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Haplotype analysis of the human α2-HS glycoprotein (fetuin) gene

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SUMMARY

Alpha2-HS glycoprotein (AHSG), which is equivalent to fetuin in other species, is a protein found in human plasma. AHSG is polymorphic with two common alleles and many variants. To examine the intragenic haplotypes and their diversity at this locus, a contiguous genomic DNA sequence (10.3 kb) was analyzed in 20 samples (40 chromosomes), and haplotypes were determined for 309 subjects. Judging from the aligned nucleotide sequences and the conserved amino acid residues comparing human and chimpanzee AHSG, it was concluded that the type 1 allele is probably older and has evolved into four major suballeles. The type 2 allele was generated from one branch of the type 1 allele. *AHSG*3* and **5* variants were each found to have a single nucleotide change in exon 7, resulting in the change of an amino acid residue from Arg_{299} to Cys and from Asp_{258} to Asn, respectively. It was noted that the *AHSG*3* mutation gives rise to an additional cysteine residue, which possibly affects the conformation of the protein. The *AHSG* gene was found to have a low mutation rate and no apparent recombination events. Furthermore, the detected substitutions were nonhomogeneously distributed at this locus. In particular, four nonsynonymous substitutions were concentrated in the carboxyl-terminal domain.

INTRODUCTION

Alpha2-HS glycoprotein (AHSG) is a 46 kDa plasma protein mainly synthesized by hepatocytes. The molecule consists of two polypeptide chains produced through post-translational cleavage from a single polypeptide by enzymatic proteolysis; the two chains are linked by a disulfide bond (Lee *et al.* 1987; Nawratil *et al.* 1996). AHSG is a member of the cystatin superfamily which includes kininogen and histidine-rich glycoprotein, the genes for which are located in the vicinity of 3q27 (Rizzu & Baldini, 1995). The AHSG molecule has three structural domains, i.e. two cystatin-like domains in a tandem arrangement which are encoded by three exons and a unique carboxyl-terminal domain encoded by the last exon (Falquerho *et al.* 1991). Fetuin, abundant in fetal calf serum, is the equivalent protein in other species (Dziegielewska *et al.* 1990). AHSG/fetuin is a multi-functional protein with an inhibitory effect on insulin receptor tyrosine kinase as a phosphorylation substrate (Auberger *et al.* 1989). AHSG/fetuin binds Ca^{2+} with high affinity, thereby preventing apatite formation in the circulation, and it is concentrated in the extra-

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cellular matrix of the bone marrow (Jahnen-Dechent *et al.* 1997). This function supposedly contributes to bone formation and metabolism. Furthermore, it has recently been revealed that AHSG/fetuin suppresses the production of tissue necrotizing factor by macrophages, thereby serving to protect the fetus (Wang *et al.* 1997).

Since Anderson & Anderson (1977) discovered the genetic variation of AHSG by two-dimensional electrophoresis, extensive polymorphisms originating from a number of alleles have been identified by isoelectric focusing (IEF). Three common phenotypes, AHSG 1, 2–1 and 2, are found in all population groups, and the frequencies of the codominant *AHSG*1* and **2* alleles, which have been used as genetic markers in anthropology and forensic hemogenetics, have been determined (Umetsu *et al.* 1983; Boutin *et al.* 1985). In addition, it has been proposed that the phenotype may involve differences in structural features such as height and bone mineral density due to an influence on physiological development (Dickson *et al.* 1994; Zmuda *et al.* 1998). Furthermore, more than 30 variants have been detected in many ethnic groups. In a previous study, to find nucleotide differences in the two common alleles, we examined the cDNA sequence obtained by the reverse transcription-PCR method, and double amino acid replacements, Thr230(ACG) → Met(ATG) and Thr238(ACC) → Ser(AGC), were detected in the transcript of *AHSG*2* (Osawa *et al.* 1997*a*). However, since the cDNAs did not cover the whole coding region, the search for mutations has not been completed. In the present study, we examined the intragenic haplotypes through analysis of the whole genomic sequence of the *AHSG* gene, including the common alleles and two variants of *AHSG*, and we demonstrate the gene diversity and linkage from an evolutionary aspect.

MATERIALS AND METHODS

Materials

Peripheral blood was obtained from 111 and 198 healthy German and Japanese individuals, respectively. Chimpanzee DNA was obtained from Dr Udono.

AHSG phenotyping

Desialyzed sera were analyzed by IEF using a polyacrylamide gel containing ampholines, pH5–6 (Pharmacia), followed by immunoblotting (Yuasa & Umetsu, 1988).

PCR amplification and sequencing

PCR amplification was performed in a volume of 50 μl from 100 ng of DNA with *Taq* polymerase (Takara). The reaction proceeded in a GeneAmp 9700 cycler (PE Biosystems) through 30 rounds of temperature cycling, at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 1 min. Overlapping segments of the *AHSG* gene were amplified using 14 pairs of oligonucleotide primers including previously reported primers (Osawa *et al.* 1997*a*, 1997*b*) and additional ones, which spanned a total of 10309 bases. After purification by means of a spin column (Qiagen), the PCR product was labeled with the BigDye terminator cycle sequencing kit, followed by sequencing using an ABI PRISM 310 autoanalyzer (PE Biosystems). This contiguous scanning was applied to 20 samples of ten type 1 homozygotes, four type 2 homozygotes, three type 2–1 heterozygotes, one type 3 homozygote and two type 5–1 heterozygotes, in which additional homozygosity was considered to be possible for four variations at nucleotide (nt) positions 1263, 2027, 9291 and 9483. The results obtained for the heterozygotes and the homozygotes were compared. The genomic sequence of the chimpanzee *AHSG* gene was also determined using this system.

Allele frequencies

To estimate the frequency of six suballeles in the common alleles and to examine the occurrence of recombination, seven specific substitutions were examined in all the samples. The nucleotide changes at nt positions 8771 and 9440 corresponding to amino acid (a.a.) positions 230

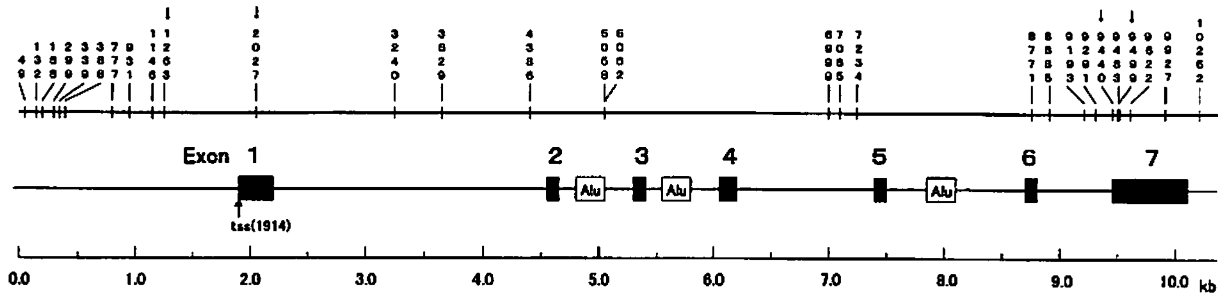

Fig. 1. Sites of variation in the human *AHSG* gene. The vertical number on the top line indicates sites of variation detected through analysis of 20 DNA samples. Arrows indicate the four known sites of variation examined to obtain assumed homozygotes for total sequencing. The structure of the human *AHSG* gene is shown in the middle. The T in the transcription start site (tss) corresponds to nt position 1914 (Banine *et al.* 1998). The nucleotide sequence of *AHSG*1A* as a representative sequence has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB038689.

and 238, respectively, were detected by the restriction fragment length polymorphism (RFLP) method as previously described (Osawa *et al.* 1997*a*). Changes at nt positions 339, 1520 and 2027 were also detected by the RFLP method using *Bst*XI, *Nal*III and *Pvu*II, respectively. To examine two other specific changes, at nt positions 1263 and 9483, we employed the amplified product length polymorphism (APLP) method (Watanabe *et al.* 1997) using mixtures of three oligonucleotide primers as follows, (5′-AGTGCTAGGAGACTCTGGTC-3′, AATGGTTTCTCTTCCTTCTCCAGAA, TCTTCTCTTCCTTCTCCAGAC) and (5′-CATGGGAGTCTGGGGGTGA-3′, AATGAAGCAGTCCCCACA, ACGGTATGAAGCAGTCCCCACC), respectively. After PCR amplification through 38 cycles of 94 °C for 15 s, 54 °C for 10 s and 72 °C for 5 s using AmpliTaq Gold (PE Biosystems), the PCR products were examined by polyacrylamide gel electrophoresis (T = 10%, C = 5%).

Sequence analysis

Multiple sequence alignment was performed using CLUSTAL W version 1.8 (Thompson *et al.* 1994). Nucleotide diversities were estimated by the method of Nei & Li (1979). A phylogenetic network was constructed by the procedures of Bandelt (1994) and Saitou & Yamamoto (1997). The number of nucleotide substitutions per site (d) was estimated by the method of Kimura (1980).

RESULTS

Determination of the haplotypes of common alleles

In previous studies of cDNA and genomic DNA, comparing the two common alleles, we found that the gene encoding AHSG type 1 and that encoding AHSG type 2 each contained several nucleotide variations in addition to those resulting in the two general substitutions at a.a. positions 230 and 238 (Osawa *et al.* 1997*a*, 1997*b*). Samples containing homozygous changes at four such variation sites were selected from the common homozygotes studied. The entire sequence of the *AHSG* gene spanning 10.3 kb was determined in the case of a total of 14 homozygotes and three heterozygotes by PCR-direct sequencing. In the sequences examined, 27 sites of nucleotide variation were found (Fig. 1). Homozygous differences were identified as non-unique polymorphic sites, which accounted for 22 of the sites of nucleotide variation. The other five sites observed in non-coding regions were unique singletons, one of which was an insertion/deletion variation consisting of a nine- or ten-times repeat of T at nt positions 768 to 777.

From the results obtained, we verified that the type 1 and 2 alleles could be divided into four and two distinct suballeles, respectively, and these were designated *AHSG*1A* to **1D* and **2A* and **2B* (Table 1). The difference between *AHSG*1B* and **1C* was a nucleotide substitution

Table 1. *Haplotypes defined on the basis of 24 identified polymorphic sites at the human AHSG locus*

Phenotype (IEF)	1				2		3	5
Allele	**1A*	**1B*	**1C*	**1D*	**2A*	**2B*	**3*	**5*
Position 49	T					G		
132	C			T	—	—	T	
299	C				—	A		
339	G		A					
1146	A					T		
1263	G			T	—	—	T	
1520	G	T	T	T	T	T	T	
2027	C	G	G		—	—		
3240	T	—			C	C		
3629	A	—			G	G		
4386	G			A	—	—	A	
5062	C					A		
6999	C	T	T		T	T		
7085	G	T	T		T	T		
7234	G	A	A	A	A	A	A	
8771	C				T	T		
8885	C	T	T		T	T		
9193	T				C	C		
9291	A	G	G	G	G	G	G	
9440	C				G	G		
9483	C					A		
9499	G							A
9622	C				—	—	T	
10252	C	T	T	T	T	T	T	

'—' indicates the same nucleotide as that in the case of the *AHSG*1A* suballele at this position.
Bold italicized numbers indicate the nucleotide position of substitutions in exons.

Table 2. *Frequencies of AHSG alleles in German and Japanese populations*

	**1A*	**1B*	**1C*	**1D*	**2A*	**2B*	**3*	**5*
Germans ($n = 111$)	0.437	0.131	0.027	0.072	0.045	0.270	0.018	ND
Japanese ($n = 198$)	0.543	ND	0.182	ND	0.091	0.179	ND	0.005

ND, not detected.

at nt position 339. The allele frequencies were estimated by detecting the seven specific nucleotides in the six haplotypes by the RFLP and AFLP methods (Table 2). *AHSG*1A* was predominant and the frequencies were similar in the German and Japanese groups. *AHSG*1B* and **1D*, however, were not encountered in the Japanese population. When the nucleotide diversity was estimated, no distinct differences were observed with the *p*-value being 0.04% in the case of both populations.

Determination of the haplotypes of variants

The specimens examined by IEF analysis included type 3 and 5 variants. We also examined a homozygote of type 3 and two heterozygotes of type 5 1. It was revealed that both contained a single nucleotide substitution in the coding region. In the case of *AHSG*3*, a C to T transition was evident at nt position 9622 in exon 7, which should be accompanied by an amino acid change from Arg to Cys at a.a. position 299. It occurred in the *AHSG*1D* suballele, as shown in Table 1. The *AHSG*5* allele had a G to A transition at nt position 9499 in exon 7 of the *AHSG*1A* suballele, which should result in a change from Asp to Asn at a.a. position 258. All amino acid substitutions detected are summarized in Table 3. In addition, in the case of the null allele (*AHSG*Q0*$_{Accura}$) reported previously (Osawa *et al.* 1999), a splice site mutation was found in the conserved 3′-acceptor site of *AHSG*1B* intron 3.

Table 3. *Nonsynonymous substitutions in the human AHSG gene*

	Amino acid residue (codon)			
	230	238	258	299
*AHSG *1*	Thr (ACG)	Thr (ACC)	Asp (GAT)	Arg (CGC)
*AHSG *2*	Met (ATG)	Ser (AGC)		—
*AHSG *3*				Cys (TGC)
*AHSG *5*			Asn (AAT)	—

' ' indicates the same residue as that in the case of the *AHSG*1* allele at this position.

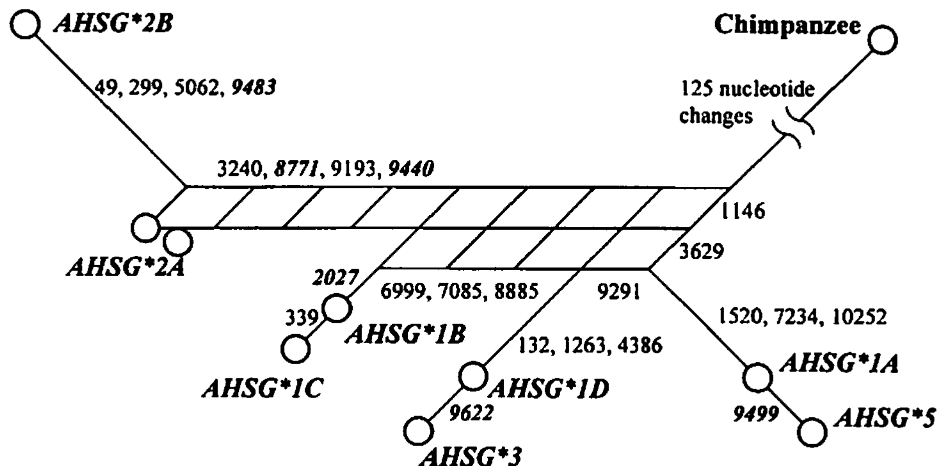

Fig. 2. The estimated phylogenetic network for the human and chimpanzee *AHSG* genes. Filled circles indicate the observed haplotypes whose designations are shown in Table 1. Numbers denote the nucleotide positions in which substitutions were found to occur, and those in bold italics denote substitutions in the coding region. The edge length corresponds to the number of nucleotide differences. Grids indicate possible parallel substitutions as judged from the nucleotides T and G corresponding to nt positions 1146 and 3629, respectively, in the chimpanzee sequence.

Analysis of the chimpanzee AHSG gene

The genomic structure and nucleotide sequence of the chimpanzee *AHSG* gene were determined and compared with the human alleles. The complete sequence, deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number AB038690, differed by 1.3% from the human alleles. In the open reading frame, nine substitutions were evident, seven of which were nonsynonymous at positions corresponding to a.a. residues 3, 5, 34, 53, 249, 293 and 315. The residues at a.a. positions 230 and 238 were conserved, compared to the human type 1 allele.

Evolutionary relationship

The phylogenetic network of the human *AHSG* gene was constructed based on the 24 nucleotide substitution events and the DNA sequence of the chimpanzee *AHSG*, as shown in Fig. 2. It includes two possible parallel substitutions at nt positions 1146 and 3629 that occurred between *AHSG*2B* and the chimpanzee gene, and between *AHSG*2A/B* and the chimpanzee gene, respectively. This phylogenetic network becomes a tree-like structure except for these two substitutions. Through evolution from the chimpanzee, the *AHSG* alleles formed three major branches, *AHSG*1A*, **1B/C* and **1D*, and the type 2 allele continuously diverged from one branch of **1B/C*. No apparent recombination events in the course of human evolution are evident at this *AHSG* locus.

Rate of nucleotide substitutions

The rate of nucleotide substitutions (λ) was estimated for *AHSG*1A*, which is the closest allele, and **2B*, which is the most distant allele,

Table 4. *Rate of nucleotide substitution ($\lambda \times 10^{-9}$) as determined through alignment of AHSG *1A and *2B with the equivalent chimpanzee sequence*

	5′-UTR (1986 bp)	Exons (1104 bp)	Introns (6681 bp)
*AHSG*1A*	1.36 (0.015)	0.73 (0.008)	1.18 (0.013)
*AHSG*2B*	1.27 (0.014)	1.00 (0.011)	1.27 (0.014)

The number in parentheses indicates the nucleotide differences per site (d).

when compared with the chimpanzee sequence. As shown in Table 4, the number of nucleotide substitutions per site (d) was calculated for the three distinct regions, the 5′-UTR, the exons and the introns. Assuming that the time of divergence (T) of humans from chimpanzees was 5.5 million years ago (Kumar & Hedges, 1998), the λ values of the 5′-UTR, the exons and the introns were estimated to be $1.27–1.36 \times 10^{-9}$, $0.73–1.00 \times 10^{-9}$ and $1.18–1.27 \times 10^{-9}$, respectively, by the formula $\lambda = d/2T$.

DISCUSSION

The *AHSG* locus is polymorphic with approximately 30 alleles, and at least *AHSG*1* to **15* have been assigned on the basis of the position to which the AHSG protein migrates in IEF (Yuasa & Umetsu, 1988). The extended polymorphism consists of two common alleles and a number of unique variants that serve as markers of certain ethnic groups. The development of molecular techniques has made elucidation of the polymorphic diversity possible by enabling the identification of the nucleotide substitutions involved. Analysis of a contiguous sequence of 10.3 kb including noncoding regions revealed that the two common alleles were apparently heterogeneous and consisted of two or four distinct suballeles. We also determined the genomic DNA sequence of the chimpanzee *AHSG*/fetuin gene by the same techniques. Our findings clearly demonstrate the linkage and evolution of the locus. Judging from the conserved Thr residues at a.a. positions 230 and 238 of the deduced amino acid sequence of the chimpanzee *AHSG*, the type 1 allele appears to be the ancestral gene in humans, and it has evolved in three major directions with divergence to the major type 2 allele. While the more recently generated type 2 allele is widespread, it is unknown if this is the consequence of any selection. In addition, the nucleotide diversity of the *AHSG* locus was calculated to be 0.04% for both the German and Japanese populations, indicating that there is no apparent difference in allele distribution between these two populations.

Rare allelic variants have a highly restricted distribution. We identified nucleotide substitutions in two such variants for which DNA was available. Both were generated relatively recently, each through a single nucleotide substitution in the coding region of the original allele. Type 3 is characterized as an anodally shifted band in IEF, and is found in Caucasians (Weidinger *et al.* 1984). The detected substitution in exon 7 should be accompanied by an amino acid change from Arg_{299} to Cys. This molecule becomes a reactive free-sulfhydryl-containing protein in addition to showing a charge alteration. This may have important effects on the conformation of the molecule, which is maintained by six disulfide bonds, and on its function (Kellermann *et al.* 1989; Araki *et al.* 1989).

We estimated the rate of nucleotide substitutions (λ) as shown in Table 4. Li *et al.* (1985) estimated the rates of nonsynonymous and synonymous substitutions in protein-coding regions as being 0.88×10^{-9} and 4.65×10^{-9}, respectively, based on 42 mammalian genomic sequences. They also estimated λ values of the 5′-UTR and intron regions as being 1.74×10^{-9} and 3.70×10^{-9}, respectively, comparing more than 10 genes in humans and rodents. Our estimates were lower than those, indicating that AHSG is a protein with a conserved sequence. AHSG/fetuin obviously plays an important role in life.

Although the lack of fetuin is not lethal, as determined through studies of null mutant mice (Jahnen-Dechent *et al.* 1997), no complete AHSG deficiency has been found in humans even in extensive population studies and clinical investigations. Among six substitutions that we detected in the coding region, all four nonsynonymous substitutions at a.a. positions 230, 238, 258 and 299 and the synonymous one at a.a. position 252 are concentrated in the carboxyl terminal domain. Comparisons of the AHSG/fetuin sequences of various mammalian species have revealed that there are fewer conserved amino acid residues in the amino-terminal portion of the terminal domain (Brown *et al.* 1992). This nonhomogeneous distribution of sites of variation suggests that a mutational hotspot is present within the region encoding the carboxyl-terminal domain of AHSG, whereas the region encoding the cystatin domains is rarely subject to mutations.

Molecular analysis of genes coding for various proteins is in progress to search for polymorphic markers of use in studies of disease and evolution. Such analysis of the genome will provide further insight into the origin and evolutionary history of various proteins in humans.

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