JDI Journal of Diabetes Investigation Open access
Official Journal of the Asian Association for the Study of Diabetes

ORIGINAL ARTICLE

Genome-wide scan for circulating vascular adhesion protein-1 levels: *MACROD2* as a potential transcriptional regulator of adipogenesis

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Keywords
Linkage analysis, MACRO domain containing 2 gene, Vascular adhesion protein-1

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J Diabetes Investig 2018; 9: 1067–1074

doi: 10.1111/jdi.12805

ABSTRACT

Aims/Introduction: Vascular adhesion protein-1 (VAP-1) is a membrane-bound amine oxidase highly expressed in mature adipocytes and released into the circulation. VAP-1 has been strongly implicated in several pathological processes, including diabetes, inflammation, hypertension, hepatic steatosis and renal diseases, and is an important disease marker and therapeutic target. Here, we aimed to identify the genetic loci for circulating VAP-1 levels.

Materials and Methods: We carried out a genomic-wide linkage scan for the quantitative trait locus of circulating VAP-1 levels in 1,100 Han Chinese individuals from 398 families in the Stanford Asian Pacific Program for Hypertension and Insulin Resistance study. Regional association fine mapping was carried out using additional single-nucleotide polymorphisms.

Results: The estimated heritability of circulating VAP-1 levels is high (h^2 = 69%). The most significant quantitative trait locus for circulating VAP-1 was located at 38 cM on chromosome 20, with a maximum empirical logarithm of odds score of 4.11 ($P = 6.86 \times 10^{-6}$) in females. Regional single-nucleotide polymorphism fine mapping within a 1-unit support region showed the strongest association signals in the MACRO domain containing 2 (*MACROD2*) gene in females ($P = 5.38 \times 10^{-6}$). Knockdown of *MACROD2* significantly suppressed *VAP-1* expression in human adipocytes, as well as the expression of key adipogenic genes. Furthermore, *MACROD2* expression was found to be positively associated with *VAP-1* in human visceral adipose tissue.

Conclusion: *MACROD2* is a potential genetic determinant of serum VAP-1 levels, probably through transcriptional regulation of adipogenesis.

INTRODUCTION

Vascular adhesion protein-1 (VAP-1), also known as amine oxidase, copper containing 3, is a membrane-bound amine oxidase that converts primary amines, such as methylamine and aminoacetone, to aldehydes with the production of hydrogen peroxide and ammonia. Possible substrates of VAP-1 include cell-surface molecules containing free NH_2 groups[1–3]. These endogenous amine substrates have been shown to exert insulin-like actions in adipocytes[4,5]. Deamination of these amines leads to hydrogen peroxide and

†These two authors contributed equally to this work.
Received 2 November 2017; revised 10 January 2018; accepted 15 January 2018
[Correction added on 7 March 2018, after first online publication: The author affiliations for Hung-Yuan Li and Tien-Jyun Chang have been corrected.].

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aldehydes production. Consequently, hydrogen peroxide and aldehydes, which trigger oxidative stress and cross-linking of biomolecules, respectively, will lead to cellular damage. VAP-1 expression is strongly increased during adipogenesis, and is highly expressed on the cell surface of mature adipocytes and vascular endothelium[6–8]. VAP-1 is also secreted into the circulation in a soluble form.

VAP-1 has been implicated in several pathological processes including diabetes, inflammation, and vascular, renal and neurological diseases. Serum VAP-1 level has been shown to predict mortality in patients with diabetes after 10 years' follow up[9]. In case–control analyses, serum VAP-1 is elevated in patients with hyperglycemia[10], hypertension[11], chronic kidney disease[12], diabetic retinopathy[13], hepatic steatosis[14] and those who are cigarette smokers[15]. In addition, inhibition of VAP-1 suppresses atherosclerosis[16], improves prognosis after stroke[17,18], ameliorates renal fibrosis[19] and hepatitis[20], and also decreases inflammation[21]. All of these show that VAP-1 is an important disease marker and therapeutic target[22].

In the present study, we sought to identify the genetic determinants of serum VAP-1 levels. We showed that serum VAP-1 levels were highly heritable. Through genome-wide linkage scans and regional association fine mapping in a large family-based cohort, we identified the strongest association signals in the MACRO domain containing 2 (*MACROD2*) gene. Knockdown of *MACROD2* in human visceral adipocytes significantly reduced *VAP-1* expression and its secretion into the medium, as well as the expression of other key adipogenic genes. Furthermore, a positive correlation between the expression of *MACROD2* and *VAP-1* were found in human visceral adipose tissue. These data showed that *MACROD2* is the genetic determinant of *VAP-1* expression.

METHODS

Stanford Asian Pacific program for hypertension and insulin resistance study cohort

The Stanford Asian Pacific Program for Hypertension and Insulin Resistance (SAPPHIRe) is a collaborative study part of the Family Blood Pressure Program of the National Heart, Lung and Blood Institute of the National Institutes of Health originally designed to investigate the genetic determinants of hypertension and insulin resistance in Chinese and Japanese patients. The study collected >1,300 sib pairs that were either concordant or discordant for high blood pressure. Detailed descriptions of the study cohort can be found in previous studies[23]. Individuals with chronic illnesses, such as diabetes, cancer, or diseases of the heart, liver or kidney, were excluded. In the present study, 1,100 individuals of Chinese origin from 398 families were recruited for analysis. The numbers of families with one to eight siblings were 57, 139, 112, 45, 29, 10, 4 and 2, respectively. Ethical approval for the present study was obtained from the institutional review board of each participating site. Informed consent was obtained from each participant. For gene expression study of adipose tissue, the study was approved by the National Taiwan University Hospital and Min-Sheng General Hospital.

Genotyping and single-nucleotide polymorphism imputation

Genomic deoxyribonucleic acid was extracted from peripheral leukocytes. Genotyping was carried out using 376 autosomal markers representing short tandem repeat polymorphisms and yielded an average map density of 10 cM. The genotyping concordance rate is 99% based on duplicate samples. An additional 1,231 single-nucleotide polymorphisms (SNPs) in the 1-unit support interval (35 cM, 41 cM) of linkage peak on chromosome 20 at 38 cM were further genotyped for regional fine mapping. SNP imputation was carried out using genotype information from the genome-wide association study from 455 individuals in the Genetics of Insulin Sensitivity consortium. SNPs with a call rate <95%, minor allele frequency (MAF) <5% and deviation from the Hardy–Weinberg equilibrium ($P < 10^{-6}$) were excluded. A total of 6,098 genotyped and imputed SNPs within the 1-unit support interval were used for fine mapping.

Individuals for measurement of gene expression in adipose tissue

We recruited 47 non-diabetic adults undergoing bariatric surgery or elective abdominal surgery, such as cholecystectomy or partial hepatectomy, in Min-Sheng General Hospital and the Yunlin branch of National Taiwan University Hospital in Taiwan. Abdominal visceral adipose tissues were sampled in a fasting state during surgery, and were placed in liquid nitrogen immediately until processing. The institutional review board of Min-Sheng General Hospital and National Taiwan University Hospital Yunlin branch approved the study. Written informed consent was also obtained from each patient. All methods were carried out in accordance with the approved guidelines with the tenets of the Declaration of Helsinki.

Human adipocyte culture

Human visceral preadipocytes, preadipocyte growth supplement and preadipocyte differentiation supplement were purchased from ScienCell Research Laboratories (San Diego, California, USA). Fetal calf serum was purchased from Gibco (Carlsbad, California, USA), and penicillin-streptomycin-amphotericin B solution was purchased from Biological Industries (Cromwell, Connecticut, USA). Human visceral preadipocytes were maintained in Dulbecco's modified Eagle's medium/F12 medium containing 5% fetal calf serum, preadipocyte growth supplement and penicillin-streptomycin-amphotericin B solution according to the manufacturer's protocol. Post-confluent preadipocytes were replaced with medium containing pre-adipocyte differentiation supplement and then continued onto differentiation for 5 days. The medium was changed every 3 days. VAP-1 concentrations in cell culture supernatants were measured using the VAP-1 human enzyme-linked immunosorbent assay

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kit according to the manufacturer's protocol (Abcam, Cambridge, UK). Relative VAP-1 levels were normalized to total cell counts.

Knockdown of *MACROD2* in induced human adipocytes

For the ribonucleic acid (RNA) interference assay, human preadipocytes were transfected with 80 nmol/L of ON-TARGET-plus SMARTpool small interfering RNA targeting *MACROD2* (#L-015258-00-0005) or non-targeting control (#D-001810-01-05; Dharmacon, Lafayette, Colorado, USA) using Dharmafect reagent (Dharmacon) according to the manufacturer's protocol. Then, 24-h post-silencing cells were changed to differentiation medium and differentiated for 5 days.

Reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was isolated using REzol C&T Reagent (Protech, Taipei, Taiwan) and reverse transcribed with Transcriptor Reverse Transcriptase (Roche Applied Science, Basel, Switzerland) according to the manufacturer's protocol. Polymerase chain reaction amplification was carried out using LightCycler FastStart DNA MasterPlus SYBR (Roche Applied Science). Each sample was analyzed and calibrated to the cyclophilin housekeeping gene in duplicates. The $2^{-\Delta C_T}$ relative quantitation method was used to present relative gene expression difference between the two groups. ΔC_t was calculated as C_t of the target gene − C_t of cyclophilin A. Primer information is provided in Table S1.

Statistical analysis

The distribution of serum VAP-1 levels is skewed, and inverse normal transformation was applied for the linkage and association analyses. The correlations between two gene expressions (ΔC_t) or between gene expression and metabolic phenotypes were calculated using Pearson's correlation.

Linkage analysis

Phenotypic variation of VAP-1 was decomposed into genetic and environmental components. Broad sense heritability of VAP-1 refers to the proportion of the phenotypic variance in a trait attributable to the genetic variance accounting for covariates. Maximum likelihood methods implemented in the SOLAR (sequential oligogenic linkage analysis routines) computing program (https://userinfo.surfsara.nl/systems/lisa/software/solar) were used to estimate the phenotypic variance components and heritability[24,25]. Multipoint linkage analysis was also carried out using SOLAR. The inverse normal transformation was made to approximate the normal distribution for VAP-1. The genetic variation was further partitioned into components for a quantitative trait locus and the residuals polygenic component[24,25].

Dividing the likelihood of the estimated variance component due to the quantitative trait locus by the likelihood of this variance component being 0 yields a likelihood ratio. The logarithm to base 10 of the likelihood ratio was calculated as a logarithm of odds (LOD) score. One-unit LOD support intervals were obtained for maximum LOD scores of ≥3.0. Age, sex and body mass index were adjusted in the analysis. A maximum empirical LOD score ≥2.0, which is regarded as evidence of "suggestive linkage," was reported[26]. Kosambi map function was used for the linkage scan.

Association analysis

Family-based association analysis was carried out using generalized estimating equations where familial correlations between family members were accounted for. A linear model was used to assess associations between the inverse normal transformed VAP-1 and individual SNPs adjusting for age, sex and body mass index. The genetic additive mode was assumed for individual SNPs in the regression analysis. *P*-values for the estimated regression coefficients for individual SNPs with MAF frequencies ≥0.05 were reported. Imputation of the entire 2.5M HapMap SNP set was carried out using MACH software (http://csg.sph.umich.edu/abecasis/mach/tour/imputation.html)[27]. A total of 2,419,983 SNPs on autosomes were imputed, and 190,379 SNPs were filtered out due to call rate <0.95, Hardy–Weinberg equilibrium $<10^{-6}$ or MAF <0.01[28].

RESULTS

The demographic and biochemical characteristics according to sex are shown in Table S2. A total of 1,100 siblings from 387 families were recruited (Table S3). The numbers of female–female, male–male and female–male sib pairs were 358, 258 and 516, respectively (Table S4).

Circulating VAP-1 levels were higher in female participants than in male participants ($P = 2.9 \times 10^{-16}$; Table S2). The heritability (h^2) of circulating VAP-1 levels was high (69%), especially in female participants (71%) compared with the male participants (54%; Table 1).

All chromosomal regions with empirical LOD scores >2.00 for circulating VAP-1 are summarized in Table 2. The highest linkage signal was observed on chromosome 20 at 38 cM (LOD 2.29, $P = 5.81 \times 10^{-4}$; Table 2, Figure 1) near the marker GGAA7E02 with 1-LOD support interval of 35–41 cM. This linkage signal appeared only in females (LOD 4.10, $P = 6.86 \times 10^{-6}$), but not in males.

Another chromosomal region identified as linked with circulating VAP-1 levels was located on chromosome 18 at 77 cM (LOD 2.15, $P = 8.22 \times 10^{-4}$) near the marker GATA6D09. We also observed suggestive linkage signals at chromosome 10

Table 1 | Heritability of serum vascular adhesion protein-1 by sex

Gender	Heritability	*P*
All	0.69 ± 0.081	1.8×10^{-17}
Female	0.71 ± 0.12	6.9×10^{-9}
Male	0.54 ± 0.16	0.00068

Values are presented as estimate ± standard error.

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Table 2 | Regions with peak logarithm of odds scores ≥2.0

	Chr.	cM	LOD	*P*-value*	Nearest marker
All	20	38	2.2907	5.81×10^{-4}	GGAA7E02
	18	77	2.1521	8.22×10^{-4}	GATA6D09
Female	20	38	4.1057	6.86×10^{-6}	GGAA7E02
	10	84	2.2514	6.41×10^{-4}	ATA24F10
Male	1	258	2.3295	5.28×10^{-4}	AFM203yg9

*Adjusted for age, sex and body mass index. Chr., chromosome; cM, centiMorgan; LOD, logarithm of odds.

at 84 cM (LOD 2.25, $P = 6.41 \times 10^{-4}$) near the marker ATA24F10 in female participants as well as another signal on chromosome 1 at 258 cM (LOD 2.32, $P = 5.28 \times 10^{-4}$) near the marker AFM203yg9 in male participants.

We carried out regional association fine mapping for the highest signal at 38 cM on chromosome 20 using additional genotyped and imputed 6,098 SNPs within the 1-LOD support interval. The strongest association signals were found in the *MACROD2* gene, especially in females (Figure 2). An imputed common intronic SNP rs1237183 (MAF 6.7%) was significantly associated with circulating VAP-1 levels in females ($P = 5.38 \times 10^{-6}$). The study-wide Bonferroni corrected *P*-value threshold was 8.20×10^{-6}. The heritability reduced from 69.3% to 66.8% with inclusion of rs1237183 as a covariate. The LOD score at 38 cM reduced from 2.3 to 0.4 after adjusting for rs1237183. Both conditional heritability and linkage analyses suggested that the effect sizes of rs1237183 were substantial.

We next examined whether the linkage signal was due to a single sibling pair with phenotypically highly discordant pair without shared alleles at the linkage locus. We defined extreme pairs as their paired VAP-1 levels were within the (≤5th, ≥95th) or (≥95th, ≤5th) percentiles (Table S5). The two phenotypically highly discordant pairs with VAP-1 levels within the (≤5th, ≥95th) or (≥95th, ≤5th) percentiles shared one and two identity by descent at the linkage peak, respectively, suggesting this linkage signal was not due to a single extreme sibling pair.

Furthermore, knockdown of *MACROD2* (knockdown efficiency 77.4%; Figure 3a) in induced primary human adipocytes significantly reduced *VAP-1* expression (relative expression 0.76 ± 11.1%, $P = 0.03$; Figure 3b) and its release into the medium (relative concentration 0.73 ± 0.05%, $P = 0.01$; Figure 3c). As VAP-1 is a marker of mature adipocytes, we examined the expression of other key adipogenic markers. Interestingly, knockdown of *MACROD2* significantly reduced the expression of adipogenic genes including *ADIPOQ* ($P < 0.0001$) and *CEBPA* ($P = 0.006$; Figure 3d). There were also trends of reduced expression of other adipogenic genes including *FABP4* ($P = 0.13$), *PPARG2* ($P = 0.12$) and *SREBP1* ($P = 0.06$; Figure 3d).

We next examined the association between the expression of *MACROD2* and *VAP-1* in 47 non-diabetic human visceral adipose tissues. *VAP-1* expression was associated with *MACROD2* expression ($r = 0.38$, $P = 0.01$; Figure 4). In addition, VAP-1 expression levels were also positively associated with body mass index, homeostatic model assessment of insulin resistance and fasting insulin levels. In contrast, VAP-1 expression levels were negatively associated with high-density lipoprotein cholesterol levels (Table S6).

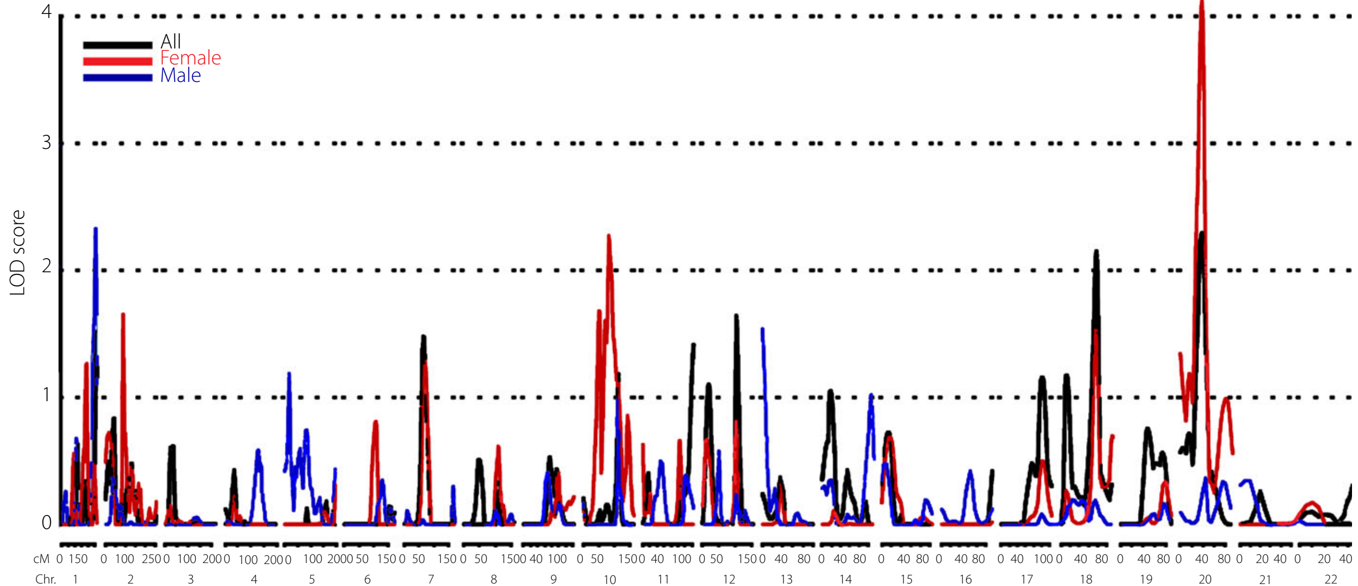

Figure 1 | Genome-wide linkage signals for circulating vascular adhesion protein-1 levels in the Stanford Asian Pacific Program for Hypertension and Insulin Resistance cohort (SAPPHIRe). Chr., chromosome; cM, centiMorgan; LOD, logarithm of odds.

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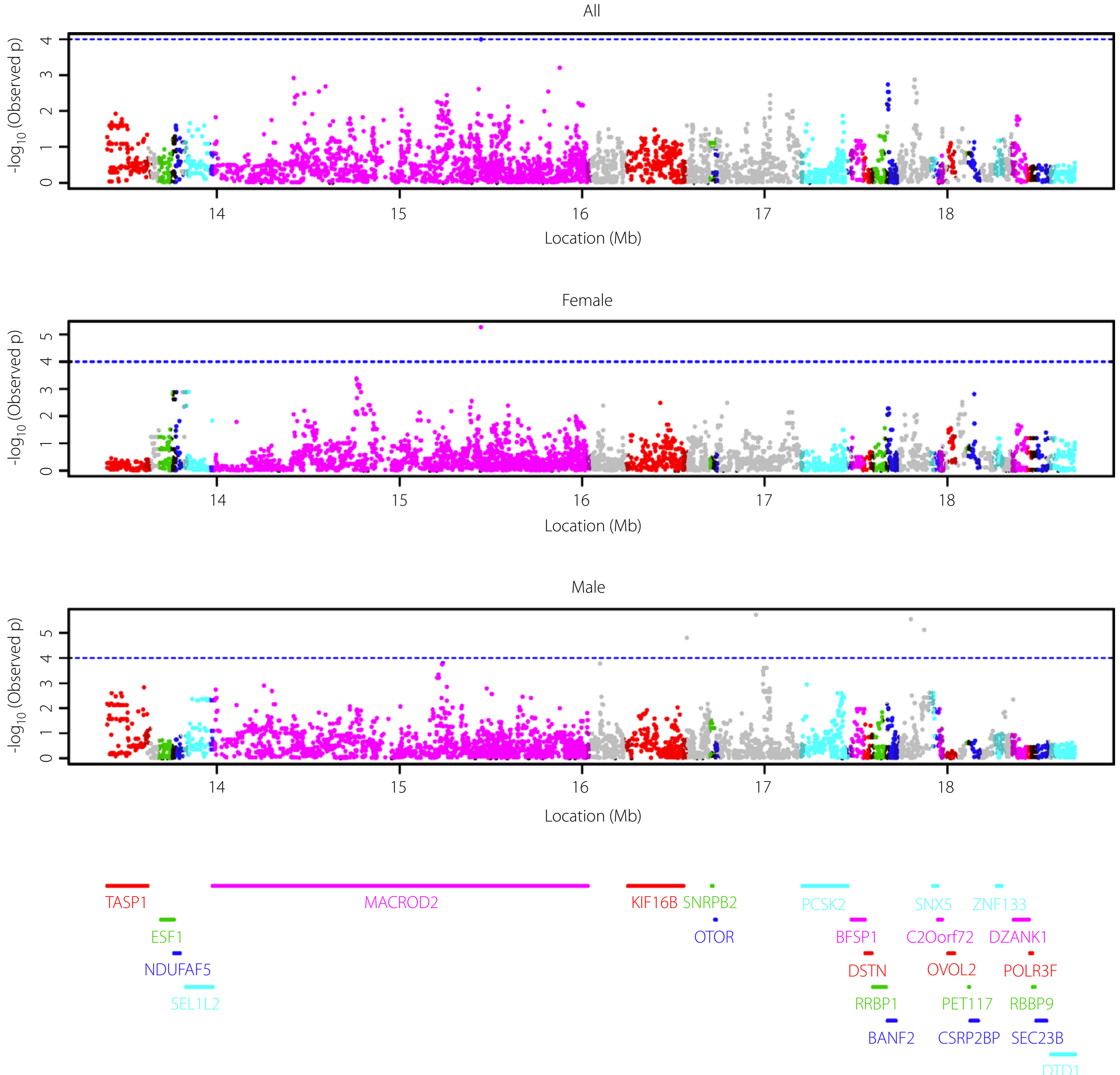

Figure 2 | Regional association fine mapping within 1-unit support region based on the generalized estimating equation approaches. Mb, megabase. [Colour figure can be viewed at wileyonlinelibrary.com]

DISCUSSION

In the present study, we found that genetic loci near the *MACROD2* gene were associated with circulating VAP-1 levels in females. Knockdown of *MACROD2* reduced *VAP-1* expression in induced human primary visceral adipocytes and its release into the culture medium. Knockdown of *MACROD2* also significantly suppressed the expression of several other key adipogenic genes. *MACROD2* expression level was found to be positively correlated with *VAP-1* expression in human visceral adipose tissue. These data indicate *MACROD2* as genetic loci regulating *VAP-1* expression, probably through modulation of adipogenesis.

Adenosine diphosphate (ADP)-ribosylation is a post-translational modification catalyzed by an enzyme family of ADP-ribosyl transferases, which uses NAD+ as a cofactor to transfer single or multiple ADP-ribose onto target proteins. This process can be reversely regulated by macrodomain-containing hydrolase, *MACROD2*, by removing mono-ADP-ribosyl moiety from proteins[28,29]. ADP-ribosylation is involved in diverse biological

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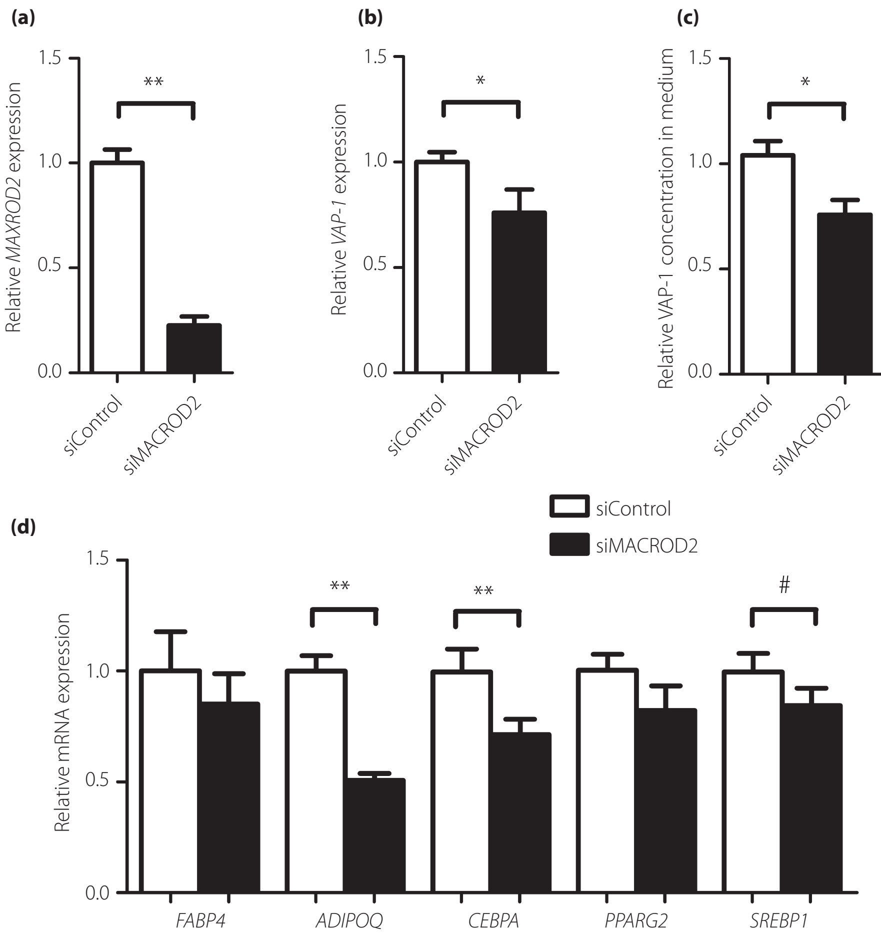

Figure 3 | The effect of knockdown of MACRO domain containing 2 (*MACROD2*; siMACROD2) on (a) *MACROD2* and (b) vascular adhesion protein-1 (*VAP-1*) gene expression in human primary preadipocytes ($n = 14$). (c) VAP-1 concentration in culture medium ($n = 10$). (d) Expression of adipogenic genes including *FABP4*, *ADIPOQ*, *CEBPA*, *PPARG2* and *SREBP1* ($n = 14$) as compared with control (siControl). Data is expressed as mean ± standard error. *$P < 0.05$; **$P < 0.01$; $^{\#}P < 0.1$. mRNA, messenger ribonucleic acid.

processes, such as immunity, insulin secretion, and regulation of chromatin structure, transcription, deoxyribonucleic acid repair and RNA metabolism, especially in response to different forms of cellular stress[30–33]. It is possible that MACROD2 removes the ADP-ribosyl moiety from transcription factors or chromatin-modifying enzymes associated with the *VAP-1* gene expression, thereby modulating its transcription.

Genome-wide genetic scans showed that genetic polymorphisms in the *MACROD2* gene are associated with hypertension[34], pulmonary function test parameters[35], Crohn's disease[36], neuropsychiatric disorders including autism spectrum disorder[37] and attention deficit hyperactivity disorder[38], severity of dementia[39], and liver cancer[40,41]. In our present study, genetic variants within the *MACROD2* region were associated with circulating VAP-1 levels. Our results suggest that *MACROD2* positively regulates *VAP-1* gene transcription in human primary visceral adipocytes and human visceral adipose tissue. Collectively, human genetic studies show the pleiotropic effects of

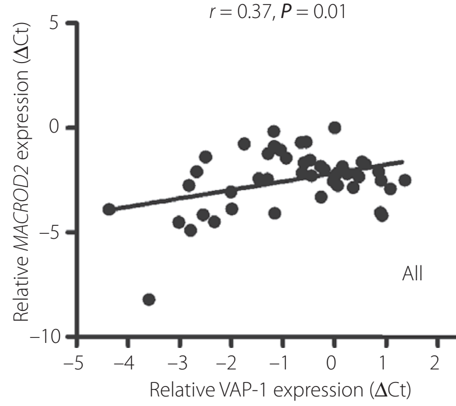

Figure 4 | Correlation between vascular adhesion protein-1 (*VAP-1*) and MACRO domain containing 2 (*MACROD2*) gene expression in human visceral adipose tissue (expressed in ΔC_t in relation to C_t of cyclophilin A). *r*, correlation coefficient.

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MACROD2 for a variety of diseases or traits, possibly mediated through the regulation of gene expression.

We also observed a higher heritability of *VAP-1* and a stronger association signaling in females (71%) than in males (54%). The reason is unclear, but environmental factors, including higher frequency of smoking in males, might interfere with the heritability difference associated with sex.

The present study is important in the aspects of the family-based design to control for population stratification, which is the most common reason for false positive findings in genetic association studies, the completeness of clinical data from the SAPPHIRe cohort and the relatively large sample size. Although the present study provides these novel findings, some limitations of this study should be recognized. First, no replication study was carried out. However, this study is currently the only study to identify the genetic determinant of serum VAP-1 levels. Second, the probands recruited in this study were hypertension patients. Therefore, the magnitude of SNP association with VAP-1 might not be representative of the general population, but for families suffering from hypertension.

In summary, we provide the first evidence for the association of variants in the *MACROD2* gene, a mono-ADP-ribosyl hydrolase, with the circulating VAP-1 levels. We also found a possible correlation between *MACROD2* and *VAP-1* genetic expression in human adipocytes and adipose tissue. The association of *MACROD2* genetic variations with several human diseases and traits in genome-wide analyses strongly suggests its pleiotropic roles.

ACKNOWLEDGMENTS

The authors thank all participants in this study. We also thank Ms Chun-Yi Lee for her computing support. This work was supported in part by grants from the Ministry of Science and Technology (MOST), Taiwan (MOST 97–2314-B-002-047-MY3 and 101-2314-B-002-069-MY3 for Dr. Lee-Ming Chuang; and 05D2-PHMOST07 for Dr. Yen-Feng Chiu), and NHRI-PH-PP03 and NHRI-PH-PP04 from the National Health Research Institutes (NHRI), Taiwan.

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 | Primers used for quantitative real-time polymerase chain reaction.
Table S2 | Baseline characteristics and serum vascular adhesion protein-1 level by sex.
Table S3 | Number of families by sibship size.
Table S4 | Numbers of female–female, male–male and female–male sib pairs.
Table S5 | Distribution of identity by descent at the linkage peak between phenotypically extremely discordant sibling pairs.
Table S6 | Association between serum vascular adhesion protein-1 gene expression in human adipose tissue and metabolic phenotypes.

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