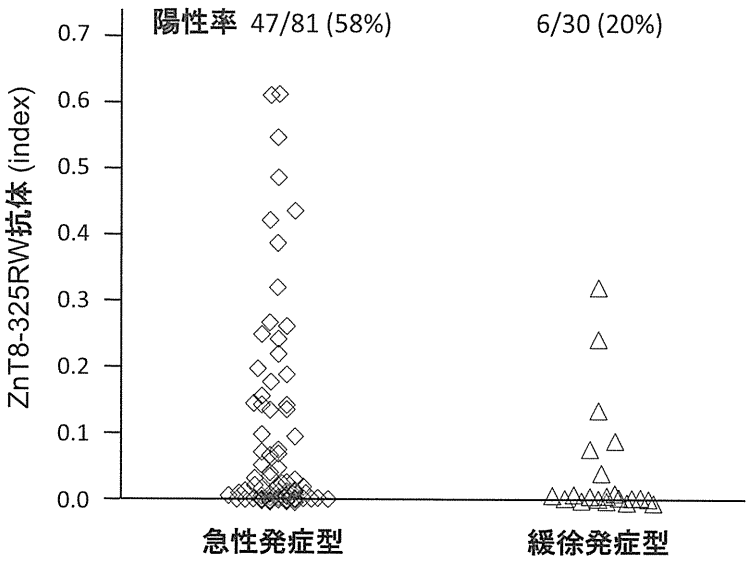

図１　発症様式別にみた日本人１型糖尿病におけるZnT8抗体の抗体価と陽性率
急性発症型におけるZnT8抗体の陽性率は緩徐発症型に比較し有意に高率であった（$p<0.0005$）。一方、ZnT8抗体陽性者における抗体価は両群間に差を認めなかった。

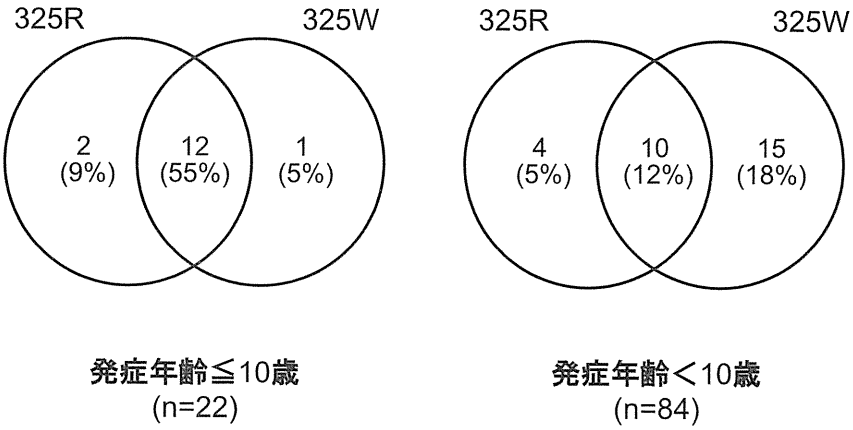

図２　発症年齢別にみたZnT8-325RおよびZnT8-325Wに対する反応
ZnT8-325RとZnT8-325Wの両者に反応する患者の割合は、発症年齢≦10歳群において有意に高率であった。

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

多施設共同研究：劇症１型糖尿病の診断マーカー同定と診断基準確立に関する研究

分担研究課題：劇症１型糖尿病患者末梢血 CD4+T 細胞における CTLA-4 発現の検討

分担研究者　島田　朗　慶應義塾大学医学部内科　講師

研究要旨

１型糖尿病の大部分は、T 細胞を主体とするリンパ球浸潤が膵島に認められることから、細胞性免疫異常が関与していると考えられているが、臨床の現場では、そのような細胞性免疫異常を的確に検出する方法が確立しておらず、専ら膵島に関連する自己抗原（GAD、インスリンなど）に対する自己抗体の検出に終始しているのが現状である。しかしながら、自己抗体の抗体価が必ずしも疾患活動性を規定しているわけではないことから、細胞性免疫異常の検出が求められている。

昨年、われわれは、細胞内サイトカイン染色のシステムを用いて抗原特異的な T 細胞の反応性を検出する方法を試み、劇症１型糖尿病を始めとする 1 型糖尿病患者において GAD 反応性の IFNγ産生 CD4 細胞（T-helper1 細胞）が検出されることを見出した。今回は、さらに、ELISPOT 法による抗原特異的 T 細胞反応の検出法についても確立し、１型糖尿病における主要抗原であるインスリンペプチドに対する反応性を検出することに成功した。また、主要な T-helper1 細胞の表面上に発現している CXCR3 をマーカーとして、それを有する免疫制御性 T 細胞についてもさらに詳細に検討するとともに、攻撃因子である effector 細胞（CD25 陰性 CD4 細胞）についても検討した。

A．研究目的

１型糖尿病の大部分は、T 細胞を主体とするリンパ球浸潤が膵島に認められることから、細胞性免疫異常が関与していると考えられている。今回、われわれは、劇症１型糖尿病における細胞性免疫異常の新規検出法を確立する目的で、GAD 反応性の IFNγ産生 CD4 細胞に加えて、ELISPOT 法による膵島抗原特異的 T 細胞反応の検出を試みた。さらに、昨年報告した、T-helper1 細胞の細胞表面上に発現している CXCR3 をマーカーとして、それを有する免疫制御性 T 細胞（CXCR3 陽性 Treg＝Hybrid Treg）につき、さらに詳細に検討した。この細胞は、動物モデルにおいて、１型糖尿病の発症制御に極めて重要であることが示されている。加えて、１型糖尿病における細胞性免疫異常において、膵島に対しての攻撃因子である effector 細胞（CD25 陰性 CD4 細胞）についても検討した。このような膵島抗原特異的 T 細胞の検出、Hybrid Treg の評価、さらには、effector 細胞の評価が、劇症１型糖尿病を始めとする１型糖尿病の新規診断法の確立に繋がることが強く期待されるのは言う迄もない。

B．研究方法

1）１型糖尿病患者（n＝200-300）のうち、（膵島関連）自己抗体（抗 GAD 抗体）陽性者をリクルートする。抗 GAD 抗体の測定は、コスミック社のキットを使用する。

2）抗 GAD 抗体陽性が確認された 1 型糖尿病患者に対して、倫理委員会の承認をすでに得

た書面を使用してインフォームドコンセントを取得し、HLA タイピングを行う。
3)患者末梢血リンパ球を採取し、CXCR3、CD4、CD25、Foxp3 の４カラーにて染色し、フローサイトメーターを用いて解析する。
4) 3)と同時に、ELISPOT 法ならびに細胞内サイトカインのシステムを用いた GAD 反応性 IFNγ産生 CD4 細胞を用いて、膵島抗原特異的 T 細胞反応の検出を試みる。具体的には、GAD、インスリンペプチドに対する反応性を中心に、IFNγ、および、IL10 産生能を検討する。
5) 抗 GAD 抗体、HLA、罹病年数と上記フローサイトメーターの解析により得られた Hybrid Treg ならびに effector 細胞との関係、さらには、膵島抗原特異的 T 細胞の反応性との関係を詳細に検討する。
6)上記の後、抗体陰性例についても同様の検討を行う。

C．研究結果

１型糖尿病患者（n＝200-300）のうち、（膵島関連）自己抗体（抗 GAD 抗体）陽性者をリクルートし、 抗 GAD 抗体陽性が確認された１型糖尿病患者、および対照としてインスリン治療中の抗 GAD 抗体陰性の確認された２型糖尿病患者に対して、倫理委員会の承認をすでに得た書面を使用してインフォームドコンセントを取得し、Hybrid Treg（CXCR3+CD4+CD25+Foxp3+）細胞、effector（CD25-CD4）細胞の検討、さらには、ELSPOT 法ならびに細胞内サイトカインのシステムを用いた膵島抗原特異的 T 細胞反応の検出を試みた。その結果、劇症１型糖尿病を始めとする１型糖尿病患者では、Hybrid Treg 数の年齢に応じた増加率が対照に比して弱いことが確認された。一方、effector 細胞数については、１型糖尿病患者において対照に比して有意に多く、さらに、年齢に応じた減少が、１型糖尿病患者でのみ認められた。ELISPOT 法を用いた膵島抗原特異的 T 細胞の反応性については、複数のインスリンペプチドに対する反応性が、新規発症の１型糖尿病患者において認められた。細胞内サイトカインのシステムを用いた GAD 反応性 IFNγ産生 CD4 細胞については、劇症１型糖尿病を含めて抗体陰性例でも反応性を認める例が存在した。ELISPOT 法を用いることにより、CD4 以外の T 細胞、多くは CD8 細胞の反応性を検出したものと考えられた。

D．考察

以上のように本研究では、１型糖尿病における細胞性免疫能の検出法の確立を試み、細胞内サイトカイン染色を用いた方法、ELSPOT 法に加えて、１型糖尿病の発症を制御するとされる Hybrid Treg、さらには、effector 細胞にも注目してその有用性を検討した。

E．結論

以上のように本研究では、１型糖尿病における細胞性免疫能の新規検出法を検討し、本年度の研究計画を予定通り終了した。

F．研究危険情報

なし

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H. 知的所有権の取得状況

1．特許取得　なし
2．実用新案登録　なし
3．その他　なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

多施設共同研究：劇症１型糖尿病の診断マーカー同定と診断基準確立に関する研究

分担課題名：劇症１型糖尿病解析における数式処理ソフトによる具体例の構成と計算

研究分担者　西村保一郎　大阪医科大学医学部　数学　教授

研究要旨

劇症１型糖尿病の統計解析に資する目的で、以下の研究を行った。数式処理ソフトを援用して、種々の非特異複素代数曲面の複素射影空間への埋め込みを解析した。

A. 研究目的

劇症１型糖尿病の統計解析に資するため、非特異代数曲面の射影空間への埋め込みの斉次イデアルを計算し、これを用いてヒルベルトスキームの斉次イデアルを構成することを通してヒルベルトスキームの性質を調べることを目的とする。

B. 研究方法

数式処理ソフトMacaulay2を用いて、曲面の埋め込みの斉次イデアルを具体的に計算する。それを用いて、曲面のヒルベルトスキームの斉次イデアルを構成する。

C. 研究結果

埋め込みを構成する技法、埋め込みの特徴を解析する技法、ヒルベルトスキームを構成する方法などの、数式処理ソフトでの複雑な計算の技法を獲得してきている。

D. 考察

曲面の斉次イデアルの研究の歴史は古く、現在まで広範な研究がなされて来ている。しかし、実際に斉次イデアルを計算するにはコンピュータが必要な場合が多く、それが可能になったのは新しいことである。実際に計算可能になったことから、更に詳細な研究が発展することが期待される。本研究はその方向の寄与を目指している。

E. 結論

最近のコンピュータの進歩に伴って、数式処理ソフトを用いた複雑な計算が行えるようになってきた。このような計算の実践を通して、代数曲面の射影空間への埋め込みに関する新たな知見を目指したい。これらの知見は劇症１型糖尿病の統計解析に有効となる可能性が期待される。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録情報

1. 特許取得　　なし
2. 実用新案登録　　なし
3. その他　　なし

Ⅳ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

該当なし

雑誌

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Ⅴ．研究成果の刊行物・別刷

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CASE REPORT

A case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency

Masaya Yamaoka · Tetsuhiro Kitamura · Hiroaki Moriyama · Yoshihito Shima · Fumitaka Haseda · Kohei Okita · Yukako Sakaguchi · Hiromi Iwahashi · Toshiaki Hanafusa · Tohru Funahashi · Masao Nagata · Michio Otsuki · Akihisa Imagawa · Iichiro Shimomura

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Abstract Several lines of evidence have suggested that pancreatic β-cell destruction is caused by inflammatory cellular responses mediated by T lymphocytes in individuals with type 1A diabetes. B lymphocytes, which play an important role in the production of autoantibodies to β-cell antigens such as insulin, glutamic acid decarboxylase (GAD) or insulinoma associated antigen 2 (IA-2) in type 1A diabetes, are also known as professional antigen-presenting cells and T-lymphocyte activators. Here, we report a case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency, which is known as a functional deficiency of B lymphocytes. A 51-year-old man was admitted to our hospital because of hyperglycemia. He had suffered from frequent bacterial infections from early childhood. At 16 years old, he was diagnosed with common variable immunodeficiency. At age 27, he experienced sudden-onset diabetic ketosis and was diagnosed with type 1 diabetes. Enzyme-linked immunospot (ELISPOT) assay recently revealed that interferon-γ-producing T lymphocytes but not interleukin 4-producing T lymphocytes, which react with GAD and insulin $B_{1\text{-}18}$, were present at increased levels in his peripheral blood at 51 years old. This case represents the longest reported interval between onset of type 1 diabetes and confirmation of cell-mediated autoimmunity against pancreatic β-cells in a patient with common variable immunodeficiency.

Keywords IDDM · CVID · ELISPOT · GAD · IA-2

M. Yamaoka · T. Kitamura · K. Okita · Y. Sakaguchi · H. Iwahashi · T. Funahashi · M. Otsuki · A. Imagawa (✉) · I. Shimomura
Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, 2-2-B5, Yamadaoka, Suita 565-0871, Japan
e-mail: aimagawa@endmet.med.osaka-u.ac.jp

H. Moriyama · M. Nagata
Department of Internal and Geriatric Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Y. Shima
Department of Respiratory Medicine and Allergy and Rheumatic Disease, Graduate School of Medicine, Osaka University, Suita, Japan

F. Haseda · T. Hanafusa
Department of Internal Medicine (I), Osaka Medical College, Takatsuki, Japan

Introduction

Several lines of evidence have suggested that pancreatic β-cell destruction is caused by inflammatory cellular responses mediated by T lymphocytes in individuals with type 1A diabetes [1, 2]. B lymphocytes, which play an important role in the production of autoantibodies to β cell antigens such as insulin, GAD or IA-2 in type 1A diabetes patients, are also known as professional antigen-presenting cells and T-lymphocyte activators.

Here, we report a case of long-standing autoimmune type 1 diabetes with common variable immunodeficiency (CVID), which is a functional deficiency of B lymphocytes.

Case report

In 2009, a 51-year-old man was admitted to our hospital because of hyperglycemia. He had suffered from frequent bacterial infections from early childhood. At 16 years old, he was diagnosed with CVID. At age 27, he had sudden-

onset diabetic ketosis and was diagnosed with type 1 diabetes. At admission to our hospital, his blood glucose level was 306 mg/dl, and urinary C peptide excretion was reduced to 8.7 μg/day. The serum IgG level was as low as 340 mg/dl (normal range 870–1,700 mg/dl), the IgA level was 57 mg/dl (normal range 110–410 mg/dl), and the IgM level was 31 mg/dl (normal range 35–220 mg/dl). Subcutaneous injection of insulin was started. He has been treated with monthly intravenous injections of γ-globulin (5,000 mg) since the age of 45. Immunoglobulin supplementation led to a marked reduction in the number of infections. Islet cell antibodies (ICA) and GAD antibodies were not detected either at 46 years old or on admission. IA-2 antibody, insulin autoantibodies (IAA), antinuclear antibody, thyroid-stimulating hormone (TSH) receptor antibody and thyroglobulin antibody tests were also negative.

Laboratory data on admission revealed normal blood count and blood chemistry. However, the CD19+ B-lymphocyte level (139/μl) but not the CD3+ T-lymphocyte level (1,160/μl) was decreased. The CD4/CD8 ratio had decreased to 30.5/46.0 (0.66). The HLA haplotypes of *DRB1-DQB1* were **04:05-*04:01* and **11:01-*03:01*. A glucagon tolerance test revealed the complete loss of endogenous insulin secretion capacity; the serum C-peptide concentrations before and 6 min after injection were undetectable (below 0.01 ng/ml).

We measured the responses of pancreatic β-cell-reactive peripheral T lymphocytes using an immunoglobulin-free enzyme-linked immunospot (ELISPOT) assay as described previously [3]. The mean number of interferon (IFN)-γ spots reactive to GAD_{65} and insulin $B_{1\text{-}18}$ peptide was 7.5 and 3.5, respectively, in a duplicate assay. Interleukin (IL)-4 spots reactive to those peptides were not detected. Neither IFN-γ spots nor IL-4 spots reacted to insulin $B_{9\text{-}23}$, $B_{10\text{-}24}$, $A_{1\text{-}15}$ and $L_{7\text{-}23}$. To compare the positivity among the other diabetic patients and control subjects in ELISPOT assay, the mean number of antigen-stimulated IFN-γ spots reactive to GAD_{65} was plotted after subtracting the background (T cells only). A significant IFN-γ response to the GAD_{65} peptide was observed in this patient (Fig. 1a). Data for other patients with type 1A diabetes and type 2 diabetes and for healthy controls were taken from our previous report [3].

Two-color flow cytometric analysis revealed decreased numbers of $CD10^-$ $CD19^+$ cells and $CD10^-$ $CD22^+$ cells (mature B lymphocytes) (Fig. 1b, c). However, the numbers of $CD10^+$ $CD19^-$ cells and $CD10^+$ $CD22^-$ cells (immature B lymphocytes) were not increased (Fig. 1b, c). The reference values of our methods were less than 1.0% for $CD10^+$ cells, 5.0–24.0% for $CD19^+$ cells and 2.0–17.0% for $CD22^+$ cells. $CD4^+$ $FoxP3^+$ regulatory T cells were 2.5% in this patient, while they were 3.6 (1.2–5.1)% [median (range)] in 20 healthy individuals [16].

Discussion

We report the first case of established T cell immunity in an autoimmune type 1 (type 1A) diabetes patient with CVID. Islet autoantibodies were not detected; however, an ELISPOT assay, a useful tool to detect T-lymphocyte-mediated autoimmunity directly with good reproducibility in type 1 diabetes patients [3, 4], revealed GAD- and insulin $B_{1\text{-}18}$-reactive Th1 cells, but not GAD- and insulin $B_{1\text{-}18}$-reactive Th2 cells among the peripheral lymphocytes in this patient. T-lymphocyte reactivity specific to beta cell

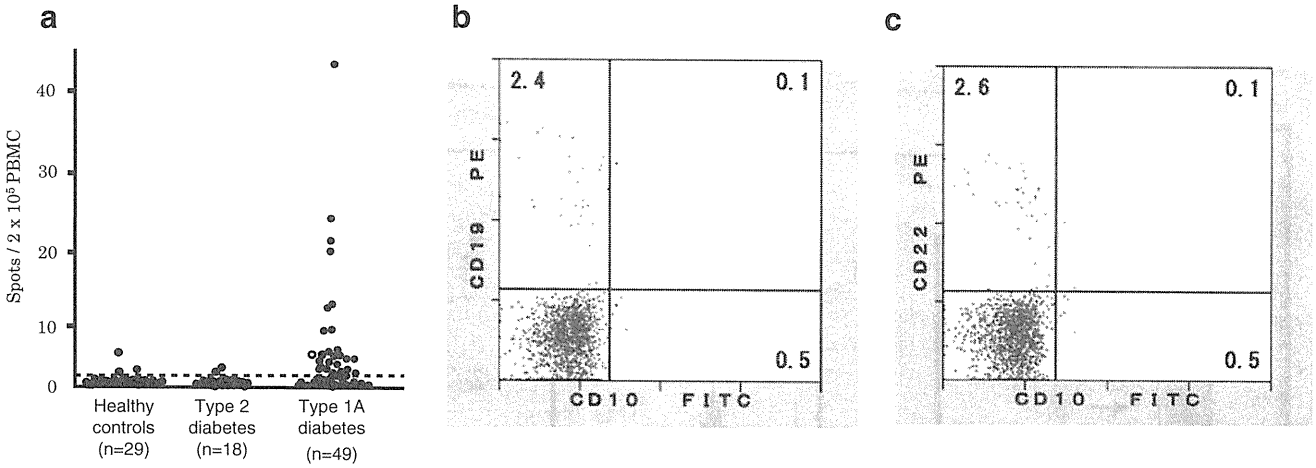

Fig. 1 **a** IFN-γ spots reactive to GAD_{65} in ELISPOT assays for subjects with type 1A diabetes or type 2 diabetes and for normal control subjects. The *open circle* represents our patient. Other data were taken from reference 3. Two-color flow cytometric analysis of our patient's PBMCs. A decreased number of $CD10^-$ $CD19^+$ cells and a normal number of $CD10^+$ $CD19^-$ cells (**b**) and a decreased number of $CD10^-$ $CD22^+$ cells and a normal number of $CD10^+$ $CD22^-$ cells (**c**) are shown

antigens of insulin B_{1-18} suggested the presence of a beta-cell-specific immune response. Higashide et al. [17] have reported that insulin B_{1-15} reactive Th1 cells are present in 6 of 18 recent-onset type 1 diabetic patients by ELISPOT assay, also suggesting the presence of insulin B_{1-18}-reactive Th1 cells in this patient indicates a long-lasting autoimmune response rather than acquired response induced by the long-lasting insulin treatment. There have already been several reports of probable type 1 diabetes with CVID, and ICA were detected in one of these patients. However, these patients did not have T lymphocyte immune reactivity to β cells at all [5–9].

CVID is a primary immune disorder characterized by hypogammaglobulinemia, antibody deficiency and recurrent infections [10]. This patient had (1) repeated infections in his childhood but not in his babyhood, (2) low levels of IgM, IgG and IgA in sera, (3) a normal number of T lymphocytes and (4) decreased but not absent B lymphocytes in his peripheral blood.

Our examination suggested that both the number and function of B lymphocytes were reduced in this patient. Laboratory data revealed a decreased $CD19^+$ B-lymphocyte level (139/μl). Flow cytometric analysis revealed no insufficient maturation of B lymphocytes in this patient. The functional deficiency of B lymphocytes was not directly observed; however, the history of repeated infections and improvement resulting from γ-globulin supplementation suggests non-specific functional loss of B lymphocytes. The lack of islet-related autoantibodies in spite of the positive reaction for his T lymphocytes against islet autoantigens might also indicate the reduction of B-lymphocyte function. On the other hand, the number of T lymphocyte including regulatory T cells, was normal in this patient.

This patient suffers from type 1A diabetes. This fact might indicate that B-lymphocyte insufficiency is not essential to the development of human type 1A diabetes despite the evidence in non-obese diabetic (NOD) mice, a rodent model [11–13]. The number of B lymphocytes in human insulitis lesions is low [2]. The effect of anti-CD20 therapy was limited to the patients with established autoimmune type 1 diabetes [14]. Type 1 diabetes has even been reported in a patient with X-linked severe agammaglobulinemia [15]. All of these findings suggest that B lymphocytes are not necessary to develop autoimmune β cell destruction in humans. Our present case with type 1A diabetes and CVID supports this concept for human type 1A diabetes. In addition, our patient had a positive reaction of T lymphocytes to islet autoantigens even 24 years after the onset of type 1 diabetes. These results might indicate that B-lymphocyte-mediated immunodeficiency was able to maintain anti-β cell autoimmunity long after disease onset.

Autoimmune diseases are more frequent in CVID patients than in general population [18, 19]. However, the prevalence of type 1 diabetes in CVID patients is not well documented. The established diagnosis of type 1 diabetes is sometimes difficult in CVID patients because islet autoantibodies are negative despite the presence of islet autoimmunity shown in the present case. It may underrepresent the prevalence of type 1 diabetes in CVID patients.

In conclusion, this case represents the longest reported interval between onset of type 1 diabetes and confirmation of cell-mediated autoimmunity against pancreatic β-cells in a patient with CVID.

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ORIGINAL ARTICLE

Genetic variations in the *CYP17A1* and *NT5C2* genes are associated with a reduction in visceral and subcutaneous fat areas in Japanese women

Kikuko Hotta[1], Aya Kitamoto[1], Takuya Kitamoto[1], Seiho Mizusawa[2], Hajime Teranishi[2], Tomoaki Matsuo[3], Yoshio Nakata[3], Hideyuki Hyogo[4], Hidenori Ochi[4], Takahiro Nakamura[5], Seika Kamohara[6], Nobuyuki Miyatake[7], Kazuaki Kotani[8], Ryoya Komatsu[9], Naoto Itoh[10], Ikuo Mineo[11], Jun Wada[12], Masato Yoneda[13], Atsushi Nakajima[13], Tohru Funahashi[14], Shigeru Miyazaki[15], Katsuto Tokunaga[16], Hiroaki Masuzaki[17], Takato Ueno[18], Kazuaki Chayama[4], Kazuyuki Hamaguchi[19], Kentaro Yamada[20], Toshiaki Hanafusa[21], Shinichi Oikawa[22], Hironobu Yoshimatsu[23], Toshiie Sakata[23], Kiyoji Tanaka[3], Yuji Matsuzawa[8], Kazuwa Nakao[1,24] and Akihiro Sekine[1,2]

Visceral fat accumulation has an important role in increasing the morbidity and mortality rates, by increasing the risk of developing several metabolic disorders, such as type 2 diabetes, dyslipidemia and hypertension. New genetic loci that are associated with increased systolic and diastolic blood pressures have been identified by genome-wide association studies in Caucasian populations. This study investigates whether single nucleotide polymorphisms (SNPs) that confer susceptibility to high blood pressure are also associated with visceral fat obesity. We genotyped 1279 Japanese subjects (556 men and 723 women) who underwent computed tomography for measuring the visceral fat area (VFA) and subcutaneous fat area (SFA) at the following SNPs: *FGF5* rs16998073, *CACNB2* rs11014166, *C10orf107* rs1530440, *CYP17A1* rs1004467, *NT5C2* rs11191548, *PLEKHA7* rs381815, *ATP2B1* rs2681472 and rs2681492, *ARID3B* rs6495112, *CSK* rs1378942, *PLCD3* rs12946454, and *ZNF652* rs16948048. In an additive model, risk alleles of the *CYP17A1* rs1004467 and *NT5C2* rs11191548 were found to be significantly associated with reduced SFA (P=0.00011 and 0.0016, respectively). When the analysis was performed separately in men and women, significant associations of rs1004467 (additive model) and rs11191548 (recessive model) with reduced VFA (P=0.0018 and 0.0022, respectively) and SFA (P=0.00039 and 0.00059, respectively) were observed in women, but not in men. Our results suggest that polymorphisms in the *CYP17A1* and *NT5C2* genes influence a reduction in both visceral and subcutaneous fat mass in Japanese women.
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Keywords: computed tomography; *CYP17A1*; Japanese subjects; *NT5C2*; sexual dimorphism; subcutaneous fat area; visceral fat area

INTRODUCTION

Metabolic syndrome is a combination of multiple risk factors, including central obesity, impaired glucose tolerance, dyslipidemia and hypertension, which increases cardiovascular disease morbidity and mortality.[1] Several studies have indicated that the intra-abdominal adipose tissue has a central role in metabolic syndrome, as the

[1]EBM Research Center, Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Center for Genomic Medicine, Unit of Genome Informatics, Kyoto University Graduate School of Medicine, Kyoto, Japan; [3]Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan; [4]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; [5]Laboratory for Mathematics, National Defense Medical College, Tokorozawa, Japan; [6]Health Science University, Yamanashi, Japan; [7]Department of Hygiene, Faculty of Medicine, Kagawa University, Kagawa, Japan; [8]Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Osaka, Japan; [9]Rinku General Medical Center, Osaka, Japan; [10]Toyonaka Municipal Hospital, Osaka, Japan; [11]Otemae Hospital, Osaka, Japan; [12]Department of Medicine and Clinical Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan; [13]Division of Gastroenterology, Yokohama City University Graduate School of Medicine, Yokohama, Japan; [14]Department of Metabolism and Atherosclerosis, Graduate School of Medicine, Osaka University, Osaka, Japan; [15]Tokyo Postal Services Agency Hospital, Tokyo, Japan; [16]Itami City Hospital, Hyogo, Japan; [17]Division of Endocrinology and Metabolism, Second Department of Internal Medicine, University of the Ryukyus Faculty of Medicine, Okinawa, Japan; [18]Research Center for Innovative Cancer Therapy, Kurume University, Kurume, Japan; [19]Department of Community Health and Gerontological Nursing, Faculty of Medicine, Oita University, Oita, Japan; [20]Division of Endocrinology and Metabolism, Department of Medicine, Kurume University, Kurume, Japan; [21]Department of Internal Medicine (I), Osaka Medical College, Osaka, Japan; [22]Division of Endocrinology and Metabolism, Department of Medicine, Nippon Medical School, Tokyo, Japan; [23]Department of Internal Medicine 1, Faculty of Medicine, Oita University, Oita, Japan and [24]Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Kyoto, Japan

Correspondence: Dr K Hotta, EBM Research Center, Kyoto University Graduate School of Medicine, Yoshida-Konoecho, Sakyo-ku, Kyoto 606-8501, Japan.
E-mail: kikukoh@kuhp.kyoto-u.ac.jp
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accumulated visceral adipose tissue leads to alterations in the plasma levels of adipocytokines, resulting in the development of dyslipidemia, hypertension and insulin resistance.[2,3] Intra-abdominal fat accumulation (central adiposity) is determined by waist circumference, waist–hip ratio, biological impedance or the visceral fat area (VFA) measured using computed tomography.[1,4,5] There is abundant evidence that body fat distribution is influenced by genetic loci.[6–8] Individual variation in waist–hip ratio is heritable, with heritability estimates ranging from 22 to 61%. Recent genome-wide association studies (GWAS) showed that genetic loci were associated with waist circumference and waist–hip ratio in the Caucasian population.[9,10] We previously reported that the rs1558902 and rs1421085 genotypes of the fat mass- and obesity-associated gene (*FTO*) were significantly associated with VFA, as well as with the subcutaneous fat area (SFA) and body mass index (BMI) in the Japanese population.[11]

Recent progress in GWAS has increased the number of known genetic susceptibility loci for obesity.[12–16] We investigated the association between the single nucleotide polymorphisms (SNPs) underlying susceptibility to obesity and fat distribution (as determined by computed tomography), and found that rs7498665 in the SH2B adaptor protein 1 (*SH2B1*) gene was associated with VFA, uncovering the genetic background of central obesity.[17]

GWAS, and meta-analysis of GWAS, have identified various disease-associated genetic variations.[18] Hypertension is one of the risk factors of metabolic syndrome and is considerably related to central obesity. Obesity-associated allele of rs1558902 and rs1421085 in the *FTO* gene were associated with hypertension, but not that of rs7498665 in the *SH2B1* gene in the Japanese population.[19] The genetic variations associated with hypertension have been identified by GWAS.[20,21] In this study, we investigate whether the recently reported hypertension-related loci are also associated with VFA, which is another important factor responsible for metabolic syndrome.

MATERIALS AND METHODS

Study subjects

We enrolled 1279 Japanese subjects from outpatient clinics; these patients agreed to undergo computed tomography testing (in the supine position) to determine VFA and SFA values at the umbilical level (L4–L5), as previously reported.[17] Both VFA and SFA values were calculated using the FatScan software program (N2system, Osaka, Japan).[22] The patients visited the hospitals to undergo treatment for obesity and/or metabolic abnormalities, such as hypertension, dyslipidemia and type 2 diabetes. Patients with secondary obesity and obesity-related hereditary disorders were excluded from this study. Patients with disease (such as cancer, and renal, heart and hepatic failure), or under treatment (such as corticosteroid and chemotherapy) that strongly affects body weight, were also excluded. Athletes were also excluded from this study. Clinical data were recorded at the first visit to the hospital. The clinical characteristics of the subjects are summarized in Table 1. Metabolic syndrome and metabolic abnormalities were diagnosed according to the criteria released by the Japanese Committee for the Diagnostic Criteria of Metabolic Syndrome in April 2005.[4,5] Written informed consent was obtained from each subject, and the protocol was approved by the ethics committee of each institution and by that of Kyoto University.

DNA extraction and SNP genotyping

Genomic DNA was extracted from the blood samples collected from each subject using the Genomix kit (Talent Srl, Trieste, Italy). We selected 12 SNPs that were previously identified as susceptibility loci for hypertension by GWAS in Caucasian populations,[20,21] and constructed Invader probes (Third Wave Technologies, Madison, WI, USA) for each. The 12 selected SNPs were as follows: rs16998073 in the fibroblast growth factor 5 (*FGF5*) gene; rs11014166 in the calcium channel, voltage-dependent, β-2 subunit (*CACNB2*) gene; rs1530440 in the chromosome 10 open reading frame 107 (*C10orf107*) gene; rs1004467 in the cytochrome P450, family 17, subfamily A, polypeptide 1 (*CYP17A1*) gene; rs11191548 in the 5′-nucleotidase, cytosolic II (*NT5C2*) gene; rs381815 in the pleckstrin homology domain containing, family A member 7 (*PLEKHA7*) gene; rs2681472 and rs2681492 in the ATPase, Ca^{2+} transporting, plasma membrane 1 (*ATP2B1*) gene; rs6495112 in the AT-rich interactive domain 3B (BRIGHT-like) (*ARID3B*) gene; rs1378942 in the c-src tyrosine kinase (*CSK*) gene; rs12946454 in the phospholipase C, delta 3 (*PLCD3*) gene; and rs16948048 in the zinc finger protein 652 (*ZNF652*) gene. The SNPs were genotyped using Invader assays, as previously described.[23] The success rate of these assays was >99.0%.

Table 1 Clinical characteristics of the subjects

	Men	*Women*	*Total*
n	556	723	1279
Age (years)	49.4 ± 12.2	52.2 ± 11.3	51.0 ± 11.8
BMI (kg m^{-2})	30.2 ± 6.1	28.1 ± 5.3	29.0 ± 5.8
VFA (cm^2)	155.3 ± 67.7	99.8 ± 53.6	123.9 ± 66.1
SFA (cm^2)	206.7 ± 108.6	241.6 ± 97.2	226.5 ± 103.7
Waist circumference (cm)	97.5 ± 11.3	91.8 ± 10.3	94.2 ± 11.1
Prevalence of metabolic disease			
Dyslipidemia	293 (53%)	244 (34%)	537 (42%)
Hypertension	379 (68%)	452 (63%)	831 (65%)
Impaired fasting glucose	177 (32%)	176 (24%)	353 (28%)
Metabolic syndrome	248 (45%)	162 (22%)	410 (32%)

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; VFA, visceral fat area.
Data are represented as mean ± s.d.

Statistical analysis

For the additive model, we coded the genotypes as 0, 1 or 2 depending on the number of copies of the risk alleles. For the recessive model, homozygosity with the risk allele was coded as 1 and the others were coded as 0. Risk alleles refer to the hypertension-associated alleles, according to previous reports.[20,21] Multiple linear regression analyses were performed to test the independent effect of the risk alleles on BMI, VFA and SFA, by taking into account the effects of other variables (that is, age and gender) that were assumed to be independent of the effect of each SNP. The values of BMI, VFA and SFA were logarithmically transformed before performing the multiple linear regression analysis. Differences in the quantities of anthropometric parameters among the different genotypes were assessed by the analysis of covariance, by taking into account the effects of other variables (that is, age and/or institute). Hardy–Weinberg equilibrium was assessed using the χ^2-test.[24] To test SNP×SNP epistasis, we used a linear regression model for each SNP1 and SNP2, and fit the model in the form of $Y=\beta_0+\beta_1\times\mathrm{SNP1}+\beta_2\times\mathrm{SNP2}+\beta_3\times\mathrm{SNP1}\times\mathrm{SNP2}+\beta_4\times\mathrm{age}+\beta_5\times\mathrm{gender}$. Although we collected the samples at the region of Hondo (Kanto, Kinki, Chugoku and Kyushu; Supplementary Table 1), we performed Wright's F-statistics[25] to evaluate the difference in the population structures of our sample using randomly selected 31 SNPs. We divided our samples into two groups (SFA >208 cm^2 and ⩽208 cm^2). Median of SFA (208 cm^2) was used as a cut-off value. The results indicated that the population structure of the two groups were almost the same in view of a very small F_{ST} value between both the groups (mean F_{ST}=0.00023). Statistical analysis was performed using R software (http://www.r-project.org/). *P*-values were assessed with a Bonferroni correction and $P<0.0042$ (0.05/12) was considered statistically significant.

RESULTS

The clinical characteristics and genotypes of the subjects are shown in Tables 1 and 2, respectively. All the SNPs were in Hardy–Weinberg equilibrium and the minor allele frequencies did not diverge from those reported in the HapMap database. The BMI, VFA and SFA values for each SNP genotype are reported in Table 3. Multiple linear regression analyses of the anthropometric parameters with respect to the 12 analyzed SNPs are shown in Table 4. The A-allele of rs1004467

Table 2 Genotypic characteristics of the subjects

SNP ID	*CHR*	*Position (Build 36.3)*	*Nearby gene*	*Allele 1/2*	*BP-associated allele*	*Genotype*	*HWE P-value*
rs16998073	4	81 403 365	*FGF5*	T/A	T	120/514/644	0.24
rs11014166	10	18 748 804	*CACNB2*	T/A	A	4/124/1151	0.73
rs1530440	10	63 194 597	*C10orf107*	T/C	C	30/296/953	0.22
rs1004467	10	104 584 497	*CYP17A1*	A/G	A	559/567/153	0.62
rs11191548	10	104 836 168	*NT5C2*	T/C	T	675/504/100	0.66
rs381815	11	16 858 844	*PLEKHA7*	C/T	T	842/381/56	0.13
rs2681472	12	88 533 090	*ATP2B1*	A/G	A	546/562/171	0.17
rs2681492	12	88 537 220	*ATP2B1*	C/T	T	168/561/549	0.19
rs6495112	15	72 619 851	*ARID3B*	A/C	A	530/575/173	0.39
rs1378942	15	72 864 420	*CSK*	A/C	C	49/410/817	0.78
rs12946454	17	40 563 647	*PLCD3*	T/A	T	34/343/901	0.84
rs16948048	17	44 795 465	*ZNF652*	G/A	G	18/326/935	0.08

Abbreviations: BP, blood pressure; CHR, chromosome; HWE, Hardy–Weinberg equilibrium.

Table 3 Mean BMI, VFA and SFA for 12 blood pressure risk variants

		Mean ± s.d.								
		BMI (kg m^{-2})			*VFA (cm^2)*			*SFA (cm^2)*		
		Genotype			*Genotype*			*Genotype*		
SNP ID	*Nearby gene*	*11*	*12*	*22*	*11*	*12*	*22*	*11*	*12*	*22*
rs16998073	*FGF5*	28.8 ± 4.7	29.0 ± 5.8	29.0 ± 6.0	126.2 ± 66.1	121.6 ± 66.5	125.3 ± 65.9	227.4 ± 98.3	224.5 ± 111.0	227.7 ± 98.7
rs11014166	*CACNB2*	27.0 ± 2.7	29.6 ± 6.1	28.9 ± 5.8	123.4 ± 82.2	136.7 ± 68.2	122.5 ± 65.8	178.6 ± 35.8	233.7 ± 106.4	225.8 ± 103.6
rs1530440	*C10orf107*	30.8 ± 6.5	28.6 ± 5.4	29.1 ± 5.9	129.8 ± 63.1	120.2 ± 66.4	124.9 ± 66.2	236.4 ± 119.2	223.0 ± 91.4	227.2 ± 106.9
rs1004467	*CYP17A1*	28.4 ± 5.6	29.5 ± 6.1	29.4 ± 5.2	117.5 ± 64.9	130.6 ± 68.5	122.5 ± 59.3	215.5 ± 92.7	231.4 ± 111.5	247.9 ± 107.9
rs11191548	*NT5C2*	28.6 ± 5.8	29.5 ± 5.9	28.9 ± 5.1	119.2 ± 65.3	130.9 ± 68.6	120.5 ± 55.7	217.2 ± 96.0	238.5 ± 113.2	228.1 ± 98.8
rs381815	*PLEKHA7*	29.2 ± 5.9	28.7 ± 5.7	27.8 ± 4.3	124.1 ± 64.2	124.3 ± 71.5	117.9 ± 55.9	229.4 ± 105.9	221.5 ± 101.6	215.3 ± 83.1
rs2681472	*ATP2B1*	29.2 ± 5.8	28.8 ± 5.4	29.0 ± 7.0	127.1 ± 67.5	121.5 ± 64.8	121.8 ± 65.8	227.4 ± 100.4	223.5 ± 100.3	233.1 ± 123.6
rs2681492	*ATP2B1*	29.0 ± 7.1	28.7 ± 5.2	29.3 ± 5.9	121.9 ± 66.3	121.4 ± 64.8	127.0 ± 67.4	234.6 ± 123.9	221.9 ± 98.3	228.7 ± 102.3
rs6495112	*ARID3B*	28.9 ± 5.8	29.0 ± 5.7	29.3 ± 6.2	122.5 ± 63.4	125.0 ± 69.2	124.9 ± 64.3	223.7 ± 106.5	229.5 ± 102.6	225.1 ± 99.2
rs1378942	*CSK*	28.0 ± 4.2	28.9 ± 6.1	29.1 ± 5.7	110.0 ± 63.3	121.8 ± 62.4	125.6 ± 67.6	222.9 ± 84.5	225.9 ± 104.3	227.0 ± 104.7
rs12946454	*PLCD3*	30.1 ± 8.2	28.6 ± 5.0	29.1 ± 5.9	137.2 ± 80.0	123.0 ± 67.4	123.7 ± 65.1	254.4 ± 105.0	216.4 ± 93.7	229.2 ± 107.0
rs16948048	*ZNF652*	28.1 ± 2.8	29.4 ± 5.9	28.9 ± 5.8	128.6 ± 73.7	124.8 ± 65.9	123.5 ± 66.1	215.0 ± 60.6	227.0 ± 96.3	226.5 ± 106.9

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area.
11, allele1/allele1; 12, allele1/allele2; 22, allele2/allele2. Allele 1 and allele 2 of each SNP is indicated in Table 2.

in the *CYP17A1* gene was significantly associated with reduced BMI (P=0.0018). The other SNPs were not significantly associated with BMI. No SNP was significantly associated with VFA. The A-allele of rs1004467 in the *CYP17A1* and the T-allele of rs11191548 in the *NT5C2* gene were significantly associated with reduced SFA. These SNPs are in linkage disequilibrium, as reported in the HapMap database (D'=0.98, r^2=0.71), and the A-allele of rs1004467 and T-allele of rs11191548 are reported to be risk alleles for increased blood pressure.[20,21]

BMI, VFA and SFA are known to be affected by gender; therefore, we compared rs1004467 and rs11191548 alleles with anthropometric parameters (BMI, VFA and SFA) in men and women independently (Table 5). Associations of both SNPs with VFA (P=0.0018 and P=0.0043) and SFA (P=0.00039 and P=0.0021) in women were significant, except the association of T-allele of rs11191548 with VFA. The VFA and SFA values of the rs11191548 genotype suggest that the recessive model would be the best-fitted model both in men and women. By using the recessive model, results revealed significant associations of the rs11191548 genotype with VFA (P=0.0022) and SFA in women (P=0.00059). These SNPs did not show any association with VFA or SFA in men, suggesting that they exhibit sexual dimorphism, as has been suggested in a recent report.[26] As both rs1004467 and rs11191548 were associated with a reduction in both VFA and SFA, we examined the association of these SNPs with total fat area. The SNPs were significantly associated with total fat area (P=0.00012 at rs1004467, P=0.00052 at rs11191548 in additive model) in women, but not in men, suggesting that risk allele for high blood pressure of these SNPs are associated with reduced adiposity in women. The very small mean F_{ST} value (0.00023) indicated no population structure in our subjects. As we collected the samples from nine institutes in four regions of Japan (Supplementary Table 1), we tested multiple linear regression analysis with age and institute as explanatory variables in men and women. Very similar results were observed. In additive model, significant associations of the

Table 4 Relationship between blood pressure-associated loci and adiposity measures

		BMI			*VFA*			*SFA*		
SNP ID	*Nearby gene*	β	*s.e.*	P-*value*	β	*s.e.*	P-*value*	β	*s.e.*	P-*value*
rs16998073	*FGF5*	−0.002	0.003	0.55	−0.003	0.010	0.78	−0.010	0.008	0.22
rs11014166	*CACNB2*	−0.005	0.007	0.48	−0.043	0.021	0.043	−0.008	0.017	0.63
rs1530440	*C10orf107*	−0.002	0.004	0.71	0.010	0.014	0.48	−0.005	0.011	0.64
rs1004467	*CYP17A1*	−0.010	0.003	**0.0018**	−0.022	0.010	0.027	−0.030	0.008	**0.00011**
rs11191548	*NT5C2*	−0.008	0.003	0.015	−0.019	0.011	0.078	−0.026	0.008	**0.0016**
rs381815	*PLEKHA7*	−0.007	0.004	0.046	−0.004	0.012	0.76	−0.015	0.009	0.10
rs2681472	*ATP2B1*	0.002	0.003	0.43	0.006	0.010	0.52	0.005	0.008	0.49
rs2681492	*ATP2B1*	0.003	0.003	0.34	0.006	0.010	0.54	0.006	0.008	0.40
rs6495112	*ARID3B*	−0.002	0.003	0.45	−0.004	0.010	0.65	−0.007	0.008	0.36
rs1378942	*CSK*	0.005	0.004	0.20	0.010	0.012	0.40	0.005	0.009	0.61
rs12946454	*PLCD3*	−0.003	0.004	0.39	0.009	0.013	0.50	−0.011	0.010	0.28
rs16948048	*ZNF652*	0.005	0.004	0.30	0.008	0.014	0.57	0.005	0.011	0.67

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area.
Data were derived from a linear regression analysis. The values of BMI, VFA and SFA were logarithmically transformed. Logarithmically transformed BMI, VFA and SFA were adjusted for age and gender. Tested alleles are risk alleles of increased blood pressure.

Table 5 Relationship between rs1004467 and rs11191548, and adiposity in men and women

			Values at each genotype			*Additive model*		*Recessive model*	
SNP ID (gene)	*Phenotype*	*Gender*	*11*	*12*	*22*	β *(s.e.)*	P-*value*	β *(s.e.)*	P-*value*
rs1004467	*n*	Men	233	259	64				
(*CYP17A1*)		Women	326	308	89				
	BMI ($kg\,m^{-2}$)	Men	29.7 ± 6.6	30.6 ± 5.9	30.1 ± 5.1	−0.011 (0.005)	0.029	−0.017 (0.006)	0.0085
		Women	27.6 ± 4.6	28.5 ± 6.0	28.9 ± 5.3	−0.010 (0.004)	0..017	−0.013 (0.006)	0.019
	VFA (cm^2)	Men	152.9 ± 67.7	160.6 ± 69.2	142.8 ± 59.9	0.004 (0.014)	0.78	−0.012 (0.019)	0.52
		Women	92.3 ± 49.2	105.4 ± 56.8	107.8 ± 54.7	−0.044 (0.014)	**0.0018**	−0.061 (0.019)	**0.0014**
	SFA (cm^2)	Men	198.6 ± 103.0	211.9 ± 113.8	215.4 ± 106.7	−0.028 (0.013)	0.037	−0.036 (0.018)	0.047
		Women	227.6 ± 82.7	248.0 ± 106.9	271.2 ± 103.3	−0.033 (0.009)	**0.00039**	−0.040 (0.013)	**0.0020**
rs11191548	*n*	Men	289	220	47				
(*NT5C2*)		Women	386	284	53				
	BMI ($kg\,m^{-2}$)	Men	30.0 ± 6.8	30.6 ± 5.4	29.4 ± 5.4	−0.007 (0.005)	0.19	−0.013 (0.006)	0.049
		Women	27.6 ± 4.7	28.7 ± 6.1	28.5 ± 4.8	−0.010 (0.004)	0.021	−0.015 (0.006)	0.0080
	VFA (cm^2)	Men	153.8 ± 68.0	161.3 ± 69.3	137.2 ± 54.8	0.007 (0.014)	0.65	−0.008 (0.018)	0.65
		Women	93.3 ± 49.3	107.4 ± 58.1	105.8 ± 52.8	−0.043 (0.015)	0.0043	−0.059 (0.019)	**0.0022**
	SFA (cm^2)	Men	202.1 ± 107.5	214.3 ± 111.3	199.9 ± 102.5	−0.023 (0.014)	0.10	−0.035 (0.018)	0.048
		Women	228.5 ± 84.9	257.4 ± 111.1	253.0 ± 89.0	−0.031 (0.010)	**0.0021**	−0.044 (0.013)	**0.00059**

Abbreviations: BMI, body mass index; SFA, subcutaneous fat area; SNP, single nucleotide polymorphism; VFA, visceral fat area.
Values are shown as the mean ± s.d. Data were derived from a linear regression analysis. The values of BMI, VFA and SFA were logarithmically transformed. Logarithmically transformed BMI, VFA and SFA were adjusted for age. Tested alleles (allele1 at both SNPs) are risk alleles of increased blood pressure.

rs1004467 and rs11191548 genotype with VFA (P=0.0015 and 0.0011, respectively) and SFA (P=0.00021 and 0.00062, respectively) were observed in women (Supplementary Table 2). Statistical analysis using analysis of covariance indicated significant associations of the rs1004467 and rs11191548 genotype with VFA (P=0.0020 and 0.0015, respectively) and SFA (P=0.00033 and 0.00042, respectively) in women (Supplementary Table 2). As some diabetes medications have an effect on adiposity,[27] we performed the analysis excluding 147 type 2 diabetic patients treated with sulfonylureas, biguanides and thiazolidinediones We found the similar significant associations of the rs1004467 and rs11191548 genotype with VFA and SFA in women (Supplementary Table 3).

We have reported that rs1558902 in the *FTO* gene is associated with both VFA and SFA,[11] and that rs7498665 in the *SH2B1* gene is associated with VFA.[17] Thus, we examined SNP×SNP epistasis in men, women and all subjects. The combination of rs1004467 and rs7498665 exhibited no epistatic effect on VFA in men (P=0.43), women (P=0.86) or all subjects (P=0.76). The combination of rs1004467 and rs1558902 did not show epistatic effect on VFA in men (P=0.99), women (P=0.53) or all subjects (P=0.60), or on SFA in men (P=0.63), women (P=0.83) or all subjects (P=0.89).

Among the SNPs tested in this study, rs16998073 in the *FGF5* gene and rs11191548 in the *NT5C2* gene were associated with increased systolic blood pressure ($P<0.05$). Rs11191548 in the *NT5C2* gene were also associated with hypertension ($P<0.05$). We could replicate the association between blood pressure and the above two SNPs that were reported to be strongly associated with blood pressure in the Japanese population (Supplementary Table 4).[28]

DISCUSSION

In this study, we showed that the A-allele of rs1004467 in the *CYP17A1* and the T-allele of rs11191548 in the *NT5C2* gene were significantly associated with reduced VFA, SFA and total fat area in women. Association of T-allele of rs11191548 in the *NT5C2* gene with increased systolic blood pressure and hypertension was replicated in our sample, as reported previously.[28] Our hypothesis was that these risk alleles would be associated with increased VFA and/or SFA as increased adiposity is a risk for hypertension;[4,5] however, these alleles affected decreased adiposity. The associations between SNPs and increased blood pressure/hypertension were evaluated after being adjusted for BMI, age and gender. Thus, the SNPs associated with visceral fat obesity-related and gender-dependent hypertension would be excluded in the screening stage. Indeed, recent analysis has shown that genetic variation near insulin receptor substrate 1 (*IRS1*) is associated with reduced adiposity and an impaired metabolic profile.[29] Thus, it is likely that rs1004467 and rs11191548 are associated with reduced VFA and SFA, as well as with hypertension in women.

The SNPs rs1004467 and rs11191548 were not associated with BMI in men or women, as reported for rs2943650 near *IRS1*.[29] As BMI represents both fat and lean body mass, our observation suggests that these SNPs influence a reduction in VFA and SFA, or influence an increased percentage of lean body mass. The significant associations of rs1004467 and rs1191548 with reduced VFA and SFA were observed in women, but not in men. The rs1004467 SNP is located in the intron of the *CYP17A1* gene. CYP17A1 is involved in the biosynthesis of glucocorticoids, mineral corticoids, androgens and estrogens.[30] The rs1004467 risk allele may reflect differences in *CYP17A1* gene expression that alter the biosynthesis of steroid hormones, leading to hypertension and reduced adiposity in women. The region of linkage disequilibrium that includes rs1004467 and rs11191548 contains a couple of genes in addition to *CYP17A1*: *NT5C2*, arsenic (+3 oxidation state) methyltransferase (*AS3MT*) and cyclin M2 (*CNNM2*). NT5C2 is a cytosolic IMP/GMP selective 5′-nucleotidase and involved in nucleic acids or DNA synthesis.[31] CNNM2 (ancient conserved domain protein, ACDP2) is a transporter of magnesium, which is required for the catalytic activity of numerous metalloenzymes.[32] Thus, these genes would be important for metabolism in adipocyte hyperplasia and hypertrophy. Further investigation is warranted to elucidate the functional SNPs and susceptibility genes.

We have previously reported that *FTO* rs1558902 is associated with VFA and SFA, and that *SH2B1* rs7498665 is associated with VFA.[11,17] Epistasis, or gene–gene interaction, has recently received much attention in human genetics.[33] In this study, the effect of these SNPs on VFA and SFA was additive, and an epistatic effect was not observed.

In summary, we showed that *CYP17A1* rs1004467 and *NT5C2* rs11191548 SNPs are significantly associated with both reduced VFA and SFA in women. Our results suggest that the region encompassing *CYP17A1* to *NT5C2* has a role in reducing visceral and subcutaneous fat mass. However, these results require confirmation in other populations.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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