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Genomic structure and mutations in adipose-specific gene, adiponectin

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BACKGROUND: Adiponectin is a collagen-like plasma protein specifically synthesized in adipose tissue. Plasma adiponectin concentrations are decreased in obesity whereas it is adipose-specific.
OBJECTIVE: To clarify the significance of the genetic variations in adiponectin gene on its plasma concentrations and obesity.
SUBJECTS: Two hundred and nineteen unrelated adult Japanese subjects (123 men and 96 women, age: 20–83 y, BMI: 16–43 kg/m^2) including 77 obese subjects (BMI > 26.4 kg/m^2).
MEASUREMENT: Human adiponectin gene was isolated from PAC DNA pools. Mutations in the adiponectin gene were screened by direct sequencing or restriction-fragment polymorphism. The levels of plasma adiponectin were determined by the enzyme-linked immunosorbent assay (ELISA).
RESULTS: Adiponectin gene spanned 17 kb on chromosome 3q27, consisting of three exons and two introns. Within 2.1 kb of the 5′-flanking region, there were two octamer elements present in the promoter of adipsin. Two nucleotide changes were identified. One was a polymorphism (G/T) occurring in exon 2, and the other was a missense mutation (R112C) in exon 3. The mean plasma adiponectin levels of the subjects carrying G allele were low (G/G: 4.5 μg/ml; G/T: 5.9 μg/ml; and T/T: 6.3 μg/ml), but were not statistically significant. The allelic frequency between the obese and the non-obese showed no significant difference. The subject carrying R112C mutation showed markedly low concentration of plasma adiponectin.
CONCLUSION: Two nucleotide changes have been identified in the adiponectin gene. G/T polymorphism in exon 2 was associated with neither plasma adiponectin concentrations nor the presence of obesity. A subject carrying missense mutation (R112C) showed markedly low plasma adiponectin concentration.
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Keywords: adiponectin; obesity; adipose-specific gene; adipocytokine

Introduction

Obesity, defined as accumulation of excess body fat, is a major health problem in the industrial countries and a major risk factor for non-insulin dependent diabetes mellitus and vascular diseases. Several lines of evidence have shown that adipose tissue not only stores a large quantity of fat as an energy source, but also secretes a variety of proteins that influence the metabolism of the body. These physiologically active molecules derived from adipocytes are 'adipocytokines'.[1,2] Adipsin was isolated from the adipocytes and proved to be one of the complement components.[3] Tumor necrosis factor (TNF) α overproduced in adipose tissue is involved in the development of insulin resistance.[4] Plasminogen activator inhibitor-1 (PAI-1), synthesized in adipose tissue, is involved in the development of thrombotic vascular diseases.[2] Further, the discovery of the *obese* gene opened up a new field in obesity research.[5] The gene product, leptin, is secreted specifically from adipocytes to regulate energy balance by reducing food intake and increasing energy expenditure. Leptin is involved in transmitting a satiety signal to the brain to reduce food intake and increase energy expenditure of the body.

Identification of adipose-specific genes should provide a clue to understand the biological significance of adipocytes. Through systematic survey of active genes in human adipose tissue using random complementary DNA (cDNA) sequencing, we have previously isolated a cDNA, apM1 (adipose most abundant gene transcript 1), which is specifically and abundantly expressed in adipose tissues.[6] The cDNA encoded a plasma protein highly homologous to collagen VIII, X and complement factor C1q. Since the gene product was predicted to be a kind of matrix protein synthesized by adipose tissue, we named it adiponectin.[7] Although the expression of adiponectin gene is specifically restricted in adipose tissue, its plasma levels

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were decreased in obesity.[7] Analysis of gene structure and identification of mutations will give rise to the understanding of the mechanism of the decreased plasma adiponectin. In this study, we analyzed the genomic organization and chromosomal localization, and identified mutations in human adiponectin gene.

Materials and methods

Isolation of the human adiponectin gene

Two P1-derived artificial chromosome (PAC) clones (PAC116-D-11 and PAC93-M-21) containing human adiponectin gene were isolated by screening human PAC DNA pools (Genome System, St Louis, MO) by polymerase chain reaction (PCR) with adiponectin specific primers (5′-ATCACCACTAACTCAGAG-CC-3′ and 5′-AGTGGGCACAAAATAGCACT-3′).

Based on the published cDNA sequence for human adiponectin,[6] three primers were designed from each exon (5′-TTCTGACTGCAGTCTGT GGTT-3′ for exon 1, 5′-CTGCTATTAGCTCTGCCCGG-3′ for exon 2 and 5′-GTTGACCAGGTGTGATGGCT-3′ for exon 3). For Southern blot analysis, 1 μg of clones PAC116-D-11 was digested with one of four restriction endonucleases, *Bam*HI, *Eco*RI, *Hind*III and *Xba*I or two-enzyme combinations of these. The DNA was separated by electrophoresis in a 0.8% agarose gel. The resolved DNA fragments were transferred to nylon membranes and hybridized with oligonucleotides previously end-labeled with [γ^{32}-P]ATP using T4-polynucleotide kinase. Hybridizations were performed in 4×SSC/0.1% SDS at 37°C overnight. The final wash conditioning was 2×SSC/0.1% SDS at 42°C for 20 min. The DNA fragments were subcloned into plasmid vector, and exon−intron boundaries were determined by sequencing with standard protocol. A 2.9-kb *Xba*I-*Bam*HI fragment containing the 5′-franking region of the adiponectin gene was entirely sequenced.

The 5′ end of human adiponectin cDNA was obtained by 5′ rapid amplification of cDNA ends (5′-RACE) with a gene-specific primer, 5′-TGAGGC-CAAAGTGGCTACAGA-3′ in combination with adaptor primer 1 (Clontech). The transcription initiation site was determined by RNase protection assay.

Radiation-hybrid mapping

Mapping was carried out using the Gene Bridge 4 Radiation Hybrid Panel (Research Genetics) according to the manufacturer's instructions, using specific primers designed to amplify the sequence between intron 1 and intron 2 containing the exon 2 region of the adiponectin gene.[8] Primers used were 5′-CGGAGTCCTTTGTAGGTCCCAACTGG-3′ and 5′-TATCAGTGTAGGAGGTCTGTGATG-3′. The amplification profile consisted of denature for 3 min at 94°C, followed by 40 cycles of denature for 30 s at 94°C, annealing for 30 s at 60°C and extension for 30 s

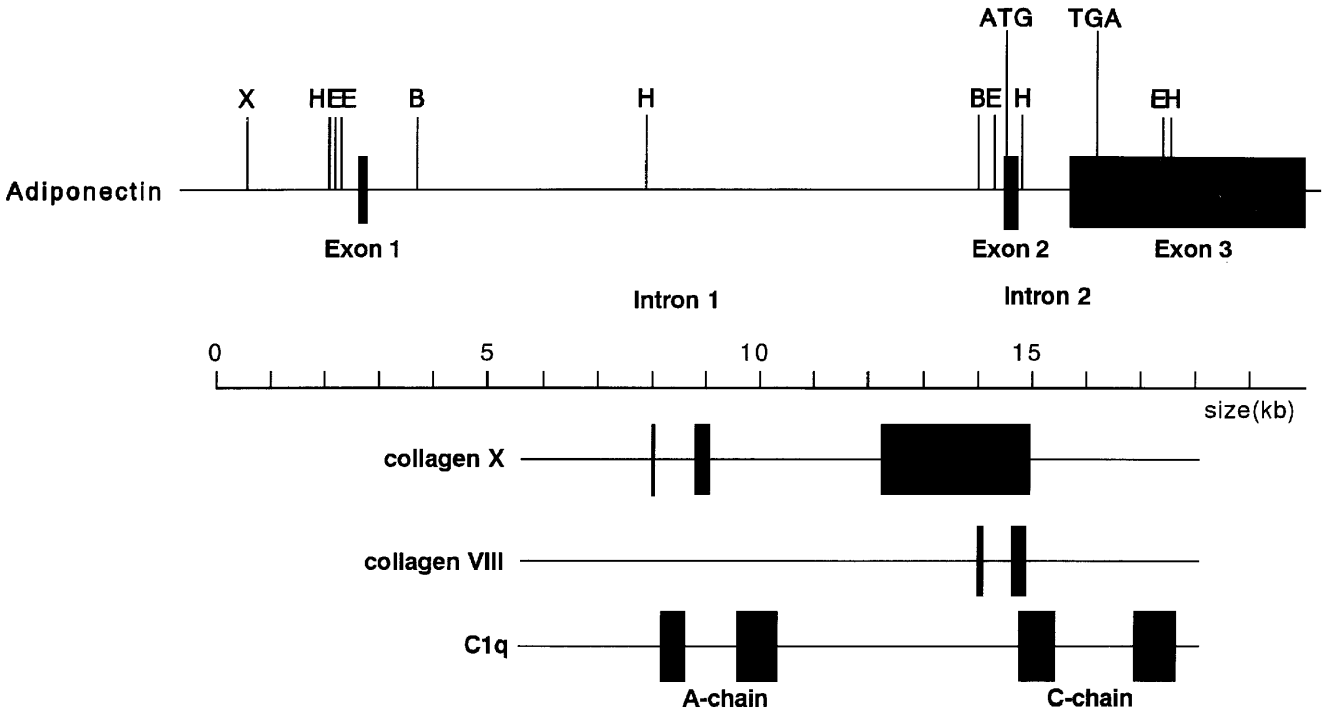

Figure 1 Genomic structure, restriction map of the human adiponectin gene and comparison with homologous genes. The organization of the human adiponectin gene and restriction site was established by Southern blotting of genomic DNA and the analysis of PAC116-D-11. Three exons are represented by solid boxes and numbered. The presumed translation initiation sites in exon 2 and the translation stop site in exon 3 are indicated by an arrow. B, E, H and X represented the endonuclease of *Bam*HI, *Eco*RI, *Hind*III and *Xba*I, respectively. The structures of collagen VIII, X and C1q genes were also shown.

at 72°C. Results were analyzed at http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper, pl.

Mutation analysis of the gene for adiponectin

We investigated the presence of mutation in human adiponectin gene in 219 unrelated adult Japanese subjects (age 20–83 y old; 123 men, average age (±s.d.) 53±14 y old, and 96 women, average age (±s.d.) 55±14 y old; BMI 16–46 kg/m^2). Seventy-seven of these subjects (43 men and 34 women) were obese on the basis of a BMI greater than 26.4 kg/m^2. The levels of plasma adiponectin were determined by the enzyme-linked immunosorbent assay (ELISA) system using monoclonal antibodies against human recombinant adiponectin as previously reported.[7]

We isolated the genomic DNA of 219 subjects from peripheral blood leukocytes. The entire translated region and part of the 5′-untranslated region of adiponectin gene (encoding exons 2 and 3) were amplified by PCR using two pairs of specific primers. One pair consisted of 5′-GAAGTAGACTCTGCTGAGATGG-3′ and 5′-TATCAGTGTAGGAGGTCTGTGATG-3′, which flank the region containing exon 2, and the other pair consisted of 5′-CAACTTCCTAACCGCACTGAAAGCC-3′ and 5′-GTAACCAACCTAGGCAGGAGTTC-3′, which flank the region containing exon 3. The PCR products were directly sequenced on an ABI 377 automatic sequencer or followed by a PCR-based assay for the analysis of restriction fragment length polymorphisms. The PCR products containing exon 2 (372 bp) were digested with *Sma*I endonuclease to yield the restriction fragment and separated on a 2% agarose gel. The gel was stained with ethidium bromide and photographed under ultraviolet illumination.

Statistical analysis

Statistical analysis were performed by means of the Mann–Whitney *U*-test or χ^2 analysis.

Results

Genomic structure and promoter sequence

Two positive P1-derived artificial chromosome (PAC) clones (PAC116-D-11 and PAC93-M-21) were isolated by screening of human PAC DNA library using the primers for adiponectin gene. Sequencing of DNA revealed that both clones contained the entire coding sequence of adiponectin gene. DNA from PAC116-D-11 was used for further analysis. The nucleotide sequences of the exon/intron boundaries and the size of the exons and introns are presented in Figures 1 and 2. Analysis of the exon–intron boundaries showed that the human adiponectin gene contained three exons within 17 kb of genomic DNA with a large first intron (Figure 1), indeed resembling the structure of human collagen X gene,[9] which has significant homology with adiponectin.[6] All of the exon–intron boundaries were consistent with the AG/GT rule[10]

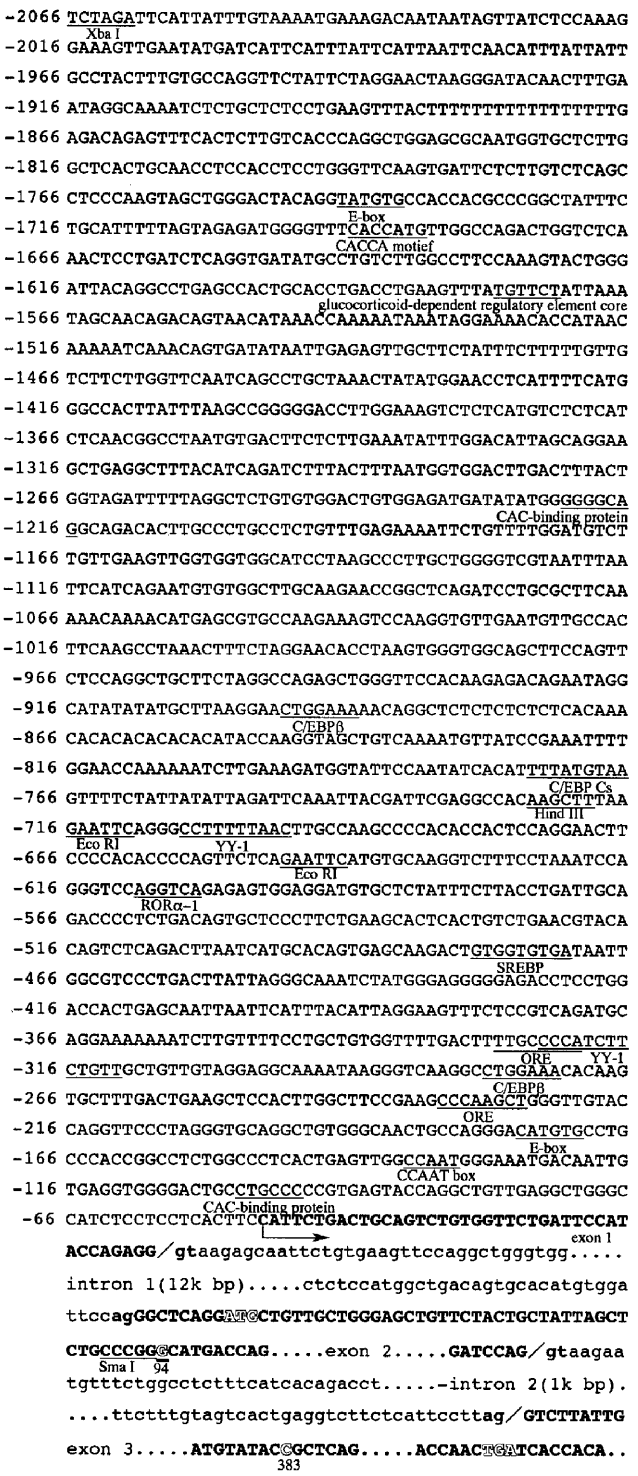

Figure 2 Sequences of promoter, individual exons and surrounding introns. Sequences of promoter, exon 1, 2 and 3, respectively, with approximately 30 nucleotids of surrounding introns are shown. All splice sites are according to the consensus rules: all introns start with gt and end with ag (shown by bold letters). The nucleotides in exons are marked by bold and capital letters. Putative binding sites and restriction sites are underlined. The translation initiation sites in exon 2, the translation stop site in exon 3 and the sites of mutation are shadowed.

(Figure 2). The translation initiation codon and the N-terminal 8 amino acids were encoded by exon 2.

To analyze the promoter region of the gene, a 2.9 kb *Xba*I-*Bam*HI fragment with exon 1 was subcloned and sequenced entirely. It contained approximately 2.1 kb of the 5′-flanking region of exon 1 (Figure 2). The 1.3 kb 5′-flanking sequence of human adiponectin/apM1 gene has been reported by Schaffler *et al.*[11] A search of the promoter region for canonical consensus sequences revealed the presence not of a consensus TATA box, but of a classical CCAAT box at −136 and several putative binding sites for transcription factors. We found some transcriptional factor binding sites such as C/EBP (CCAAT enhancer binding protein) and RORα1 (RAR-related orphan receptor) known to regulate the differentiation and adipogenesis of the adipocytes as previously described.[11] Further, we found another E box at −1742 and two consensus sequences in the region between −1.3 and −2.1 kb. A CACCA motif, one of the common short elements present in the promoter of genes, such as lipoprotein lipase participating in the differentiation of the adipocytes was found at −1691.[12] We also identified a core sequence of glucocorticoid-dependent regulatory element at −1578.[13] These elements in the promoter also seem to influence the fat-specific expression of adiponectin gene. Adipsin is a gene exclusively expressed in the adipocytes and negatively regulated in obese animals.[14] Compared to the promoter of the adipsin gene, we identified two octamer elements at −233/−226 CCCAAGCT, and −328/−321 TTGCCCCA, corresponding perfectly to the sequences in the 56 bp fragment ORE (obesity regulatory element). ORE has been proven to be necessary for negative regulation of adipsin gene.[15]

Chromosomal localization

We determined a precise localization of the human adiponectin gene by means of the Gene Bridge 4 Radiation Hybrid Panel using specific primers designed to amplify the sequence containing the exon 2 region. The data are shown in Table 1. The human adiponectin gene was mapped between markers D3S1571 and D3S1580. We assumed that the LOD score of 15 is faithful enough to assume their linkages. The gene was placed 8.23 cR from D3S1571 of the chromosome 3q27. The genes for thrombopoietin[16] and 100 kDa complement-activating components of Ra-reactive factor[17] were mapped to chromosome 3q27. The deletion and translocation of 3q27 have been reported in minor facial dysmorphism[18] and non-Hodgkin's lymphoma,[19] respectively.

Genotype analysis of the gene for adiponectin

Since adiponectin is a secretory protein present in plasma, we have established a enzyme-linked immunosorbent assay (ELISA) system for the determination of plasma adiponectin concentrations. We found that its plasma levels were decreased in obesity while the expression of adiponectin gene was exclusively restricted in adipose tissue.[7] Plasma adiponectin level may also be regulated by genetic variation.

We measured plasma concentrations of adiponectin in 219 unrelated Japanese subjects including 123 men (mean (± s.d.) age 53 ± 14 y old; BMI 16−46 kg/m^2) and 96 women (mean (± s.d.) age 55 ± 14 y old; BMI, 17−46 kg/m^2). Of these subjects, 77 (43 men and 34 women) were obese (defined as those with a BMI greater than 26.4 kg/m^2). The plasma insulin levels (mean ± s.d.) were higher in obese subjects (men 12.0 ± 8.4 μU/ml, women 11.6 ± 6.3 μU/ml) than non-obese subjects (men 6.9 ± 4.1 μU/ml, women 8.0 ± 5.9 μU/ml). Plasma adiponectin concentrations varied from 0.42 to 27.3 μg/ml and decreased in the obese as previously reported.[7] No significant correlation was found between plasma adiponectin concentrations and plasma insulin levels.

To find out the genetic variations associated with decreased plasma concentration, we amplified and sequenced entire coding regions of the adiponectin genes in all subjects. Two nucleotide changes have been identified in 438 alleles of adiponectin gene. One was a G to T substitution at nucleotide 94 in exon 2 (Figures 2 and 3b and Table 2). With the glycine codon GG*G* in position 15, the nucleotide exchange to GG<u>T</u> causes no amino acid conversion (G15G). Thus, it was a silent polymorphism. The other was a C to T substitution at nucleotide 383 in exon 3 (Figures 2 and 3d). With the arginine codon <u>C</u>GC in position 112, the nucleotide exchange to <u>T</u>GC predicts the amino acid conversion from arginine to cysteine. Thus, it was a missense mutation (R112C).

We examined whether the mutations in adiponectin gene are associated with its plasma level or obesity. For G15G polymorphism, a PCR-based assay for the analysis of restriction fragment length polymorphism was performed (Figure 4). There was a *Sma*I recognition site surrounding G nucleotide at 94. When the PCR-products of exon 2 (372 bp) obtained from

Table 1 Gene Bridge 4 Panel Radiation Hybrid mapping data

Gene/locus	*Data vector*	*LOD score*[a]	*Flanking markers*
Adiponectin	00020000020100002000100 01 01220010012001000100000 00 10001000010100100000100 11 00001000000001210 0	15	D3S1571 D3S1580

[a]The LOD scores quoted are the highest for which these linkages are supported.

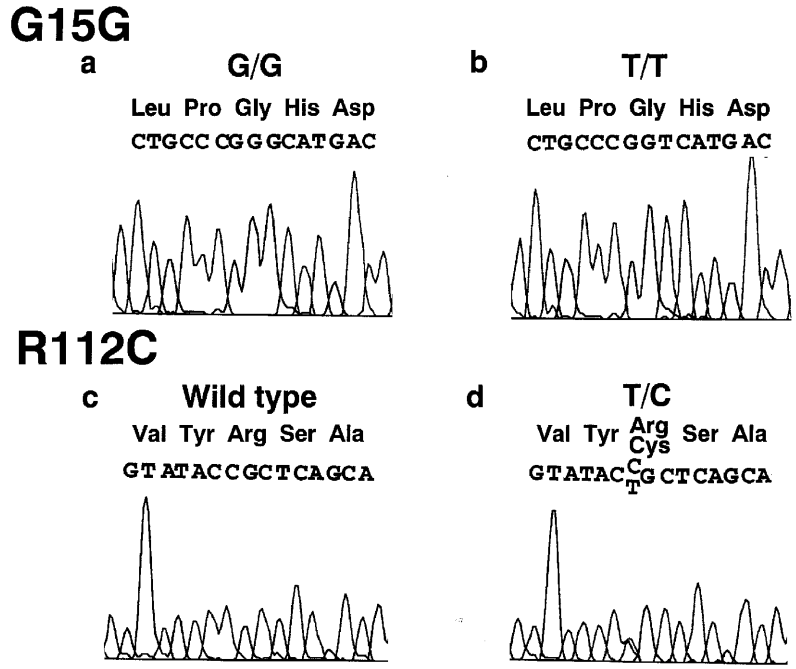

Figure 3 Electropherograms showing the sequences surrounding the mutation in human adiponectin gene. The sequences surrounding the mutations in human adiponectin gene are shown. (a,b). A frequent G to T substitution in exon 2, which caused no amino acid change (G15G). (c,d) R112C missense mutation in exon 3, in which arginine codon (CGC) in position 112 was converted to (TGC). The sequences from a wild type (c) and a heterozygote of the mutation (d) were shown.

Table 2 Human adiponectin polymorphism and plasma concentrations

	G/G	*G/T*	*T/T*
Non-obese	10 (7%)	61 (43%)	71 (50%)
Adiponectin (μg/ml)	5.1 ± 3.1	6.7 ± 5.0	7.2 ± 4.7
Obese	6 (8%)	33 (42%)	38 (50%)
Adiponectin (μg/ml)	3.5 ± 2.3	4.4 ± 3.4	4.6 ± 3.6
Total	16 (7%)	94 (43%)	109 (50%)
Adiponectin (μg/ml)	4.5 ± 2.9	5.9 ± 4.3	6.3 ± 4.5

homozygotes for G/G allele was digested with *Sma*I, two restriction fragments of 216 bp and 156 bp were yielded (Figure 4, lane 1). In DNA from a homozygote for T/T allele, the PCR products was not cleaved, remaining as a 372 bp single band (Figure 4, lane 3). Among 142 non-obese subjects, 71 (50%) were homozygous for T/T, 61 (43%) were G/T heterozygotes, and 10 (7%) were homozygous for the G/G (Table 2). The frequency of the T allele was 71%, indicating that this was a frequent polymorphism and T allele was predominant in the population.

The mean (± s.d.) plasma adiponectin levels of the subjects with G/G, G/T and T/T allele were 4.5 ± 2.9 μg/ml, 5.9 ± 4.3 μg/ml, and 6.3 ± 4.5 μg/ml, respectively. Mean plasma adiponectin level in the subjects having G allele was low, but it was not statistically significant. Next, we compared the frequency of T allele in non-obese and obese subjects. Among 77 obese subjects, 33 (42%) were homozygous for T/T, 38 (49%) were G/T heterozygotes, and 6 (8%) were homozygous for the G/G (Table 2). Allelic frequency of T was 71%, respectively, resulting in no significant difference between non-obese

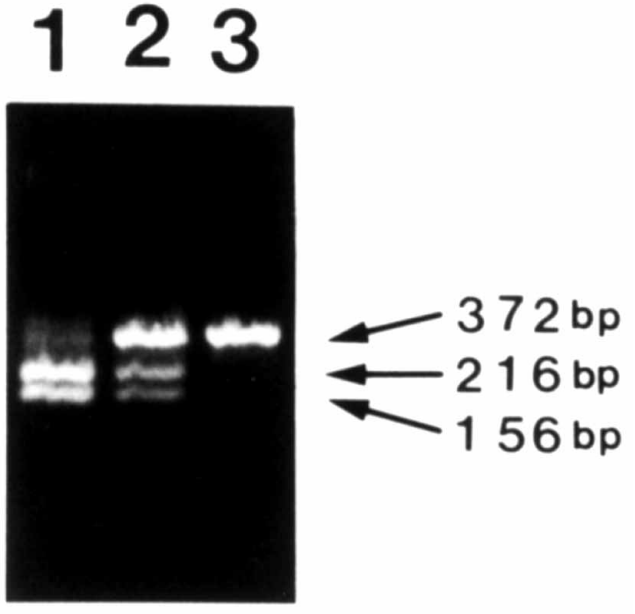

Figure 4 Results of polymerase-chain-reaction (PCR) screening for polymorphism in the gene for adiponectin. PCR amplification of DNA, followed by restriction-enzyme digestion and ethidium bromide-stained agarose-gel electrophoresis, was performed as described in the Materials and Methods section. When applied to a DNA sample that was homozygous for the G/G alleles at nucleotide 94 (lane 1), the restriction endonuclease *Sma*I cut the entire DNA sample into two fragments. In DNA from a sample heterozygous for the G/T alleles, fragments of three sizes were observed (lane 2). In DNA from a sample homozygous for the T/T alleles, the fragment amplified by PCR was not cleaved by the restriction enzyme, resulting in a single band (lane 3).

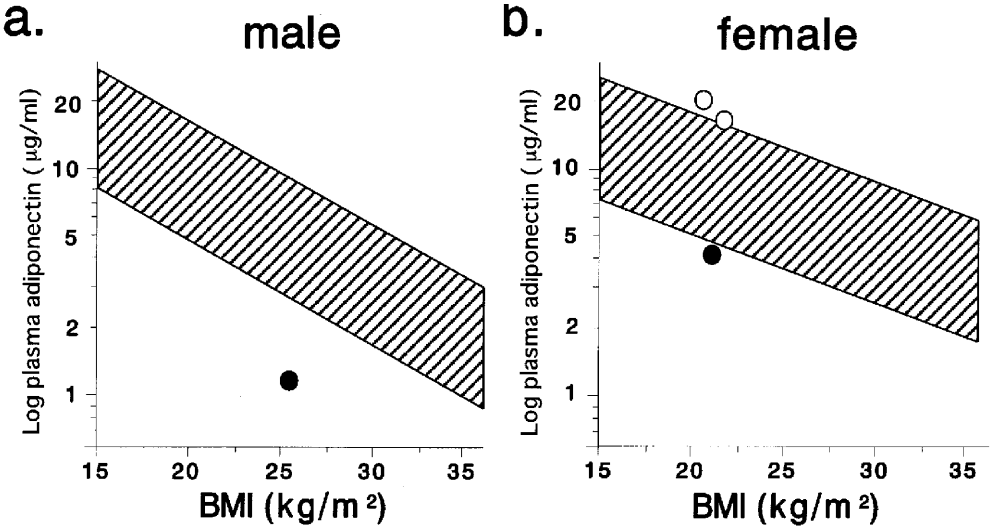

Figure 5 The range of plasma adiponectin concentrations. The hatched area represents the range of plasma adiponectin concentrations corresponding to BMI in clinically normal subjects (mean ± s.d.); a, male, and b, female. (a) The closed circle indicates the proband. (b) Closed and open circles indicate a daughter with carried R112C mutation and two daughters with wild type, respectively.

and obese subjects. We also analyzed the relationship between the polymorphism and the metabolic parameters affected by adipose tissue functions such as plasma levels of glucose, insulin, cholesterol, triglyceride and HDL-cholesterol. These parameters were not significantly different among the three groups.

The R112C proband carried the mutation with a heterozygous form. His plasma adiponectin concentration was as low as 1.16 μg/ml compared with the average concentration of non-obese men of 7.7 ± 3.1 μg/ml as previously reported,[7] whereas his BMI was normal as 25.3 kg/m^2. He suffered from coronary artery disease, lung thrombosis and autoimmune disease. He had one son and three daughters. Among them, only the second daughter carried the R112C mutation. She showed the low concentration of plasma adiponectin as 4.3 μg/ml in spite of her normal BMI (21.5 kg/m^2), whereas the first and third daughters carrying no mutation showed 20.0 and 14.6 μg/ml, respectively (Figure 5).

Discussion

The human body is composed of various types of cells. Each cell expresses a large number of housekeeping genes, required for cells to live, and fewer cell-specific genes to exert the specialized functions of the cell. Analysis of cell-type-specific genes will provide us with valuable information to understand the function of the cells. Several adipose-specific genes including aP2, adipsin[3] and leptin[5] have been isolated. Adiponectin is a novel gene exclusively expressed in human adipose tissue. In the current study, we have shown that adiponectin gene spanned 17 kb on chromosome 3q27, consisting of three exons and two introns. It shares significant homology with collagen X, VIII and complement factor C1q, and contains a collagenous structure at the NH2 terminus and a globular domain at the COOH terminus.[6] The translated region was interrupted by a single intron resembling the situation of human collagen X,[9] VIII[20] and C1q[21] genes (Figure 1). C1q protein forms a characteristic superstructure in which three protomers trimerize to form a collagen triple helix, and three trimers multimerize to form a 'bouquet'.[22] When viewed under the electron microscope, C1q protein appeared to bend at Gly36 in the middle of triple-helical chains corresponding to the point divided into two exons. Accordingly, it is possible that the boundary of two exons in the adiponectin gene corresponds to a bend in the collagen triplex formation.

The expression of adiponectin is especially limited to adipose tissue. Adiponectin had homology to mouse adipoQ/Acrp30 with 85% at cDNA level and with 83% at amino acid level, respectively. It has been reported that AdipoQ[23] or Acrp30[24] mRNA was induced more than 100-fold during differentiation of 3T3 preadipocytes[24] and AdipoQ mRNA levels in adipose tissue were markedly reduced in the obese.[23] We observed that the adiponectin concentrations in plasma negatively correlated with the degree of human obesity.[7] DNA fragment containing −1332 bp of adiponectin gene possessed promoter activity in 3T3-L1 adipocytes.[11] Adipsin is a protein predominantly produced by adipose tissue.[3] Expression of adipsin mRNA in adipose tissue is negatively regulated and its plasma concentrations are markedly decreased in obese rodents[14] but not in humans.[25] A comparison with promoters of the two genes would be important to understand the mechanism of obesity-induced downregulation. Platt *et al* reported that a 56-bp DNA fragment between −687 and −743 upstream from the start of the adipsin gene contained the suppressive element of expression in adipose tissue of obese mice.[15] Further, this fragment was bound by

proteins less abundantly present in nuclear extracts from obese mice than lean mice. They called the fragment ORE (obesity regulatory element). In comparison with the promoter of the adipsin gene, we identified two octamer elements at −233/−226, CCCAAGCT, and −328/−321, TTGCCCCA, perfectly corresponding to the sequence in ORE. The two octamers may be *cis*-elements of negatively regulated genes in the development of obesity.

Previously, several mutations in the adipose-specific genes have been described in humans with obesity. Genetic disruption of leptin causes obesity in humans as well as rodents.[26] Peroxisome-proliferator-activator receptor (PPAR)γ2 is a nuclear receptor regulating the differentiation of adipocytes. Two missense mutations in the human PPARγ2 gene have been reported. Constitutively active Pro 115 Gln mutation affects the Ser 114 phosphorylation site and is associated with obesity.[27] Some reports suggest the association of the Pro 12 Ala substitution with obesity,[28,29] although the participation of the mutation in the development of obesity still remains controversial.[30–32] Thus, mutations in adipose-specific genes may lead to obesity. In this study, we identified two nucleotide changes in human adiponectin gene in 219 unrelated subjects. One was a G to T mutation at nucleotide 94 in exon 2 without any amino acid conversion and we found no association between this polymorphism and obesity. The other was a C to T mutation at nucleotide 383 in exon 3 with significant amino acid conversion from arginine to cysteine (R112C) found in the non-obese subject with decreased plasma adiponectin level. The mutation was derived from the C to T transition occurred in CG dinucleotides regarded as hypermutable nucleotide in the human genome.[33] Although this mutation was rare, one daughter carrying this mutation also showed distinguishably decreased plasma adiponectin compared to the other siblings. The reason why this mutation causes the reduced plasma adiponectin level has been obscure in spite of their normal body mass indexes. This amino acid substitution will give rise to a change in ionic charge and conformation of the adiponectin protein, because the SH group has superseded an NH2 group. It is likely that the R112C substitution leads to the conformational change resulting in the reduction of plasma adiponectin concentration. In order to obtain definitive proof that the mutation presented here is responsible for the decreased plasma level, *in vitro* expression studies will be required.

The physiological function of adiponectin remains unclear to date. Adiponectin is a matrix-like protein with homology to collagens,[6] suggesting that it is secreted and anchored in the adipocyte milieu. This protein also has a structural similarity to complement factor C1q and thus belongs to the collectin family. It is well known that adipocytes secrete a variety of the factors in complement system including factor B,[34] C3,[35] adipsin (factor D), and acylation stimulating protein (ASP)/C3a.[36] Adiponectin may interact with these proteins and may promote the function of mature adipocytes such as triglyceride synthesis. The entrapment of adiponectin in the vicinity of adipocytes may be one of the mechanisms for decreased plasma concentration in obesity. Adiponectin is secreted into blood circulation may interact with vascular endotherial cells. Recently we found that adiponectin reduced the expression of adhesion molecules in endothelial cells.[37] Attachment of monocytes to endothelial cells is the first step of atherogenic vascular changes. Decreased plasma adiponectin may contribute to the development of atherogenic vascular diseases in obesity. It is notable that the subject carrying R112C mutation with low levels of plasma adiponectin level suffered from coronary artery disease and lung thrombosis. We could not conclude the significance of the mutation because it was found in only one patient. Further genetic study in a larger population will address the answer.

Acknowledgements

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