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Determination of genomic breakpoints in an epileptic patient using genotyping array

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Abstract

Recent advances in DNA microarray technology have enabled the identification of small alterations throughout the genome. We used standard karyotype analysis, followed by DNA microarray analysis and PCR to precisely map the chromosomal 4p deletion and determine the deletion breakpoints in the genome of an epileptic patient. The karyotype of the patient was 46,XY,del(4)(p15.2p15.3) as determined by G-banding analysis. We used a high-density oligonucleotide genotyping array to estimate the size of the deletion (4.5 Mb) and to locate the breakpoints within a 9-kb region on one side of the deletion and a 100-kb region on the other side. We amplified by PCR and sequenced the genomic region encompassing the breakpoints, and mapped the deletion to regions extending from 21648457 to 26164287 and from 26164505 to 26167493, respectively (chromosome 4 of NCBI Homo sapiens Genome Build 35.1). The deletion involves 18 genes, one of which (*CCKAR*) is partially deleted.

Keywords: Genomic breakpoints; DNA microarray; PCR; Epilepsy

Recent advances in DNA microarray technology have enabled the identification of small alterations throughout the genome. The DNA microarray techniques have been used to analyze gene dosage in cancer and congenital disorders [1–4]. The role of large-scale copy number variations in the evolution of the human genome has become a focus of research attention because of their recent discovery in the genome of healthy individuals [5,6]. Genome-wide screening for DNA copy number changes can be carried out by array-based comparative genomic hybridization. Its highest map resolution is less than 100 kb, with approximately 30,000 arrayed BAC clones spanning the entire human genome [7]. Another genome-wide DNA screening approach uses high-density oligonucleotide microarrays, originally designed to genotype more than 10,000 SNPs in the entire human genome [8–10]. These array analyses have some clear advantages. They follow simple protocols that are easily reproducible. Moreover, analyses can be easily carried out with established assay systems that are commercially available. However, copy number analysis can be complicated by poor signal-to-noise ratio caused by various experimental biases. Although several effective algorithms/software for copy number analysis have been reported [11–13], the currently available ones do not support the analysis using the new oligonucleotide array, namely, GeneChip Mapping 500K Set. Here, we report a simple method that eliminates background noise from data obtained by the oligonucleotide microarray analysis of gene dosage. We have used this method to locate deletion breakpoints in the genome of an epileptic patient.

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Materials and methods

Clinical case report. The patient was the second of three children. The parents and the other children were phenotypically normal. The infant was born at 40 weeks gestation, did not demonstrate asphyxia, and weighed 2250 g. The multiple minor congenital anomalies observed at birth were intestinal adhesion, atrial septal defect, pulmonary stenosis, and cryptorchidism. These anomalies were operated on until the patient was 10 years old.

The patient was diagnosed with mental retardation (MR) at 2 years of age. Owing to this, he received elementary and junior high schooling at a class for the handicapped (TIQ 40; based on WAIS-R, examined at 17 years of age). After graduation from junior high school, he easily engaged himself in dairy work that was a part of the family business. His language skills were always delayed. The first episode of complex partial seizures (CPS) was at 16 years of age; these were accompanied with oral automatism, motion arrest, and gelastic seizures. These epileptic episodes included a simple partial seizure (SPS) along with auditory hallucinations.

The patient's first medical contact with Miyazaki Medical College Hospital was when he was 16 years old. On physical examination, his height was recorded as 164 cm, and his weight was 48 kg. He had myopia and a distinct triangular face with hypoplastic malar areas, forehead, and low jaw. These CPS and SPS seizures occurred at a frequency of 1–2 seizures per week and were not controllable with valproic acid (VPA) monotherapy (600–800 mg/day); however, a combination therapy of clobazam (CLB; 10 mg/day) and VPA, i.e., a CLB+VPA combination therapy decreased the frequency of the seizures to 1–2 per month. Written informed consent was obtained from the patient and his parents. This study was approved by the Ethics Committees of the Miyazaki Medical College, the Medical School of Hirosaki University, and the RIKEN Yokohama Institute.

Twenty milliliters of non-fasting venous blood was collected from the subject, and genomic DNA was prepared from isolated peripheral leukocytes according to standard protocols.

Cytogenetic analysis. High-resolution chromosome analysis was performed on methotrexate-synchronized peripheral blood lymphocytes, using G-banding with trypsin-Giemsa [14].

Microarrays. Genome-wide DNA screening on Affymetrix GeneChip Mapping 250K Sty EA array and Mapping 50K Hind array was performed using the GeneChip Instrument system according to the standard protocols from the manufacturer (Affymetrix, Santa Clara, CA, USA). Briefly, Genomic DNA was digested with restriction endonuclease, ligated to an adaptor, and subjected to PCR amplification with adaptor-specific primers. The PCR products were digested with DNase I and labeled with a biotinylated nucleotide analogue using terminal deoxynucleotidyl transferase. The labeled DNA fragments were hybridized to the microarray, the hybridized DNA probes were captured by streptavidin–phycoerythrin conjugates, and the array was scanned.

Calculation of signal intensity on DNA microarrays. Signal intensity (SI) for each probe set was calculated as follows: SI from the perfect match probe for the A allele (PA) added to SI from the perfect match probe for the B allele (PB) minus SI from the mismatch probe for the A allele (MA) added to the SI from the mismatch probe for the B allele (MB):

$$\mathrm{SI} = (\mathrm{PA} + \mathrm{PB}) - (\mathrm{MA} + \mathrm{MB}).$$

The reference signal intensity (SIR) for each probe set was calculated using SI from five arrays hybridized with DNA from individuals not affected by epilepsy, as

$$\mathrm{SIR} = \mathrm{Mean}\Big(\sum(\mathrm{SI}) - \mathrm{Max}(\mathrm{SI}) - \mathrm{Min}(\mathrm{SI})\Big).$$

The relative signal intensity (RSI) from hybridized DNA of the subject with epilepsy was calculated for each probe as

$$\mathrm{RSI} = \mathrm{SIS}/\mathrm{SIR},$$

where SIS denotes subject SI.

The RSI for each SNP from arrays hybridized with DNA of the subject (RSIS) was then calculated as

$$\mathrm{RSIS} = \mathrm{Mean}\Big(\sum(\mathrm{RSI}) - \mathrm{Max}(\mathrm{RSI}) - \mathrm{Min}(\mathrm{RSI})\Big).$$

Each RSIS value was mapped onto the NCBI Homo sapiens Genome Build 35.1 according to the physical position of the corresponding SNP. To facilitate the analysis, we also calculated a moving average of RSIS (MARSIS) across five adjacent SNPs as:

$$\mathrm{MARSIS} = \mathrm{Mean}\Big(\sum(\mathrm{RSIS}) - \mathrm{Max}(\mathrm{RSIS}) - \mathrm{Min}(\mathrm{RSIS})\Big).$$

PCR and sequencing analysis. PCR primers were designed to amplify DNA at 2-kb intervals in the genomic breakpoint region. The genomic region encompassing the breakpoints was PCR-amplified with combinations of the different primers. The amplified DNA was sequenced using the BigDye Terminator cycle sequencing kit and an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA, USA), and the sequence was compared with the human genome sequence using the BLASTN program [15].

Results

Cytogenetic analysis

Banding analysis of the patient's chromosomes showed 46 chromosomes and a deletion of chromosome 4p. The karyotype was initially designated as 46,XY,del(4)(p15.2p15.3) (Fig. 1).

Microarray analysis and localization of breakpoints

Genome-wide DNA screening to define the region encompassing the breakpoints was performed by microarray analysis. Upon mapping the RSIS value onto the NCBI Homo sapiens Genome Build 35.1, we identified a region of weak signal from 4p15.2 to 4p15.3, which matched the karyotyping data (Fig. 2). We estimated the deletion to span 4.5 Mb and localized the breakpoints within a 9-kb region on one side and a 100-kb region on the other side. We amplified the genomic region encompassing

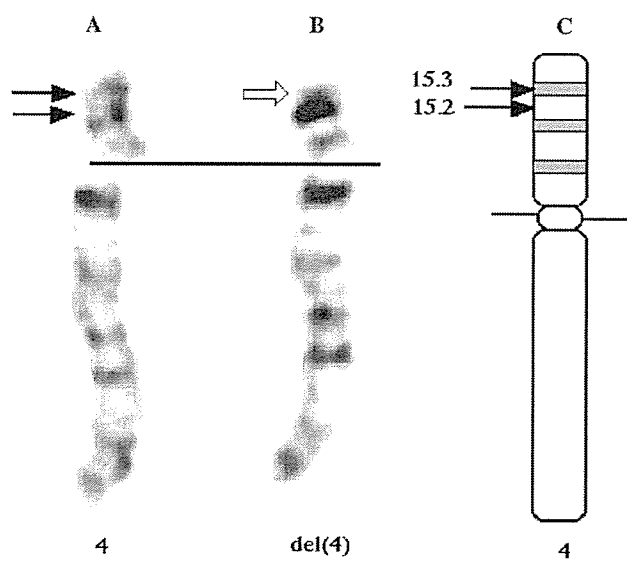

Fig. 1. G-banded chromosomes showing the deletion in chromosome 4 on the right (B) and the normal homolog on the left (A). This region is indicated with arrows. (C) is the ideogram of chromosome 4.

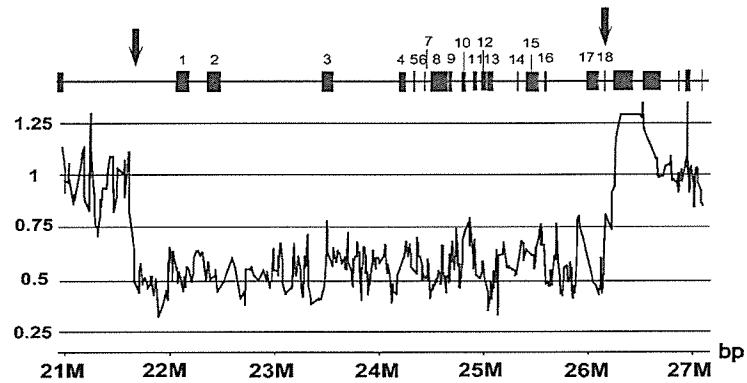

Fig. 2. A 4.5-Mb deletion in chromosome 4 identified by microarray analysis. The moving average of the RSIS (see the Materials and methods) across five adjacent SNPs obtained from Mapping 250K Sty EA array analysis was mapped onto the NCBI Homo sapiens Genome Build 35.1 according to the physical position of the corresponding SNP. Each RSIS value was normalized such that their average was 1. Closed rectangles represent the genes. The numbers on the rectangles correspond to the gene numbers in Table 1. The arrows indicate the break points determined by sequencing analysis.

the breakpoints by PCR, using combinations of primers designed for amplification at 2-kb intervals in the genomic breakpoint region (4 primers in the 9-kb region and 50 primers in the 100-kb region). We could amplify the relevant genomic region with two primer combinations, f4 (5′-tgcacactgaacttgtcactgc-3′) and r41 (5′-ctggcacttatgatgcactcg-3′), and f4 and r42 (5′-acactctattgagtcaatcgttc-3′). The two PCR products differed by 2 kb (f4-r41 was 7 kb and f4-r42 was 5 kb). The sequence of the amplified genomic region was compared with the corresponding human genome sequence using the BLASTN program. The regions deleted spanned DNA sequences from 21648457 to 26164287 and from 26164505 to 26167493 in chromosome 4 of NCBI Homo sapiens Genome Build 35.1. The deletion involves 18 genes, one of which (*CCKAR*) is deleted partially (Table 1). The region between the deletions (from 26164288 to 26164504 in chromosome 4), which remains intact in the affected subject, is located in the second intron of the *CCKAR* gene, whereas the second deleted region (26164505–26167493) involves the second exon of that gene. As a result, only the first exon of the *CCKAR* gene is unaffected by the deletion in chromosome 4 (Fig. 3).

Discussion

We have delineated the deletion in the genome of an epileptic patient suffering from complex partial seizures, hence generating a list of possible candidate genes involved in the pathogenesis of epilepsy (Table 1). Among the 18 genes listed that are located within the deleted region, the *LGI2* gene is one of the strongest candidates because it belongs to the same family as *LGI1*, a gene implicated in epileptogenesis [16–21]. Members of the LGI1 family are predicted to be secreted proteins and consist of an N-terminal leucine-rich repeat region and a C-terminal EAR (epilepsy-associated repeat) region [21,22]. Kalachikov et al. [18] and Morante-Redolat et al. [19] found that mutations in the *LGI1* gene are responsible for autosomal dominant lateral temporal lobe epilepsy. However, a phenotype consistent with this type of epilepsy, as in our patient (data not shown), is

Table 1
Genes involved in the deleted regions

Number	Gene symbol	Cyto	Gene name
1	GPR125	4p15.31	G protein-coupled receptor 125
2	GBA3	4p15.31	Glucosidase, beta, acid 3 (cytosolic)
3	PPARGC1A	4p15.1	Peroxisome proliferative activated receptor, gamma, coactivator 1, alpha
4	DHX15	4p15.3	DEAH (Asp-Glu-Ala-His) box polypeptide 15
5	ATP5LP3	4p15.2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit g, pseudogene
6	LOC391640	4p15.2	Similar to heterogeneous nuclear ribonucleoprotein A1, isoform a
7	SOD3	4p16.3-q21	Superoxide dismutase 3, extracellular
8	DKFZp761B107	4p15.2	Hypothetical protein DKFZp761B107
9	LGI2	4p15.2	Leucine-rich repeat LGI family, member 2
10	SLA/LP	4p15.2	Soluble liver antigen/liver pancreas antigen
11	PI4K2B	4p15.2	Phosphatidylinositol 4-kinase type-II beta
12	ZCCHC4	4p15.2	Zinc finger, CCHC domain containing 4
13	ANAPC4	4p15.2	Anaphase promoting complex subunit 4
14	SLC34A2	4p15.3-p15.1	Solute carrier family 34 (sodium phosphate), member 2
15	KIAA0746	4p15.2	KIAA0746 protein
16	LOC389203	4p15.2	Hypothetical gene supported by BC032431
17	RBPSUH	4p15.2	Recombining binding protein suppressor of hairless (*Drosophila*)
18	CCKAR	4p15.1-p15.2	Cholecystokinin A receptor

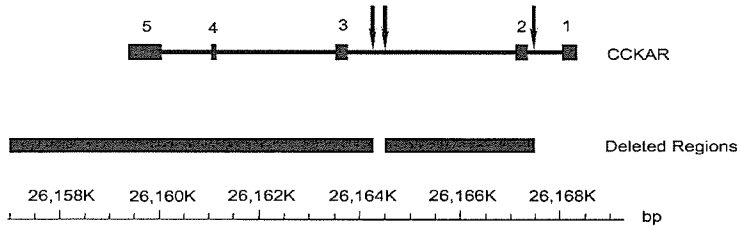

Fig. 3. Schematic diagram of the *CCKAR* gene showing the location of the deleted regions. Closed rectangles, exons; horizontal lines between closed rectangles, introns; thick horizontal lines, deleted regions. The arrows indicate the break points determined by sequencing analysis.

not always associated with identifiable mutations in *LGI1*, and dysfunction in other members of the LGI1 family including *LGI2* could also be responsible for epileptogenesis in patients with this type of epilepsy. Further research on epileptic patients is required to evaluate whether other *LGI1* family members are implicated in epileptogenesis. It should also be noted that the functions of some of the genes comprised in the deleted region are still not understood, and their absence might be epileptogenic.

Using the GeneChip Human Mapping 250K Sty EA Array and the Mapping 50K Hind array, which are capable of genotyping on average 300,000 SNPs, we could locate the genomic breakpoints within a 9-kb region on one side of the deletion and a 100-kb region on the other side. We can now also use the Mapping 500K Array Set, a SNP typing system which comprises two arrays (250K Sty and 250K Nsp), to genotype an average of 500,000 SNPs. This system allows genome-wide screening for copy number alterations with about 6-kb resolution, a size small enough to be amplified by PCR. As the procedures that follow (PCR and DNA sequencing) are rather simple, we can complete the entire analysis within a two-week period. Using this method, we were able to identify a 1.4-Mb deletion and locate its breakpoints in one subject and trisomy 21 in another subject.

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ORIGINAL ARTICLE

Analysis of growth hormone receptor polymorphism in Japanese semisuper centenarians

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Background: Recent studies have demonstrated a significant association between mutations in genes involved in the GHR/IGF1 signaling pathway and extension of the lifespan of model organisms. Exon 3 insertion or deletion is one common polymorphism in the growth hormone receptor (*GHR*) of humans. The exon 3 deletion allele is reported to have stronger signaling in the GH/GHR pathway, which may correlate to short lifespan.

Methods: We investigated the common polymorphic variation in 119 Japanese semisuper centenarians (SSC; older than 105) compared with 104 healthy younger controls via the polymorphism-based polymerase chain reaction method.

Results: The frequency of exon 3 deletion variation of *GHR* in SSC was found to be higher than controls, although this was not significant statistically. Also, the single nucleotide polymorphism genotype frequency and allele frequency exhibited no differences between SSC and controls.

Conclusions: These results show that SSC in Japan do not tend to have the allele of GHR, which has a lower signaling capacity.

Keywords: aging, centenarian, gene polymorphism, GHR, longevity.

Introduction

Human growth hormone (GH) is produced and secreted by the anterior pituitary and has long been known to be an important determinant of bone and soft tissue growth, influencing carbohydrate, lipid and protein metabolism throughout the entire lifespan.[1–3] Recent research continues to reveal the potential role of GH in the regulation of aging and immunity. The therapeutic uses of GH are expanding beyond treatment for short children and adult GH deficiency. Human recombinant GH is marketed to the public as a substance that reverses various symptoms of aging while results of aging research in lower animals and patients suggest that GH deficiency extends lifespan.[4]

The effects of GH are mediated via the GH receptor (GHR), a single membrane-spanning protein that belongs to the large family of cytokine receptors. Part of the GHR corresponding to its extracellular domain is cleaved and present in the circulation as GH binding protein (GHBP). The signaling of GH/GHR contains one molecule of GH binding to two molecules of receptors sequentially, activation of Janus kinase 2 and downstream signal pathways followed by increases in

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the expression of insulin-like growth factor 1, which mediates the indirect effect of GH and other hormone-dependent genes. Studies in genetically-altered mice have provided strong evidence that alterations in GH signaling and the following insulin-like growth factor (IGF)-1 production can delay aging and prolong life.[5] It has been reported that homozygous *GHR/BP* gene-disrupted mice, the mouse model for Laron syndromes, grow more slowly, have a nearly 1-year increase in lifespan with a reduced IGF-1 level, and are significantly smaller than their heterozygous and normal partners.[6,7]

The human *GHR* gene is a single copy gene that spans 300 kb of chromosome 5. It contains nine coding exons (2–10) (Fig. 1): exons 2–7 encode the extra cellular domain; exon 8 encodes the single transmembrane domain; and exons 9 and 10 encode the intracellular domain. There are two isoforms in the human transcripts of GHR differing in exclusion or retention of exon 3 during splicing, a full-length isoform *GHRfl* and exon 3 exclusion isoform *GHRd3*, whereas in lower animals only GHRfl is expressed. A recent investigation revealed that the responsiveness to GH therapy is much different across genotypes among short children. GHRd3 allele was associated with 1.7–2.0 times more growth acceleration than full-length isoform and its signaling transduction was 30% higher than full-length homodimers.[8] This indicates that the polymorphism in exon 3 of *GHR* is important in growth hormone physiology.

In an attempt to understand the role of GHR in longevity, we examined 114 Japanese semisuper centenarians (SSC; older than 105) compared to 104 healthy younger controls.

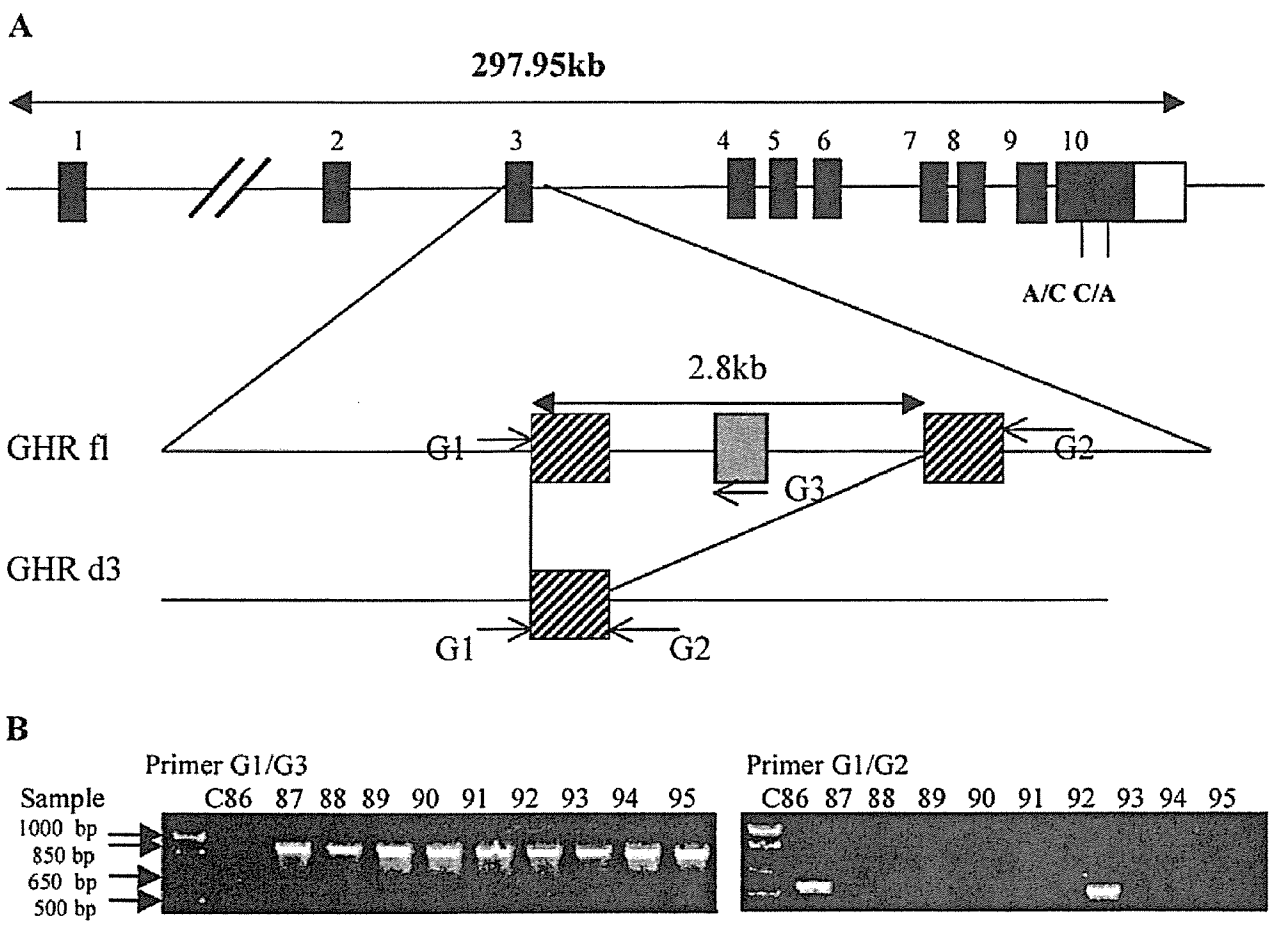

Figure 1 Genomic organization of the growth hormone receptor (GHR) and exon 3 region, and electrophoresis of polymerase chain reaction (PCR) amplified segments. (A) Indel variation in GHR. Black block with numbers indicate exons and block with dashes indicate repeat sequence around exon 3. Arrows with character G1–3 indicate the PCR primers to detect the variation of exon 3. GHRfl, full length with exon 3; GHR d3, GHR with exon 3 deletion. A/C and C/A are two single nucleotide polymorphism (SNP) sites in exon 10. (B) Agarose electrophoresis. The length of 934 bp indicates fl while 532 bp indicates d3 allele. Samples of C86, d3/d3; C87-91, C93-95, fl/fl; C92, fl/d3.

Materials and methods

Subjects

A total of 119 Japanese SSC aged 105–110 (104 female, 15 male; mean age 106.81) were recruited in 2002 to participate in this study. Forty-six SSC were living at home and 76 were institutionalized. None were in an acute care situation, and none were receiving tube feeding. Past disease history of the SSC was: 27 coronary artery diseases; 38 strokes; four diabetes and 19 cancers. The control subjects comprised 104 healthy volunteers aged 20–59 (33 female, 71 male; mean age 29.67) recruited from hospital and institutional workers, medical and nursing school students, and bank clerks. The control subjects were free from diseases and disease history such as coronary artery disease, stroke, diabetes and cancer. All subjects enrolled in this study were Japanese. Twenty milliliters of non-fasting venous blood was collected from all subjects, and genomic DNA was prepared from peripheral leukocytes according to standard protocols. Written informed consent was obtained from all participants directly or by proxy. This study was approved by the ethics committees of the medical school of Keio University.

Genotyping

Human genomic DNA were isolated from 50 μL of peripheral blood using GenElute Mammalian Genomic DNA Miniprep Kit (Lot 014K6019, Sigma Aldrich, Munich, Germany) and then amplified by the GenomiPhi DNA amplification Kit (Lot 309403, Amersham Biosciences, Piscataway, NJ, USA). To determine genotype of *GHR* exon 3, we applied one revised polymerase chain reaction (PCR)-based assay method.[9] The pair of primers G1 (5′-GTTGGTCTGCTGGTCT GCTT-3′) and G3 (5′-GTGCTCTGCTAAGGATAGC TG-3′) allowed amplification of the exon 3 allele only, whereas primers G1 and G2 (ACTTTAGCCAGTCGT TCCTG) amplified *GHRd3* allele under specific conditions thereby discriminating the three possible alleles in this locus (i.e. homozygous of *GHRfl/fl*, *GHRd3/d3* and combination heterozygous *GHRfl/d3*) (Fig. 1a). Parameters of PCR were the initial step of 5 min at 94°C, followed by 35 cycles consisting of 30 s at 94°C, 30 s at 55°C, 1 min 30 s at 72°C, and the last extension of 5 min at 72°C. The expected length of 934 bp indicates the *GHRfl* allele and 532 bp indicates the *GHRd3* allele. Primer sets of single nucleotide polymorphism (SNP) in exon 10 (1627A/C) were TCTGTGAGGCAGATGCC AAAAAATG and GAGAAACTCTTTGTCAGGCAA GGGC. PCR cycles were as follows: initial denaturation at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min; annealing at 60°C for 1 min; extension at 72°C for 2 min; and a final extension of 72°C for 5 min. Fragments were automatically sequenced by a capillary sequencer (Beckman Coulter Fullerton, CA, USA).

Statistical analysis

The Hardy–Weinburg equilibrium was applied to evaluate the representation of samples from the local population who met the genetically or inherited stable equilibrium when $P > 0.05$. The χ^2 test was performed in the comparison of allele frequency and genotype frequency between SSC and control subjects using SPSS software version 10.5. A value of $P < 0.05$ was accepted as the level of significance. We estimated linkage disequilibrium (LD) as $D = x11\text{-}p1q1$, where $x11$ is the frequency of haplotype A1B1, and $p1$ and $q1$ are the frequencies of alleles A1 and B1 at locus A and B, respectively. A standardized LD coefficient, r, is given by $D/(p1p2q1q2)1/2$ where $p2$ and $q2$ are the frequencies of the other alleles at locus A and B, respectively.[9] Lewontin's coefficient D' is given by $D/D\text{max}$, where $D\text{max} = \min[q1p2, p1q2]$ when $D > 0$.[10,11] Haplotype frequencies for multiple loci were estimated by the expectation–maximization method.

Results

Hardy–Weinburg equilibrium

Our 223 subjects were distributed in or around Tokyo, Japan. We assessed the quality of the genotype data for the Hardy–Weinburg equilibrium in these samples using the χ^2 test. No significant deviation from the Hardy–Weinberg equilibrium was observed for this indel polymorphism in the study (data not shown).

Allele and genotype frequency in Japanese centenarians and younger controls

The genotype frequency distribution at exon 3 of *GHR* was studied in the two groups (Table 1). The subjects of homozygous exon 3 deletion in SSC and controls were 6 and 2, respectively. The subjects of heterozygous exons deletion in SSC and controls were 27 and 20, respectively. These results indicate that SSC have a tendency to delete exon 3. However, statistically there were no differences in the distribution of full-length (fl/fl), exon 3 deletion allele (d3/d3) or both (fl/d3) between centenarians and younger controls ($P = 0.143$). These results indicate that, contrary to lower animals, GHR signaling in SSC may be higher than in the control group.

Next, we extended our research to include whether other loci of *GHR* polymorphisms were different between SSC and controls. We first chose the SNP locus, which might have a low level of LD. Two SNP sites exist (1627A/C and 1734C/A) in exon 10 by ensemble database (Fig. 1). However, the 1734A/C site did not fit this analysis because one allele frequency was very low in our samples (data not shown). We therefore chose to analyze 1627A/C SNP. The LD between exon 3

Table 1 Allele and genotype frequency among semisuper centenarians (SSC) and control

Genotype frequency	SSC	Control	X^2	P
(1) Exon 3				
fl/fl	0.723 (86)	0.789 (82)		
fl/d3	0.227 (27)	0.192 (20)		
d3/d3	0.050 (6)	0.020 (2)		
Total	1.000 (119)	1.000 (104)	2.139	0.333
(2) 1627A/C				
A/A	0.198 (20)	0.242 (23)		
A/C	0.465 (47)	0.463 (44)		
C/C	0.337 (34)	0.295 (28)		
Total	1.000 (101)	1.000 (95)	0.706	0.703
Allele frequency	SSC	Control		
(1) fl	0.836 (199)	0.885 (184)		
d3	0.164 (39)	0.115 (24)		
Total	1.000 (238)	1.000 (208)	2.151	0.143
(2) A	0.431 (87)	0.474 (90)		
C	0.569 (115)	0.526 (100)		
Total	1.000 (202)	1.000 (190)	0.731	0.393

P-value < 0.05 was considered statistically significant.

and 1627, D′ is 0.417 and r^2 is 0.026. As shown in Table 1, both genotype frequency and allele frequency showed no differences between SSC and controls. These results indicate that at least SSC in Japan exhibit no tendency to have the allele of *GHR*, which have a lower signaling capacity.

Discussion

In order to learn genetic associations involved in human longevity, the SNP analysis provide some clue. Here, we analyzed SSC subjects of more than 105 years old. SSC people are the uppermost age group at the moment, although the number is limited for statistical analysis. We found a higher frequency of homozygous exon 3 deletion of *GHR* in SSC than in controls. These results were unexpected, because exon 3 deletion of *GHR* has a stronger signaling capacity than full *GHR*. Transfection experiments have shown that homozygous exon 3 deletion has 30% higher signaling than full-length carriers.[8] Our results contradict those of the mouse model. In mouse models, the lifespan of the Laron mouse ($GHR^{-/-}$) was extended 37–55%.[5] What causes this discrepancy? The $GHR^{-/-}$ mouse correlates to the human Laron syndrome, which is different from SSC. There are no differences between SSC and controls in other parts of the SNP of *GHR* (Table 1). These results show that at least SSC in Japan show no tendency to have the allele of *GHR*, which has a lower signaling capacity. Because longevity is a long-term complex process, it is highly likely that some mechanisms compensate for the differences in the effect of signaling generated by *GHR* exon 3 variants. This signaling variation might act as a temporary event in response to a short-term stimulus free from involvement in the long-term goal.

Biological functions of GH can be directly or indirectly mediated through IGF-1 that is produced in many organs (mostly in the liver) in response to growth hormone.[12] In studies on the aging process using lower organisms like *Caenorhabditis elegans* and yeast, mutants of daf-2 homologue IGF-1 receptor (*IGF-1R*), have been found able to extend their lifespans.[13] A current investigation into polymorphic variants of *IGF-1R* in Italian populations indicates the potential association of one specific allele of IGF-1R with longevity accompanied by a lower level of plasma IGF-1.[14] In Japanese populations, one insulin-like receptor (INSR) gene haplotype was more frequent in the same SSC subjects used in this paper.[15] Our results here suggest that the GHR in humans might not have a significant effect on longevity, although the sample size is rather small to make the conclusion statistically. Taken together in humans, IGF-1R/INSR but not GHR may have a relation to longevity.

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Original Article

Interaction between Serotonin 2A Receptor and Endothelin-1 Variants in Association with Hypertension in Japanese

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Serotonin has been implicated in the pathogenesis of hypertension because of its ability to induce vasoconstriction *via* stimulation of serotonin 2 (5-HT2) receptors. Recently, an association between the T102C functional polymorphism of the serotonin 2A (5-HT2A) receptor gene and hypertension in the UK has been reported. Another association study, however, failed to replicate this association in a Chinese population. We therefore investigated the possible association between the 5-HT2A T102C polymorphism and hypertension in two large Japanese populations (n=2,968 total). We also investigated the possible interaction between the 5-HT2A T102C polymorphism and the G/T (Lys198Asn) polymorphism of the endothelin-1 (ET-1) gene, based on robust biological evidence for the existence of an interaction between the serotonin and endothelin systems. The results showed that there was no significant difference in the frequencies of the alleles and genotypes between the hypertensive and normotensive subjects. However, a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension (p=0.0040) and with diastolic blood pressure (p=0.0013) was revealed. A marginally significant interaction in the association with systolic blood pressure was also shown (p=0.045). The associations of the 5-HT2A T102C polymorphism with hypertension and diastolic blood pressure in ET-1 T allele carriers were significant (p=0.0056 and 0.021, respectively). The association of the 5-HT2A T102C polymorphism with systolic blood pressure in ET-1 T allele carriers was marginally significant (p=0.054). Thus, the present study suggests that the 5-HT2A T102C and ET-1 G/T polymorphisms are interactively associated with hypertension. (*Hypertens Res* 2006; 29: 227–232)

***Key Words*: serotonin receptor, endothelin, hypertension, genetics, polymorphism**

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) is a naturally occurring vasoactive monoamine and is widely distributed in the human organism (*1*). Serotonin executes diverse cardiophysiological actions, which are mediated by different subtypes of serotonin receptors. Currently, serotonin receptors are divided into seven groups (5-HT1–7). Among these groups, 5-HT2 receptors mediate the vasoconstrictive actions of serotonin, and these are further categorized into three subtypes (A, B, and C). Among these three subtypes, the 5-HT2A

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Table 1. Characteristics of Participants According to Hypertension Status

Variable	Population 1		Population 2	
	Normotensive (n=1,364)	Hypertensive (n=852)	Normotensive (n=502)	Hypertensive (n=250)
Sex (male %)	84.8	89.4*	77.1	78.2
Age (years)	49.5±9.0	53.8±6.6*	52.4±8.8	57.2±8.3*
Body mass index (kg/m^2)	22.6±2.8	24.2±3.2*	22.5±2.8	23.8±2.7*
Systolic blood pressure (mmHg)	122.7±10.7	148.7±12.7*	112.4±10.6	143.4±17.0*
Diastolic blood pressure (mmHg)	72.3±7.3	87.3±8.4*	72.1±8.9	89.4±9.3*
Total cholesterol (mg/dl)	195.4±32.1	203.2±31.6*	198.5±30.9	201.8±36.3
High density lipoprotein cholesterol (mg/dl)	60.8±13.3	60.8±13.2	54.1±14.7	52.1±14.8
Triglyceride (mg/dl)	127.0±75.9	155.3±85.4*	107.9±76.3	137.3±126.8*

Data are mean±SD. *$p<0.05$ *vs.* normotensives. Blood pressure readings before the start of antihypertensive medication were not available for 705 hypertensive subjects whose values were measured under treatment.

receptor is the primary receptor mediating vasoconstriction under conditions of normal blood pressure (*2*). Thus, the 5-HT2A receptor may play an important role in the regulation of blood pressure.

The 5-HT2A receptor gene is located on chromosome 13. Given the biological evidence for a relation between the 5-HT2A receptor and blood pressure, it is important to evaluate how variations in the 5-HT2A receptor gene are associated with blood pressure as genetic factors. In this context, a functional polymorphism (T102C) of the 5-HT2A receptor gene has been investigated in relation to hypertension. An initial study showed that increased frequency of the 102C allele was significantly associated with hypertension in female UK residents (*3*). Another association study, however, failed to show a significant association between the 5-HT2A T102C polymorphism and hypertension in a Chinese population (*4*).

Generally, inconsistent associations could result from various factors, including racial difference, insufficient statistical power, and interactions of polymorphisms with other genetic and environmental factors (*5*). In this context, it may be of significance that the serotonin system has been shown to biologically interact with the endothelin system (*6–14*). This interaction could modify the association between the 5-HT2A T102C polymorphism and hypertension. However, whether genetic interactions between polymorphisms corresponding to the biological interaction significantly influence blood pressure in the general population remains to be assessed. We therefore analyzed the association between the 5-HT2A T102C polymorphism and hypertension in two large Japanese populations, with consideration of the interaction between the 5-HT2A T102C polymorphism and the G/T (Lys198Asn) polymorphism in exon 5 of the endothelin-1 (ET-1) gene, because the ET-1 system (*15*), especially the ET-1 G/T polymorphism (*16*), has been shown to be involved in the development of hypertension.

Methods

Subjects

The clinical characteristics of the subjects included in the study are shown in Table 1. Population 1 (n=2,216) originated from the Ehime region of Japan, and population 2 (n=752) from the Hyogo region of Japan (*17*). All subjects were Japanese urban residents. Subjects in population 1 participated in medical check-ups 1–11 times (average 6.2 times per person), and the mean values of variables in their personal health records were used in the analyses. Subjects in population 2 also underwent a medical check-up, and the values of variables in their personal health records were used in the analyses. All subjects provided informed consent for participation in the molecular-genetic studies. The ethics committee of Ehime University approved the study.

Diagnostic Categories

Each subject was assigned to one of the blood pressure diagnostic categories defined by the following criteria. Hypertensive subjects had a previous diagnosis of hypertension and were being treated with antihypertensive medication, or their systolic/diastolic blood pressure (SBP/DBP) was >140/90 mmHg. Normotensive subjects had never been treated with medication for hypertension, and their SBP/DBP was <140/90 mmHg. Blood pressure was measured in the sitting position with the use of a standard sphygmomanometer during medical check-ups.

DNA Analysis

The TaqMan chemical method, which is an established and frequently used method (*18–21*), was used to detect the 5-HT2A T102C polymorphism. The forward primer was 5′-AAATGATGACACCAGGCTCTACAGT-3′, the reverse

Table 2. 5-HT2A Genotype and Allele Frequencies in Hypertensive and Normotensive Subjects

Genotype and allele	Population 1			Population 2			Populations 1 and 2		
	Normo-tensive	Hyper-tensive	*p* value	Normo-tensive	Hyper-tensive	*p* value	Normo-tensive	Hyper-tensive	*p* value
5-HT2A genotypes (*n* (%))									
CC	344 (25.2)	230 (27.0)		123 (24.5)	68 (27.2)		467 (25.0)	298 (27.0)	
CT	645 (47.3)	409 (48.0)		254 (50.6)	108 (43.2)		899 (48.2)	517 (46.9)	
TT	375 (27.5)	213 (25.0)	0.38	125 (24.9)	74 (29.6)	0.15	500 (26.8)	287 (26.0)	0.48
5-HT2A alleles (*n* (%))									
C	1,333 (48.9)	869 (51.0)		500 (49.8)	244 (48.8)		1,833 (49.1)	1,113 (50.5)	
T	1,395 (51.1)	835 (49.0)	0.17	504 (50.2)	256 (51.2)	0.71	1,899 (50.9)	1,091 (49.5)	0.30

5-HT2A, serotonin 2A.

primer was 5′-TGTCCAGTTAAATGCATCAGAAGTG-3′, the T-allele specific probe was 5′-FAM-AACTCTGGAGAA GCT-MGB-3′, and the C-allele specific probe was 5′-VIC-AACTCCGGAGAAGC-MGB-3′. The person who assessed the genotype was blinded to the clinical data of the subjects from whom the samples originated. The ET-1 G/T polymorphism was previously determined in our populations (*17*).

Statistical Methods

Comparisons of categorical variables were performed using the χ^2 test. Analysis of variance was used to assess differences in the means and variances of continuous variables. Because of a skewed distribution of data, logarithmically transformed plasma triglyceride values (TG) were used in the analysis. Logistic regression models were used to assess whether the 5-HT2A T102C polymorphism made a statistically significant contribution to the prediction of hypertension, with consideration of the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms. General linear models were used to assess whether the 5-HT2A T102C polymorphism made a statistically significant contribution to the prediction of blood pressure, with consideration of the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms. *p* values less than 0.05 were considered statistically significant. Statistical analysis was performed with SPSS statistical software.

Results

Association of the 5-HT2A T102C Polymorphism with Hypertension

Table 1 presents the clinical characteristics of the participants in populations 1 and 2. In population 1, the relative frequencies of the CC, CT and TT genotypes were 25.9%, 47.6% and 26.5%, respectively. In population 2, the relative frequencies of the CC, CT and TT genotypes were 25.4%, 48.1% and 26.5%, respectively. In population 1, the allele frequencies were 49.7% and 50.3% for the C and T alleles, respectively. In population 2, the allele frequencies were 49.5% and 50.5% for the C and T alleles, respectively. These results are consistent with the Hardy-Weinberg equilibrium. There was no significant difference in the frequencies of the alleles (p=0.17) and genotypes (p=0.38) between the hypertensive and normotensive subjects in population 1 (Table 2). There was no significant difference in the frequencies of the alleles (p=0.71) and genotypes (p=0.15) between the hypertensive and normotensive subjects in population 2. Finally, there was no significant difference in the frequencies of the alleles (p=0.30) and genotypes (p=0.48) between the hypertensive and normotensive subjects in the combined group of populations 1 and 2.

Interaction between the 5-HT2A T102C and ET-1 G/T Polymorphisms in Association with Hypertension

We next analyzed the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. This analysis showed a significant interaction in population 1 (p=0.012, odds ratio [OR]=0.74, 95% confidence interval [95% CI]=0.58–0.94) and failed to show a significant interaction in population 2 (p=0.14, OR=0.73, 95% CI=0.48–1.11). Finally, analysis combining populations 1 and 2 yielded a lower p value of 0.0040 (OR=0.74, 95% CI=0.60–0.91) for the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. The interaction was also significant after adjustment for sex and age (p=0.0022), and for sex, age, body mass index (BMI), plasma total cholesterol, high density lipoprotein (HDL)–cholesterol, and TG (p=0.050). Table 3 shows the opposite directions of the association of the 5-HT2A T102C polymorphism with hypertension between the ET-1 genotypes. The association of the 5-HT2A T102C polymorphism with hypertension in ET-1 T allele carriers was significant (p=0.0056). The association of the ET-1 G/T polymorphism with hypertension also showed opposite directions between the 5-HT2A genotypes (Table 4). The association of the ET-1 G/T polymorphism with hypertension in 5-HT2A CC homozygotes was significant (p=0.028). The

Table 3. 5-HT2A Genotype Frequency in Hypertensives and Normotensives According to ET-1 Genotype

ET-1 genotype	5-HT2A genotype	Genotype frequency (*n* (%)) Normotensive (*n*=1,866)	Hypertensive (*n*=1,102)	*p* value	OR	95% CI
GG	CC	256 (26.4)	139 (24.7)			
	CT	460 (47.4)	258 (45.8)			
	TT	255 (26.3)	166 (29.5)	0.20	1.10	0.95–1.26
GT+TT	CC	211 (23.6)	159 (29.5)			
	CT	439 (49.1)	259 (48.1)			
	TT	245 (27.4)	121 (22.4)	0.0056	0.81	0.70–0.94

ET-1, endothelin-1; 5-HT2A, serotonin 2A; OR, odds ratio; CI, confidence interval.

Table 4. ET-1 Genotype Frequency in Hypertensives and Normotensives According to 5-HT2A Genotype

5-HT2A genotype	ET-1 genotype	Genotype frequency (*n* (%)) Normotensive (*n*=1,866)	Hypertensive (*n*=1,102)	*p* value	OR	95% CI
CC	GG	256 (54.8)	139 (46.6)			
	GT+TT	211 (45.2)	159 (53.4)	0.028	1.39	1.04–1.86
CT	GG	460 (51.2)	258 (49.9)			
	GT+TT	439 (48.8)	259 (50.1)	0.65	1.05	0.85–1.31
TT	GG	255 (51.0)	166 (57.8)			
	GT+TT	245 (49.0)	121 (42.2)	0.064	0.76	0.57–1.02

ET-1, endothelin-1; 5-HT2A, serotonin 2A; OR, odds ratio; CI, confidence interval.

association of the ET-1 G/T polymorphism with hypertension in 5-HT2A TT homozygotes was also marginally significant (p=0.064).

Interaction between the 5-HT2A T102C and ET-1 G/T Polymorphisms in Association with Blood Pressure

Given the marginally significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension, we next analyzed the possible interactions between these polymorphisms in their association with blood pressure in the combined group of populations 1 and 2. This analysis showed a marginally significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with SBP (p=0.045). This interaction was also marginally significant after adjustment for sex and age (p=0.045), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG (p=0.058). Moreover, there was a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with DBP (p=0.0013). This interaction was also significant after adjustment for sex and age (p=0.0018), and for sex, age, BMI, plasma total cholesterol, HDL-cholesterol, and TG (p=0.0023). Table 5 shows the opposite directions of the association of the 5-HT2A T102C polymorphism with blood pressure between the ET-1 genotypes. The association of the 5-HT2A T102C polymorphism with SBP in ET-1 T allele carriers was marginally significant (p=0.054), and the association of the 5-HT2A T102C polymorphism with DBP in ET-1 T allele carriers was significant (p=0.021). Table 6 again shows the opposite directions of the association of the ET-1 G/T polymorphism with blood pressure between the 5-HT2A genotypes. The association of the ET-1 G/T polymorphism with DBP in 5-HT2A CC homozygotes was significant (p=0.0013).

Discussion

Given the biological evidence for a relation of the 5-HT2A receptor to blood pressure, a functional polymorphism (T102C) of the 5-HT2A receptor gene has been investigated in relation to hypertension. An initial study showed that increased frequency of the 102C allele was significantly associated with hypertension in female UK residents (*3*). A subsequent study failed to show a significant association between the 5-HT2A T102C polymorphism and hypertension in a Chinese population (*4*). Consistent with the results of the latter study, the present study failed to show a significant association, although increased frequency of the 102C allele was non-significantly associated with hypertension in the combined group of populations 1 and 2, in line with the results of the former study.

This failure could be attributable to racial difference. How-

Table 5. Blood Pressure for 5-HT2A Genotype According to ET-1 Genotype

BP	ET-1 genotype	5-HT2A genotype			*p* value	
		CC	CT	TT	For regression	For interaction
SBP (mmHg)	GG	129.9±18.2	129.3±19.1	130.4±17.7	0.61	
	GT+TT	132.0±18.0	130.3±18.1	128.8±17.9	0.054	0.045
DBP (mmHg)	GG	77.4±10.6	77.7±11.7	78.5±10.6	0.33	
	GT+TT	79.9±11.2	77.6±10.9	77.4±10.7	0.021	0.0013

Data are mean±SD. 5-HT2A, serotonin 2A; ET-1, endothelin-1; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP.

Table 6. Blood Pressure for ET-1 Genotype According to 5-HT2A Genotype

BP	5-HT2A genotype	ET-1 genotype		*p* value	
		GG	GT+TT	For regression	For interaction
SBP (mmHg)	CC	129.9±18.2	132.0±18.0	0.11	
	CT	129.3±19.1	130.3±18.1	0.29	
	TT	130.4±17.7	128.8±17.9	0.21	0.045
DBP (mmHg)	CC	77.4±10.6	79.9±11.2	0.0019	
	CT	77.7±11.7	77.6±10.9	0.98	
	TT	78.5±10.6	77.4±10.7	0.14	0.0013

Data are mean±SD. ET-1, endothelin-1; 5-HT2A, serotonin 2A; BP, blood pressure; SBP, systolic BP; DBP, diastolic BP.

ever, genetic effects are usually consistent across human populations (*22*). Therefore, the failure might be rather attributable to gene–environmental and/or gene–gene interactions, because such interactions could modify or mask associations. In this respect, the present study revealed a statistically significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with hypertension. Consequently, increased frequency of the 5-HT2A 102C allele is significantly associated with hypertension in ET-1 T allele carriers, consistent with the initial study (*3*). However, it should be noted that this interaction was significant in population 1 and in the combined group of populations 1 and 2, but not in population 2, despite the fact that the OR for the interaction were very similar between the two populations. This implies that studies with modest sample sizes can fail to detect interactions, and a combination of samples will be required to achieve adequate statistical power.

Moreover, the present study showed a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with blood pressure. In particular, the interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in their association with DBP was significant in both populations 1 and 2 (data not shown), constituting strong evidence in favor of the existence of this interaction.

A genetic interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms is also supported by persistent biological evidence for the existence of interactions between the serotonin and endothelin systems. For example, subthreshold concentrations of ET-1 amplify the vasoconstrictor effect of serotonin in human arteries (*6*, *7*, *9*, *10*) and in the guinea pig trachea (*11*). Preincubation of a platelet suspension with ET-1 has been shown to inhibit the serotonin-mediated platelet response (*8*). ET-1 has been shown to inhibit serotonergic amplification of epinephrine-induced aggregation of platelets (*8*). Pre-treatment of rabbit platelets with ET-1 has been shown to enhance serotonin-promoted protein tyrosine phosphorylation (*12*). In the rabbit platelet membrane, ET-1 has been shown to enhance serotonin binding and inhibit its internalization (*12*). On the other hand, serotonin also potentiates ET-1–induced vascular smooth muscle cell proliferation (*13*).

The T102C polymorphism is located in the coding sequence in exon 1 of the 5-HT2A receptor gene and does not change any amino acid, and thus it is a silent polymorphism in that both nucleotides result in a codon that encodes Ser at amino acid position 34 (*23*). Nevertheless, the T102C polymorphism results in a differential gene expression (*24*). This functionality of the 5-HT2A T102C polymorphism also increases the plausibility of a genetic interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms.

In conclusion, the present study revealed a significant interaction between the 5-HT2A T102C and ET-1 G/T polymorphisms in the pathogenesis of hypertension in two large Japanese populations. This interaction was supported by several lines of molecular biological evidence. Nevertheless, association studies are often irreproducible, warranting further studies in large populations to investigate the interactions between the serotonin and endothelin systems, with consideration of various gene–environment and gene–gene interactions.

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Aging and high-density lipoprotein metabolism

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With the recent explosion of the elderly population, identification of factors involved in extended life with good health is particularly important for both individuals and societies. Epidemiological and interventional studies have demonstrated the importance of high-density lipoprotein cholesterol as a negative risk factor for cardiovascular disease. Also, certain genetic polymorphisms that are involved in high-density lipoprotein metabolism are proposed to be associated with longevity. These findings raise the possibility that enhancing high-density lipoprotein cholesterol levels by pharmacological intervention could prevent cardiovascular disease and promote healthy aging. The aim of this review is to summarize the current knowledge of high-density lipoprotein and its metabolism in relation with cardiovascular risk as well as frailty, a syndrome characterized by multisystem decline in the oldest old.

Keywords: caloric restriction, centenarians, cholestery ester transfer protein, frailty, high-density lipoprotein, inflammation, longevity, nutrition, paraoxonase, reverse cholesterol transport

future medicine

With the rapid increase in the oldest old population, the identification of factors that are involved in extended life with good health is becoming a major target for healthcare providers. In the elderly population, cardiovascular disease (CVD) is not only the leading cause of death, but also an aggravating factor for disability and frailty. Numerous epidemiological and clinical studies have established that a low level of high-density lipoprotein cholesterol (HDL-C) is a strong risk factor for CVD in the general population [1]. Recently, the importance of HDL, not only as a risk factor for CVD but also as an indicator for all-cause mortality, has been emphasized, especially in the oldest old. Low HDL-C levels, but not high low-density lipoprotein cholesterol (LDL-C) levels, were demonstrated to be associated with an increased risk of CVD and stroke in individuals aged over 85 years [2]. In addition, low HDL-C combined with low levels of serum albumin predicted higher mortality among the elderly population [3]. By contrast, high HDL-C levels have been proposed to be associated with healthy aging and longevity. For example, familial hyperalphalipoproteinemia was reported to be associated with longevity [4], and higher HDL levels were observed among healthy individuals aged 85–89 years as compared with middle-aged subjects [5]. The associations between HDL and healthy aging have been attributed mainly to the antiatherosclerotic and cardioprotective effects of HDL. However, recent studies revealed that the diverse effects of HDL, other than its antiatherosclerotic properties, may be associated with overall health status in the oldest old, and research in this area is rapidly expanding. Based on these studies, HDL and molecules involved in HDL metabolism seem attractive candidates for longevity-promoting factors. In this article, we attempt to review the current status of our knowledge of HDL metabolism and its associations with healthy aging and longevity.

HDL cholesterol & its subpopulation in the oldest old

In general, levels of HDL-C as well as total cholesterol (TC) decrease in older age [6]. Menopause, change in body mass index (BMI) and fat distribution, and age itself may be important determinants of the decline in HDL-C levels among the elderly. However, results from studies in the oldest old, including centenarians, have been conflicting. Some investigators reported that centenarians showed similar lipid profiles in comparison with a middle-aged population [7], but others demonstrated lower TC and HDL-C in centenarians [8,9]. This discrepancy may be attributable to the differences in sampling, and the nutritional and functional status of the centenarians.

HDL particles are heterogeneous in apolipoprotein (apo) composition, density, particle size and electrophoretic mobility, so that not only the absolute value, but also parameters on HDL subclasses were examined as indicators for CVD risk assessment. To fractionate HDL into subpopulations, various procedures including ultracentrifugation, polyanion precipitation and gradient gel electrophoresis have been applied.

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According to the ultracentrifuge procedure, HDL comprises two subclasses; the larger, more lipid-rich HDL_2 ($1.063 < d < 1.125$), and the smaller, denser HDL_3 ($1.125 < d < 1.210$). Lipoprotein profiles and HDL subclasses among elderly people have been extensively studied. Ettinger and colleagues demonstrated that HDL_2-C, but not HDL_3-C increased slightly with age in both men and women aged over 65 years [10]. Although the analytical procedure was different, the predominance of HDL_2 subfraction was observed among octogenarians [11] and centenarians [7,9]. Recently, using nuclear magnetic resonance (NMR) spectroscopy, Barzilai and colleagues analyzed lipoprotein particle size among individuals aged over 95 years and their offspring [12]. Although the differences in lipoprotein cholesterol levels were modest, it was found that particle sizes of both HDL and LDL were remarkably larger in the long-lived subjects compared with controls with a mean age of 70 years. In addition, the offspring of long-lived subjects had intermediate sizes of HDL and LDL particles, indicating that these lipoprotein phenotypes are heritable, and may be a causal biological candidate of longevitypromoting factors. Based on these epidemiological findings, the predominance of larger particles in HDL (and possibly LDL) subclasses appears to be the most reproducible phenotype among subjects who have reached extreme old age.

Antiatherogenic properties of HDL

Reverse cholesterol transport

Reverse cholesterol transport (RCT), which enhances cholesterol efflux from the arterial wall and transport to the liver for re-use or excretion into bile, has been proposed as one of the major antiatherogenic mechanisms of HDL. Recently, a growing body of evidence has supported the association between polymorphisms of candidate genes in the RCT pathway and CVD risk; therefore, extensive research has focused on genetic variations in RCT as human longevity genes.

Cholesteryl-ester transfer protein

Cholesteryl-ester transfer protein (CETP) is a principal carrier protein in RCT, which mediates the transfer of cholesteryl esters from HDL to apoB-containing lipoproteins. Genetic polymorphisms of CETP have been extensively studied in relation to CVD risk and longevity.

Several mutations at the *CETP* gene locus, which cause depletion of CETP activity and consequently high HDL-C levels, have been described, especially in Japanese subjects [13–15]. CETP deficiency caused by a G-to-A mutation at intron 14 was originally proposed as a longevity factor due to its antiatherogenic lipoprotein profile, including high levels of HDL_2 subclass [13]. However, results from epidemiological studies on CETP deficiency have been conflicting. In the Honolulu Heart Program, including 3469 Japanese–American elderly men, CETP mutations exhibited proatherogeneity when combined with a serum HDL-C level of less than 60 mg/dl [16]. Recently, a successive follow-up study of this cohort demonstrated a lower risk of CVD in individuals with CETP mutations when their HDL-C levels were 60 mg/dl and higher [17]. However, in the Omagari study, where CETP deficiency by a G-to-A mutation at intron 14 is extremely frequent, prevalence of the mutation was higher in patients with CVD, and lower in older subjects aged over 80 years, suggesting a negative impact of the mutation on longevity [18]. *Taq1B* genotype is the best examined polymorphism in the *CETP* gene in terms of CVD risk, and a recent meta-analysis, including 13,677 subjects, confirmed that the genotype is associated with HDL-C levels, and accordingly with the risk of CVD [19]. We investigated CETP deficiency and *Taq1B* genotype in 256 centenarians as a candidate longevity-promoting gene [20]. Although heterozygous CETP deficiency and B2 allele of *Taq1B* polymorphisms were consistently associated with lower CETP mass and higher HDL-C concentrations in centenarians, neither of these allelic variations were associated with longevity. By contrast, the I405V polymorphism in the *CETP* gene was shown to have strikingly higher impact on longevity. Barzilai and colleagues demonstrated that long-lived Ashkenazi Jews and their offspring had a dramatically higher frequency of VV genotype [12]. In addition, VV genotype was associated with lower CETP concentrations and increased HDL and LDL particle size, suggesting that this variant could be one of the genetic components for this unique lipoprotein phenotype among long-lived subjects. However, the association of I405V genotype and longevity was not observed in Italian [21] or Japanese (unpublished observation) centenarians. Despite extensive investigations, we have not reached a definitive consent on the roles of *CETP* gene polymorphisms on longevity. One possible explanation is that the effects of CETP are

dependent on its metabolic context, for example, HDL-C levels and interaction with other molecules of RCT.

Hepatic lipase, lipoprotein lipase & ABCA1

In addition to CETP, hepatic lipase (HL), lipoprotein lipase (LPL), and ATP-binding cassette transporter 1 (ABCA1) are important components of RCT. HL, an enzyme secreted by the liver, regulates the lypolysis of triglyceride-rich lipoproteins (TRL) and the catabolism of HDL. Among numerous polymorphisms in the *HL* gene, the C(-514)T polymorphism has been repeatedly investigated in terms of its effects on HDL-C levels and CVD risk, yet the results have been conflicting [22–24]. ABCA1, a membrane protein that mediates the transport of phospholipid and other metabolites from cell to apo A1 in an ATP-dependent manner, was proposed to play a pivotal role in RCT. Since *ABCA1* mutations have been demonstrated to cause Tangier disease, a rare recessive disease characterized by extremely low HDL-C levels and progressive atherosclerosis, numerous studies have focused on genetic variance of ABCA1 as a CVD risk factor. The R219K polymorphism in the *ABCA1* gene was associated with a reduced severity of CVD in French–Canadians [25] and a lower risk of premature CVD in related hypercholesterolemic subjects [26], but not in Japanese populations [27].

Since CETP, hepatic and lipoprotein lipases, and ABCA1 may regulate cholesterol efflux synergistically, we simultaneously investigated associations of genetic variations involved in RCT and longevity [20]. In a study of 256 centenarians, we demonstrated a significant association between *Pvu II* (-/-) variants of the *LPL* gene and higher HDL-C concentration in centenarians; however, we found no association of these genetic polymorphisms with longevity. Neither *ABCA1* R219K genotype nor *HL* C(-514)T promoter polymorphism had significant associations with longevity or lipid profiles in centenarians or controls. While a phenotypic trait in HDL particle size is specified, most of the studies that investigated polymorphisms of genes involved in HDL metabolism did not find positive associations with longevity (Table 1). There are several possible explanation for these negative results. First, a few gene polymorphisms may play pivotal roles in determining lipoprotein trait and longevity, so that a comprehensive analysis of many candidate genes in a particular pathway may be more efficient. Second, as demonstrated in recent studies, the effects of gene polymorphisms on HDL-C levels and CVD risk were modulated by confounding factors such as dietary fat and BMI [28,29]. Further studies with sample sizes large enough to calculate gene–gene as well as gene–environment interactions are required.

Antioxidative & anti-inflammatory properties of HDL

Other than RCT, antioxidative and anti-inflammatory effects of HDL are proposed to be a constituent of its antiatherogenic property. HDL has been shown to inhibit the oxidation of LDL by antioxidative property of apo A1 and paraoxinase (PON); an enzyme carried by HDL particles [30]. As oxidized LDL contributes to atherogenesis in the critical phase, numerous studies have been conducted to examine whether PON activity and/or genetic variations contribute to CVD risk. Lines of evidence from cross-sectional and prospective studies suggest that low PON activity in serum is predictive of CVD [31] even in the elderly [32]. Since the variance in serum PON activity is largely explained by certain polymorphisms in the *PON1* gene, including Q192R, L55M, and T(-107)C genotype, many studies examined these *PON1* genotypes in relation to CVD risk. However, the results have been conflicting and a recent meta-analysis of 43 studies, including 11,212 CVD patients and 12,786 controls, did not demonstrate a significant association between *PON1* genotypes and CVD [33]. Possible associations of PON activity and its gene polymorphisms with longevity were also studied. It was reported that serum PON activity decreased with aging [34]; however, in healthy octogenarians with no signs of CVD, PON activity was significantly higher compared with middle-aged subjects [35]. *PON1* G192 R allele, which is suggested to attribute to higher hydrolysis activity for paraoxon, was associated with longevity in 308 Italian centenarians, and a similar trend was observed in 296 Irish octo- and nonagenarians [36]. By contrast, in a population aged 85 years and older, Heijmans and colleagues found no significant associations between G192R polymorphism and all-cause mortality or fatal CVD in a cross-sectional and prospective design, except a higher all cause mortality in RR carriers in a subset of diabetic patients [37]. It is known that numerous factors, such as antioxidants, alcohol, dietary fat, and physical and pathological state modulate PON activity. *PON1* genetic polymorphism was also demonstrated to be associated with Parkinson's disease [38], in which oxidative stress plays a major role in the pathogenesis. Therefore, inconsistent results from epidemiological studies on *PON1* gene polymorphism can be

Table 1. Associations between genetic variations that modulate high-density lipoprotein metabolism and longevity.

Genes	Polymorphisms	Population	Results	Ref.
APOA1	*Msp*	Italian cohort	P allele dominant in the oldest old aged 81–109 years	[52]
CETP	Intron 14 G to A	Japanese cohort	Intron 14 splicing defect less frequent in subjects >80 years old	[20]
CETP	Intron 14 G to A	Japanese centenarians	No association with longevity	[22]
CETP	G442A	Japanese centenarians	No association with longevity	[22]
CETP	*Taq1B*	Japanese centenarians	No association with longevity	[22]
CETP	I405V	Ashkenazi Jews aged 95–107 years	VV genotype dominant in subjects with exceptional longevity	[14]
CETP	I405V	Italian centenarians	No association with longevity	[23]
LPL	*Pvu II*	Japanese centenarians	No association with longevity	[22]
LPL	*Hind III*	Japanese centenarians	No association with longevity	[22]
HL	C(-514)T	Japanese centenarians	No association with longevity	[22]
ABCA1	R219 K	Japanese centenarians	No association with longevity	[22]
PON1	T(-107)C	Sicilian octogenarians	CC genotype dominant in healthy octogenarians	[33]
PON1	Q192R	Leiden aged 85 years and over	No association with survival after the age of 85	[35]
PON1	Q192R	Italian centenarians Irish octo- and nonagenarians	R allele more frequent in centenarians and octo/nonagenarians	[34]

Modified from Arai Y and Hirose N. J. Atheroscler. Thromb. *11, 246–252 (2004).*
ABCA1: ATP-binding cassette transporter 1; APO A1: Apolipoprotein A1; CE: Cholesteryl ester; CETP: Cholesteryl ester transfer protein; HL: Hepatic lipase; LPL: Lipoprotein lipase; PON: Paraoxonase.

explained partially by those modifiable factors and subclinical conditions, which are occasionally underestimated in the very old.

HDL cholesterol potentially exerts anti-inflammatory effects on the vasculature. HDL was demonstrated to inhibit expression of E-selectin, vascular cell adhesion molecule-1 (VCAM)-1, and intercellular adhesion molecule (ICAM)-1 induced by interleukin (IL) 1β in human umbilical vein endothelial cell [39]. However, experimental data suggest that during systemic inflammation, HDL composition changes to become pro-inflammatory. In an acute-phase rabbit model, HDL particles were remodeled and apo A1 was displaced by serum amyloid A (SAA), an acute-response protein. Proinflammatory HDL enhanced monocyte chemotactic activity induced by LDL [40]. The association between systemic inflammation and HDL-C is also being investigated in humans. Observational studies suggest a strong relationship between C-reactive protein (CRP) and cardiovascular mortality [41,42]. A recent clinical trial demonstrated that intensive treatment with a statin reduced the progression of atherosclerosis in CVD patients in addition to reducing levels of both atherogenic lipoproteins and CRP [43]. In addition, an inverse correlation between CRP and HDL-C levels was observed among the elderly population, and CRP levels significantly predicted 10-year total and cardiovascular mortality [44]. The mechanism underlying the association between the anti-inflammatory properties of HDL-C and atherosclerosis remains to be clarified; however, the association should be concomitantly investigated from a geriatric perspective, especially in the oldest old (Figure 1).

Practical use of HDL-C as a marker of overall health status in the oldest old

HDL-C as a marker of frailty in the oldest old

In clinical setting of the oldest old, CVD and risk factors are often associated with frailty, a geriatric syndrome characterized by multisystem decline with high risk of disability and mortality. Recent research has shed light on the biological underpinnings of frailty, especially in relation to metabolic imbalance and cytokine dysregulation [45,46]. For the purpose of prevention and management of frailty, numerous studies have focused on biomarkers of frailty, suggesting increased levels of inflammatory cytokines such as IL-6, hypercoaguability, and poor nutrition as possible candidates [47,48]. A predictive value of HDL-C for frailty in combination with serum albumin and inflammatory markers was also proposed. In a prospective