood varies depending on the genetic and/or physiological causes, and has been implicated in the development of chronic inflammatory diseases.
Method: The *cis*-regulatory effects of genetic variations on the transcription of interleukin-6 receptor gene, *IL6R*, were studied by assessing allelic transcriptions in the immortalized lymphocytes derived from unrelated and familial samples.
Results: The assays for allelic transcription in the cells from unrelated subjects demonstrated an extensive and variable range of allelic transcriptional imbalances, suggesting an operation of multiple *cis*-regulations with varying degrees on the locus. Analysis of the familial samples illustrated the Mendelian inheritance of allelic transcriptions, enabling us to assign each haplotype allele into one of the 3 transcriptional strengths. A comparison of the allele structures based on the transcriptional attributes highlighted 2 SNP variations, rs952146 and rs4845617, as being associated with higher allelic transcription. Consistently, lymphocytes that were homozygous for the 2 SNPs exhibited differences in their transcript levels depending on the haplotypes.
Conclusion: Inheritance assessment of allelic transcription of *IL6R* identified 2 SNPs that are associated with transcriptional variation *in cis*.
General significance: Our results not only demonstrate genetic variations that are associated with *IL6R* transcription *in cis* but also demonstrate an effective genetic approach for isolating *cis*-regulatory variations.

1. Introduction

Interleukin-6 (IL-6) signaling plays a pivotal role in the resolution of inflammatory episodes by mediating the transition of innate to adaptive immunity at sites of inflammation [1]. This regulatory function involves the recruitment, activation, and clearance of leukocytes [1–4]. The multi-faceted functionality of IL-6 signaling is rendered largely by the selective utilization of different casts of the cognate receptor. The expression of *IL6R* gives rise to 2 isoforms of the IL-6 receptor (IL-6R): the membrane-bound receptor and the soluble receptor. The membrane type normally transduces the IL-6 signal in cells that express it, such as hepatocytes and a subset of leucocytes. This "classical signaling" activates the expression of acute phase proteins such as C-reactive protein (CRP) during an early immune response. The soluble receptor (sIL-6R) is generated either via translation of the alternatively spliced mRNA or via proteolytic cleavage of the membrane type receptor termed shedding [2,5]. Unlike other soluble receptors that act as signal antagonists, sIL-6R when it forms a complex with IL-6 mediates "trans-signaling" via ubiquitously expressed gp130 protein in cells where *IL6R* expression is absent, which leads to pro-inflammatory events such as leukocyte infiltration and rescuing T cells from apoptosis.

An elevated plasma level of sIL-6R has been associated with high levels of circulating inflammatory markers such as IL-6 and CRP and is a characteristic feature of many inflammatory diseases, including Crohn's disease, rheumatoid arthritis, and asthma [4,6,7]. Selective blockade of trans-signaling using soluble gp130, sgp130, or its engineered version, sgp130FC, has been reported to ameliorate inflammatory conditions in model animals, and thus is considered for a therapeutic target for many inflammatory diseases [4,8,9]. On the other hand, the Asp358Ala (rs2228415) variant of *IL6R*, which enhances the shedding rate of the receptor and hence contributes to the increase of circulating sIL-6R in a per-allele manner, was recently associated with a low risk of coronary heart disease [10–14]. These studies strongly indicate not only the importance of properly modulating the IL-6 signaling to ensure the proper resolution of an inflammatory episode but also the causality between a person's genetic makeup and risk of disease. In light of this, it is also equally provable that the genetic variation that affects the *IL6R* transcriptional rate also affects the risk of developing inflammatory diseases.

In the present study, we attempted to identify genetic variations that affect the *IL6R* transcriptional rate in the *cis* configuration in human immortalized lymphocytes. Since a heterozygous configuration of the *cis*-regulatory variation gives rise to imbalanced allelic transcription (iBAT) of the affected gene, the quantitation of iBAT and correlation of

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the measurements with genomic variations has been a method of choice for carrying out this type of work [13,15–17]. We therefore utilized this approach on the basis of our initial observation of an extensive and variable range of allelic imbalances in *IL6R* transcription in human immortalized lymphocytes. The state of allelic balance in the cDNA was determined by taking advantage of the high accuracy and precision of melting analysis for the allele quantitation method [18]. Analysis of the genetic basis with respect to *IL6R* iBAT using the biological samples of a large family resulted in the identification of 2 SNPs that were associated with high *IL6R* transcriptional levels.

2. Materials and methods

2.1. Lymphocytes, gDNA, and cDNA

Lymphocytes and gDNAs that were derived from unrelated individuals and from members of a family were obtained from KCM Bank at the Korea Institute of Oriental Medicine, Korea. The study was approved by the Institutional Review Board (I-2008/010-001). The cells were cultured in RPMI media supplemented with 15% FBS at 37 °C. Total RNA extraction from the cells using TRIzol® reagent (invitrogen™, Carlsbad, CA, USA) and cDNA synthesis from 1 μg of total RNA using an iScript™ cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA) were performed according to the manufacturer's instructions.

2.2. Quantitative competitive PCR

Dimorphic DNA or a mixture of target and competitor templates was amplified using primers that were common to both competing templates until the plateau phase was reached. The fractions of the competing DNAs in the amplicons were subsequently determined by melting analysis for allele quantitation as described previously [18,19]. Briefly, 5 μl of the PCR amplicons was mixed with same volume of the probe solution (10 mM EDTA and 0.4 μM of corresponding fluorescent resonance energy transfer probes). DNA melting was performed with a Roche LightCycler 2.0 (Roche, Mannheim, Germany) under the following conditions: denaturation at 95 °C for 30 s; probe annealing at a given temperature for each PCR amplicon at 49–55 °C (see supplementary table1) for 5 min; and 0.1 °C stepwise increments in temperature until the probe's melting was completed. The background-subtracted fluorescence values (640/Back-530) from the Cy5 dye for every sample were extracted with the aid of LightCycler Software 4.05 and then submitted for calculation to yield relative allelic abundance in each sample. For the calculation, the melting curves were first normalized in a way that the initial and the final fluorescence level were converted to 1 and 0, respectively, and those remaining were proportionally. The relative abundance of an allele to the other in a sample was then obtained by calculating and bias-correcting the ratio of the area that formed by the melting curves of two allelic references to the area that formed by the melting curves of the sample and the reference of opposite allele.

2.3. Intergenic relative quantitation

As previously described, the competitor templates for the target sequences from *IL6R*, *POLH*, and *PARP16* that were to be quantitatively compared were made to contain single nucleotide variations [20]. They were placed in a single plasmid through repetitive cloning to form a competitor array (CA), which rendered the absolute quantitative relationships among the competitor templates (see supplementary table2 for sequence information). The cDNA that was added with the CA after liberating each template by restriction digestion was subjected to quantitative competitive PCR using the appropriate primer sets. The quantities of cDNA relative to each other were determined by comparing the relative quantities of the targets to their corresponding competitor templates. This was done by considering the absolute relative quantities among the competitor templates in the CA.

2.4. Genotyping

The SNPs that were used for genotype analysis were selected in the 160-kb region of the *IL6R* locus, including 57 kb of the region that is upstream to the gene and 50 kb that is downstream. Application of pairwise tagging method ($r^2 > 0.9$) has selected 22 SNPs to capture all of the 71 SNPs in the region of which minor allele frequencies are greater than 0.1 in the East Asian panel (JPT + CHB) of the Hapmap database (Fig. 1a). Genotyping of the given SNPs was carried out by applying the unlabeled oligonucleotide probe (UOP) to the dilute SNP-spanning amplicons as described previously [20]. Four-fold diluents of the PCR amplicons at the plateau phase were mixed with 5 volumes of UOP solution that contained 5 nM Syto® 9 (Life Technologies), 12.5 mM EDTA, and 5 mM Tris (pH 8.0). The mixtures then underwent a thermal reaction using a LightCycler® 2.0 instrument (Roche Diagnostics) as follows: denaturation at 95 °C for 5 s, annealing at 57 °C for 1 min, and melting with a gradual increase in temperature of 0.1 °C/s until each probe was melted. The melting profiles of the UOPs were obtained by reading the fluorescence emission at 430 nm during probe melting. The genotype of each PCR product was then determined based on the temperature wherein the corresponding UOP melted away.

3. Results

3.1. Extensive allelic imbalance of the IL6R transcript

In this study, we utilized two SNPs in *IL6R* gene, rs4845617 in exon 1 and rs2228145 in exon9, separately or together for the assessment of relative allelic abundance measurement in mRNA since they exhibit relatively high heterozygosity in East Asian population (0.500 and 0.496, respectively) and are transcribed into the *IL6R* mRNA. To qualitatively configure the nature of iBAT in *IL6R* transcription, amplicons that span the SNP, rs4845617, were generated using genomic DNA (gDNA) or lymphocyte cDNA as templates that were prepared from 8 arbitrarily chosen subjects who were heterozygous for the given SNP (Fig. 1a and b). We subsequently acquired the normalized melting curves of the corresponding fluorescent probe for the amplicons along with those for 3 control amplicons each of which represented one of the 2 alleles or the equimolar mixture of the 2 alleles. The principle of this assay is that the melting curve of a sample with a biased allelic composition shifts from the reference curve with equal allelic representation toward the reference curve corresponding to the dominant allele in that sample [19]. Following this, we compared the patterns on the basis of the template origin (Fig. 1b).

The melting curves for all of the gDNA-derived amplicons coincided with that of the control amplicon in terms of equal allele contribution, which demonstrates not only balanced contributions of both alleles in the gDNA but also the reliability of the assay. On the contrary, those for the cDNA amplicons exhibited various allele compositions with half of the subjects being consistent for equal allelic contribution and the others deviating from the allelic balance to a slight (S8), moderate (S4, S6), or large (S2) extent. These observations suggest the involvement of more than one *cis*-factor, each of which forms a polymorphism that influences the transcriptional rate.

3.2. Association of iBAT with local SNPs

The variable range of *IL6R* iBAT in a large portion of the subjects signifies the isolation and characterization of individual genetic or epigenetic variation that may constitute a part of the regulatory components of transcription. To address this issue with an emphasis on the genetic cause, we quantitatively assessed the allelic transcriptional patterns and subsequently associated the measures with the genotypes of the local SNPs at the *IL6R* locus. Allelic quantitation and genotyping were performed using the lymphocyte cDNA and gDNA, respectively, which were prepared from the 70 subjects who are heterozygous for

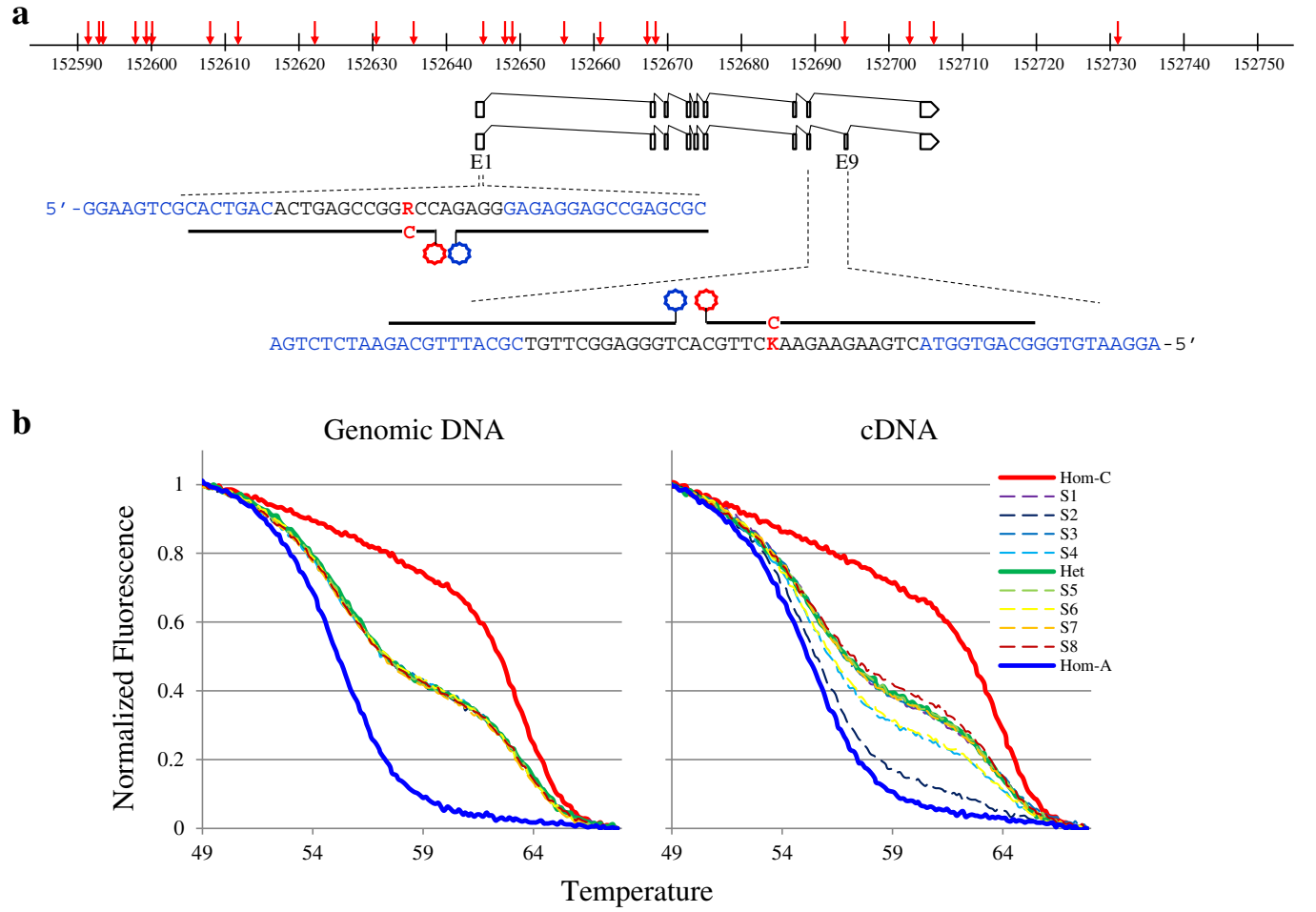


Fig. 1. Extensive and variable degrees of allelic imbalances in *IL6R* transcription. a. The amplicons for the assessment of *IL6R* allelic transcription were designed to contain one of the exonic SNPs on exon1 (E1) and exon9 (E9). Their sequences are depicted along with the genomic structure of the gene. The PCR primers are denoted with blue letters while the SNPs are denoted using the red letters G and K to indicate G/A and C/T polymorphisms, respectively. The fluorescent resonance energy transfer probes for each amplicon are depicted with a solid bar over or below the sequences to indicate the sense or anti-sense orientation, respectively. The fluorescent dye that was attached to one end of each probe is denoted with a red (Cy5) or blue circle (FAM). The probe's nucleotides that correspond to the polymorphic loci are indicated on the bar with red letters. The locations of the SNPs for associating with transcriptional levels are indicated with red arrowed bars over the scale. b. The normalized melting curves that were generated with the E1 amplicon using gDNA (right) or lymphocyte cDNA (left) from 8 subjects (S1–S8) are depicted along the melting curves of 3 standards that represent each of 2 allelic versions (Hom-C and Hom-A) and an equimolar mixture of 2 alleles (Het).

the rs8192284 on exon9. The allelic ratio of the *IL6R* transcript and the genotypes of the given SNPs for each subject are given in the Supplementary Table3.

An iBAT in a given subject is likely to be a functional consequence of a dimorphic regulatory element involving sequence variation such as an SNP. The SNP as either the causal variation itself or a surrogate of the causal variation should be heterozygous in the subject. Therefore, we tried to select the SNPs for which heterozygous subjects were preferentially allocated to the subject that exhibited iBAT. In Fig. 2, we present the heterozygous genotypes of all of the tested SNPs for each subject along with the allelic transcriptional pattern of *IL6R*.

A set of SNPs exhibited a certain level of biased distribution of their heterozygous subjects such that larger portions of the heterozygous subjects were allocated into the iBAT group. Although preferential allocation of heterozygous subjects for rs12025518 to the iBAT group was evident, however, no SNP was found to enrich their heterozygous subjects such that they were allocated to the iBAT group in an exclusive manner. The absence of the absolute association of iBAT with a heterozygous genotype of any SNP supports the notion that multiple layers of *cis*-regulation operate at the locus. For instance, 2 independent and physically separated regulatory variants can be recombined in a way that the transcriptional effect of one variant is concealed by the opposing effect of the other. Any of the regulatory SNPs in this recombinant allele could form a heterozygous genotype with another allele that is oppositely recombined while 2 alleles represent a BAT. We therefore attempted to analyze the iBAT in a more precise way by employing an analysis of SNPs in haplotype structures using familial samples. The SNPs that resulted in allocating the heterozygous subjects to the iBAT group more than to the BAT group were selected for further analysis.

3.3. Mendelian inheritance of IL6R iBAT

To investigate the regulatory nature of *IL6R* iBAT in further detail, we analyzed the allelic transcription levels in association with the haplotype structures that were formed by a set of SNPs by using the subjects of a large family that consisted of 76 informative members. Allelic transcription was measured by utilizing 2 exonic SNPs from 42 subjects that were heterozygous for any of the 2 SNPs. After determining the genotypes for the selected SNPs (supplementary table4), the haplotype structure in each subject was built by analyzing the segregation of the SNP alleles in generations (supplementary figure1). Haplotype alleles with identifiable allelic origins were then named to denote the carrier's

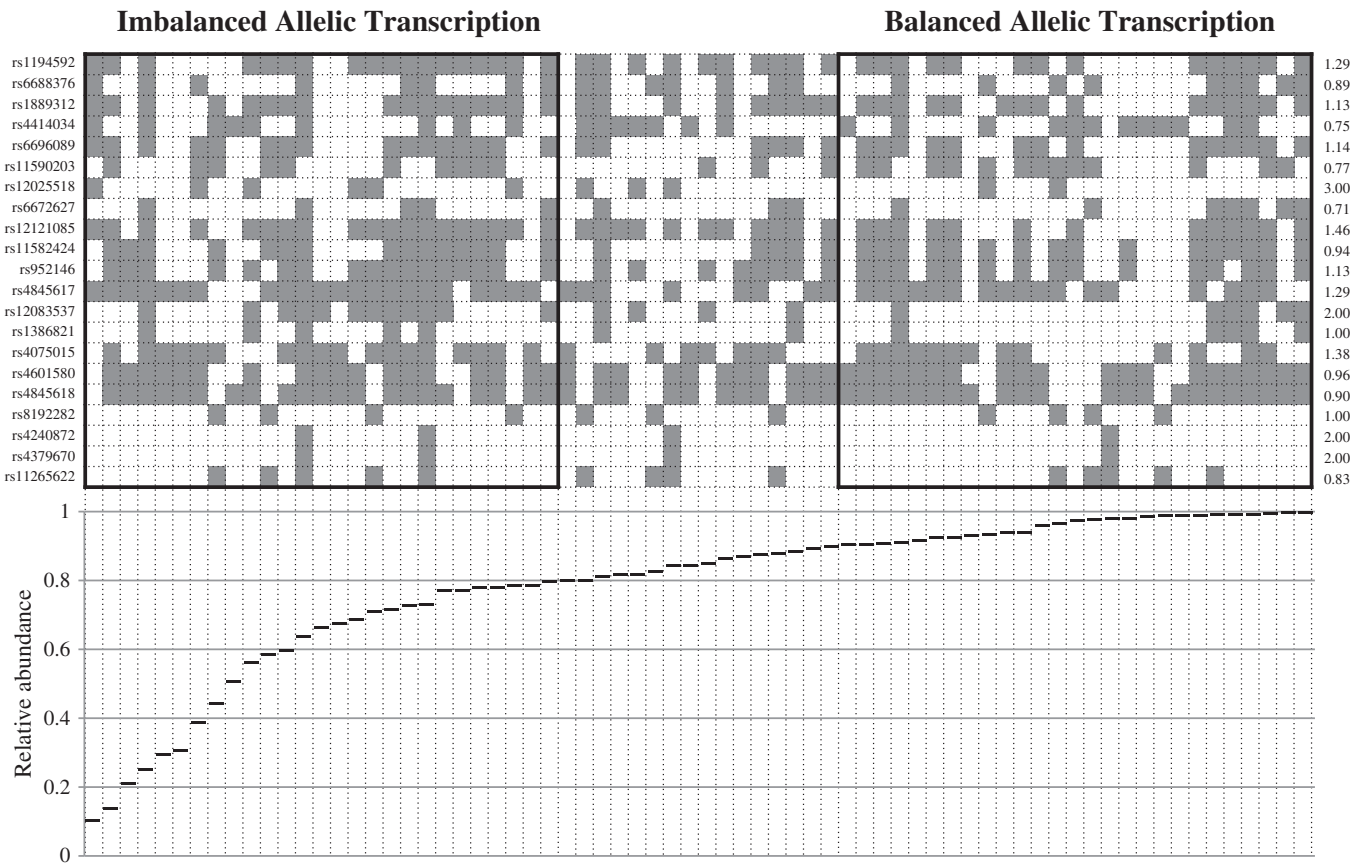


Fig. 2. Association of allelic transcriptional imbalances with local SNPs. The information of 72 subjects with respect to the heterozygous genotypes for local SNPs (top, gray boxes) and the extents of allelic imbalances in IL6R transcription (bottom) are presented in a way that the subject with wider variation in terms of allelic transcription comes first in the columnar array by lining up the matched subjects. Each bar on the bottom represents the relative transcriptional ratio of the less expressed allele to the more expressed allele in the lymphocytes for a given subject. The subjects in which the transcriptional level of one allele was less than 80% of the other were designated as belonging to the iBAT group while the subjects that showed transcriptional differences of less than 10% between the 2 alleles were allocated to the balanced allelic transcription (BAT) group as indicated with solid borderlines. The frequency ratios of the heterozygous genotype for a given SNP between the imbalanced allelic transcription and the balanced allelic transcription subjects are denoted on the right.

identity after the letter by indicating it as being of paternal origin (p), or maternal origin (m). The same haplotype alleles that were identical by decent were given the same names, which gave rise to a recovery of 47 independent alleles in the family.

The transcriptional strength of each haplotype allele that its structure bears was subsequently analyzed. A matrix of haplotype alleles was constituted to present the relative transcriptional ratio of one haplotype allele to the other in each of the informative lymphocytes (Fig. 3). For instance, the haplotypes pII3 and pII1 were found to comprise heterozygotes in 4 subjects and exhibited equivalent allelic transcription levels as the relative ratio of the pII3-derived transcript to the pII1 derivative was 1, 0.96, 0.96, or 0.92 in each subject (supplementary figure 1). These 2 haplotypes and those that also exhibited transcriptional levels that were equivalent to any of the 2 haplotype alleles through direct or indirect comparisons were assigned to the medial-type alleles that carried the medial transcriptional strength (green box). Other haplotypes that could be compared to any of the medial alleles were subsequently assigned as being either the strong (orange) or the weak (blue) type depending on the transcriptional level of each relative to the medial type. The haplotypes that could not be compared with any of the designated haplotypes were left unassigned.

The matrix analysis of the haplotypes supported the notion that the transcriptional strength of *IL6R* is inherent on the haplotype structure. To represent more intuitively the Mendelian inheritance of iBAT, we present a part of the pedigree with the names of the haplotype alleles and the allelic transcription patterns for the constitutive members in Fig. 3b. In this pedigree, each haplotype allele is color-coded to denote one of the 3 transcriptional strengths that they bear. As it was determined to be a weak allele, the mII1-derived transcript accounted for a smaller proportion of *IL6R* transcripts in the subject I2 in which mII1 formed a heterozygote with a medial-type allele. The descendants (II4 and III2) who were transmitted with this allele also exhibited iBAT with more allelic contributions to the transcript via the counter alleles. Interestingly, the allelic proportions of the mII1-derived transcript

Fig. 3. Allelic comparison matrix for transcriptional strength and Mendelian inheritance of allelic transcription. a. A comparison of haplotype alleles with respect to the transcriptional strength was made by coordinating 2 haplotype alleles in the matrix and presenting all of the relative allelic transcription from a haplotype (Y axis) to another (X axis) when a direct or indirect comparison was available for the subjects in the family. The state of allelic balance in the coordination of haplotype alleles are color-coded green, blue, and orange to denote that the transcription from the haplotype on the Y axis is equivalent to, less than, or more than that on the X axis, respectively. The haplotype alleles were similarly color-coded to indicate the relative transcriptional strengths with green, blue, and orange for the alleles that are equivalent to, weaker than, or stronger than either pII3 or pII1, respectively. b. A part of a family pedigree is presented. The color-coded haplotype alleles indicate transcriptional strength, and the genotypes of the SNPs on exon 1 and exon 9 that each haplotype carries are sequentially presented under the symbol for each family member. The relative transcriptional ratios of *IL6R* between the haplotype alleles of each member are graphically represented.

were similar in the subjects I2 and II4, in which the mII1 allele paired with one of the medial-type alleles. On the other hand, for subject III2, in which mII1 allele paired with a strong-type allele, the proportion of the mII1-derived transcript shrunk further and thus confirmed the allelic specificity of the transcriptional strengths that are inheritable.

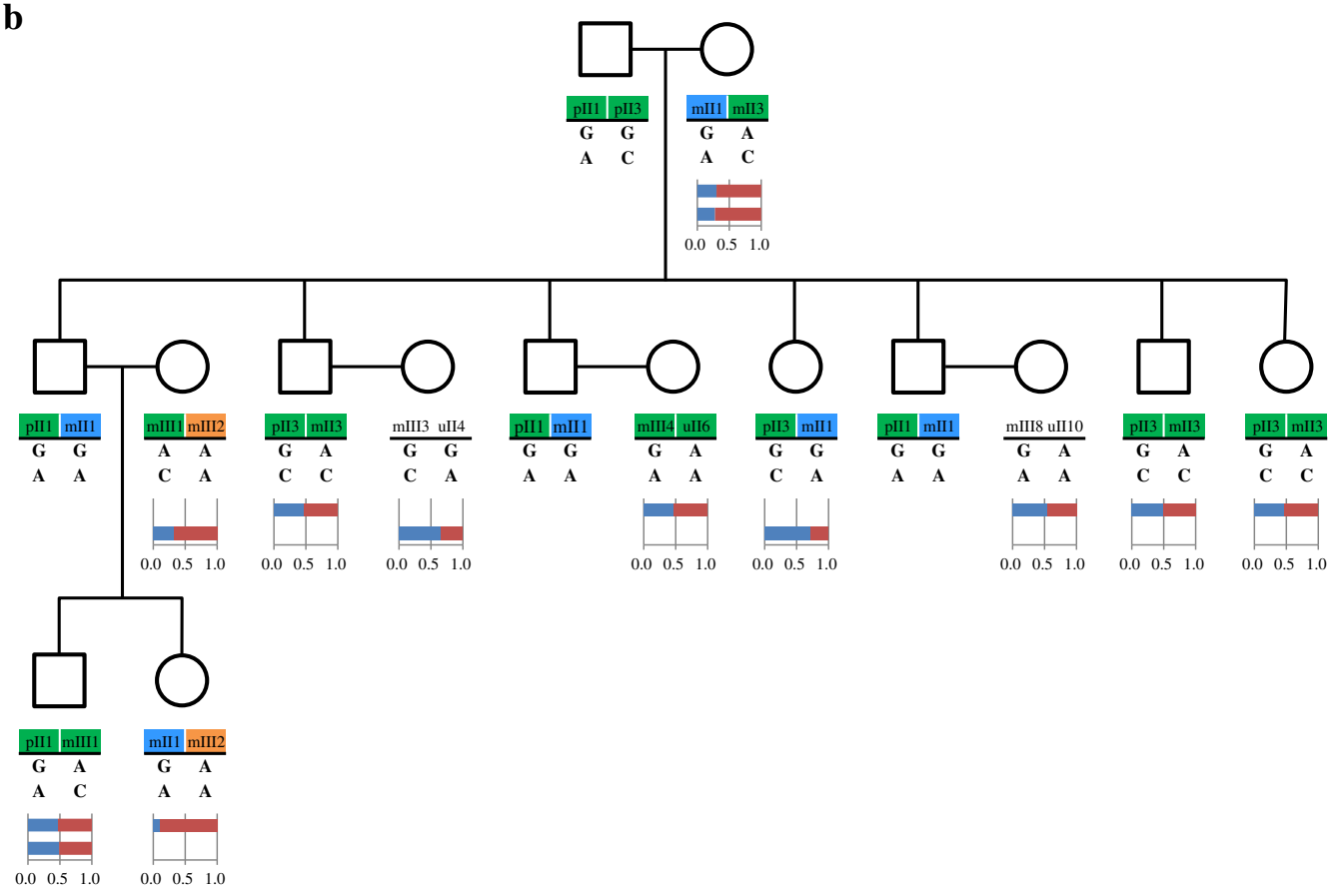
3.4. Regulatory variations of IL6R transcription

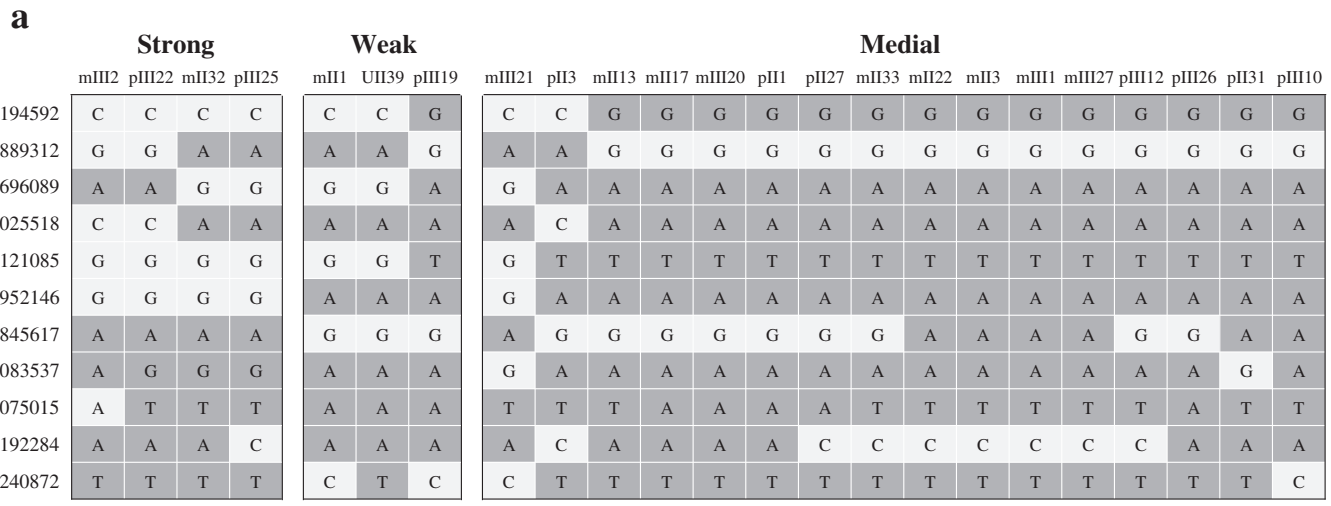
We next tried to delineate the specific variations that can account for the transcriptional strength of each haplotype allele. Of the 23 informative haplotype alleles, those that carry equivalent

a

	pII1	mII13	mII3	mII29	pII3	mII2	mII1	mII17	mII22	mII32	pII10	mII27	pII22	pII26	mII21	uII6	uII19	uII4	uII10
mII8																			1.17
pII14		0.85																	
mII16		1.17															1.5		
mII3																		1.94	
mII4																0.89			
uII39												0.67							
pII19									0.59										
mII21									1.17										
pII20				1.13 1.08															
pII29				0.89															
mII22	1.02																		
pII31	0.96																		
mII20									0.96						1.04				
pII12		1.08									0.92								
pII25	1.22													1.33					
mII1	1.07					0.49													
mII33	0.92													1.13					
pII27	0.92												0.56						
mII1			0.41		0.37	0.12													
pII3	1 0.96 0.96 0.92	1.08 0.96 1	0.92 0.96 0.89				2.7	1.04	1.04	0.43	1.11	0.89							

b





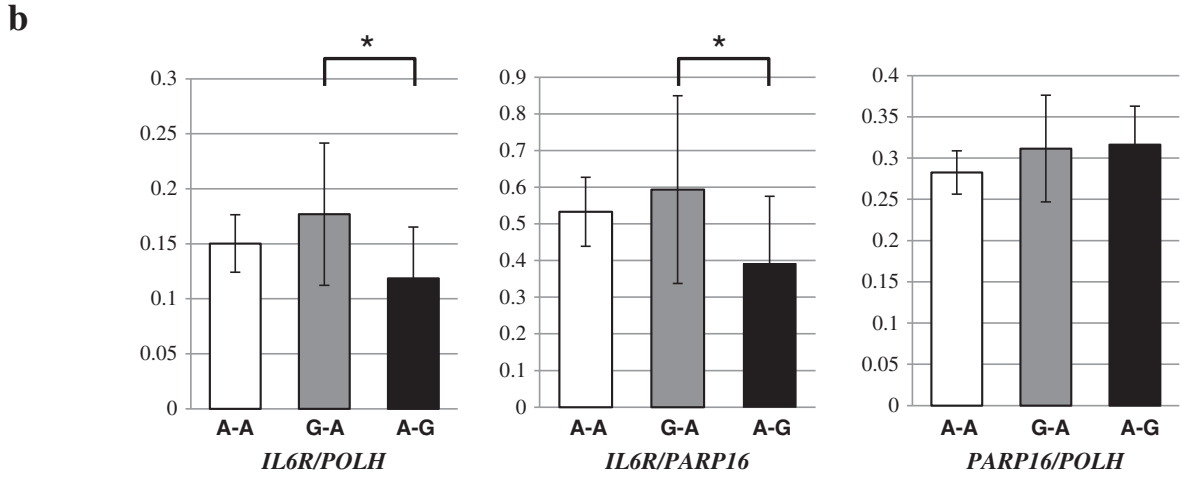


Fig. 4. Identification of regulatory variations and their effect on IL6R transcription. a. Haplotype structures of the same transcriptional strength were aligned to identify the variations that are specific to transcriptional strength. b. The average intergenic relative transcriptional ratios among *IL6R*, *POLH*, and *PARP16* in the lymphocytes are graphically presented based on the haplotype structures that were formed by 2 SNPs, rs952146 and rs4845617. The error bars represent the standard deviations. N: AA, 5; GA, 8; AG, 22. * $p < 0.05$.

transcriptional strength were clustered and aligned together to show the genotypes of all of the tested SNP loci (Fig. 4a). The strong haplotype alleles carried the same genotype in each of 6 SNP loci. The weak haplotypes also presented the 6 SNP loci that carried same genotypes. Notably, 2 of the SNPs among them, rs952146 and rs4845617, exhibited reciprocal genotypes between the strong and weak allelic types. In addition, the haplotype structure of the strong allele that was formed by these 2 SNPs, the G-A haplotype, appeared to be very specific with the only exception in the medial haplotype allele, that being mII21. This observation might confer this haplotype structure as being a functional biomarker that is associated with a high level of *IL6R* transcription.

To address this notion further, we assessed the transcriptional level of *IL6R* in the lymphocytes that were homozygous for these 2 SNPs, which enabled us to associate the transcriptional level with the haplotype alleles that were formed by the 2 SNPs. In order to compare the transcriptional levels in different cell lines, we employed a qcPCR method, whereby we adapted a competitor array (CA) in which the competitor templates for the sequences of *IL6R* and the control genes *PARP16* and *POLH* were arranged to have the same copy number. In this assay, the CA provided a means for measuring the amount of the *IL6R* transcript in relation to the amounts of the control genes [20]. The control genes were selected because their expression levels in 4 different lymphocyte cell lines when measured using an expression microarray were relatively constant, and thus they were expected to be expressed at constant levels in all the tested cell lines. The relative cDNA level of *IL6R* to each control gene are presented in the supplementary table5 and summarized on the basis of the haplotype structures in Fig. 4b. The relative transcript levels of *PARP16* to *POLH* in the tested cells appeared to be relatively constant with the differences in the minimum and maximum values being within a 2-fold range. No association of these values with any of the haplotype structures was evident. On the contrary, the relative transcript levels of *IL6R* to *PARP* (I/P) or *POLH* (I/H) in these cells varied over a wide range up to 6.2 or 4.6 fold of the maximum to minimum value ratios, respectively, while exhibiting a certain level of association with the haplotype structures (G–A vs. A–G, $p = 0.023$ for I/P or $p = 0.011$ for I/H) or with the genotypes of rs4845617 (G vs. A, $p = 0.012$ for I/P or $p = 0.009$ for I/H). This analysis demonstrated that a strong haplotype drives the transcription of *IL6R* 1.5 times more than those that represent both weak and medial haplotypes and thus supports the notion that the haplotype structure of the 2 SNPs or, at least, the genotype of rs4845617 is an important biomarker for predicting the transcriptional level of *IL6R*.

4. Discussion

In this study, we observed an extensive and variable degree of *IL6R* iBAT in immortalized human lymphocytes. The variable allelic

transcription was inherent to the allelic structure of the *IL6R* locus, as determined via a pedigree analysis of allelic transcription, and suggests the operation of multiple *cis*-regulations to varying degrees. Three types of haplotype alleles with respect to the transcriptional strength they bear were identified for the family that we examined. The strong-type alleles invariably carried G and A variants, thus forming the G–A haplotype, in the 2 SNP loci rs952146 and rs4845617, respectively. In addition, the occurrence of the G–A haplotype was exclusive for the strong-type alleles, except for one case in medial-type alleles. The weak-type alleles also exhibited invariable genotypes in several SNP loci but not in an exclusive way: they also appeared in a large fraction of the medial-type alleles.

The association of the G–A haplotype with a higher transcriptional level was further validated by the fact that the lymphocytes that were homozygous for the G–A haplotype exhibited *IL6R* transcription that was 1.5-fold greater than that for lymphocytes that were homozygous for the A–G haplotypes. It should be noted that, since the A–G haplotypes consist of all weak-type and many medial-type alleles in the family samples, the difference of the transcriptional levels between the strongest and weakest alleles could be far greater than 1.5 fold. In our family samples, the difference in transcriptional strength was in the range of 1.2–2.4-fold and 0.4–0.7-fold of the medial-type alleles when strong-type and weak-type alleles were compared to the medial-type, respectively. If no feedback regulation for *IL6R* transcription occurred, this result estimates up to a 10-fold difference in the unstimulated *IL6R* transcription between the lymphocytes depending on the allelic constitutions. However, it is yet to be addressed whether this genetic effect can be extrapolated to other tissues that express *IL6R* or to conditions that lead to *IL6R* expression being stimulated or repressed. In this regard, it is notable that the rs4845617 is located on the exon 1, the region to which DNase hypersensitivity and binding sites for multiple transcription factors were mapped in cells of various origins through the ENCODE project [21].

Our results also indicate that the regulatory variant that is in tight linkage with the G–A haplotype influenced the regulation to allow greater transcription of *IL6R* compared to the other haplotypes. In addition, the effect of the strong-type alleles on transcription likely contributes to a high plasma level of sIL-6R, which is consistent with a previous report [22], and consequently to the risk of developing chronic inflammatory diseases. Together with the SNP rs2228415 affecting the circulating sIL-6R level through the differential susceptibility of two allelic proteins to the shedding, the regulatory variations that differentiate the transcript level would give rise to a complicated but broad and dense spectrum of sIL-6R levels in the population. It would be interesting to delineate the clinical manifestations that combinations of two types of genetic effects will bring out. In this circumstance, our finding provides an additional parameter for making finer measurements of the risk of developing chronic inflammatory diseases.

The medial-type alleles may include those that are formed by the recombination of weak-type and strong-type alleles if the responsible regulatory elements are separate entities. It is therefore less likely that a medial-type allele would have a representative genotype or haplotype. The unavailability of a representative genetic makeup for the weak-type alleles in this study could also be attributed at least in part to multiple regulatory elements having variants that independently reduced the transcriptional rate of the gene. In this case, weak-type alleles would be a mixed population of alleles each of which would carry a transcription-reducing variant in any of multiple numbers of elements. Further analysis by employing a larger family would address this issue.

Genetic variations that are attributed to differential transcriptional rates underlie large parts of the phenotypic variations within and between species [23]. This signifies the need to search for regulatory variations, define transcriptional functions of the sequences that they belong to, and identify their effects on phenotypes. Regulatory variations have been sought using approaches that associate gene expression levels or correlate allelic transcriptions with particular genetic variation(s) by utilizing various platforms such as expression microarrays, SNP chip analysis, and RNA sequencing [17,24]. Each approach has its own merits and limitations. For example, associating expression levels with genetic variation need to control for environmental factors whereas an allelic transcriptional approach generally requires correct phasing of genomic variations and accurate quantitation of allelic transcription [16]. Both approaches can also be compromised through involvement of multiple layers of regulatory variations in gene expression. A good example of this is our attempt to determine the correlation of *IL6R* iBAT with the prevalence of the heterozygous genotypes in the iBAT group, which was not successful (Fig. 2). In this regard, our approach of adapting allelic transcriptional measurements for subjects from a large family is a practical and efficient solution to these limitations. Combined with pedigree analysis, our approach provides strong analytic tools to gain insight into the genetic effects of regulatory variations on the transcription, and to identify the genetic makeups that are in tight linkage with the causes of transcriptional variation.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2013.10.029.

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