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ORIGINAL ARTICLE

# A functional SNP in *FLT1* increases risk of coronary artery disease in a Japanese population

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**Coronary artery disease (CAD) including myocardial infarction is one of the leading causes of death in many countries. Similar to other common diseases, its pathogenesis is thought to result from complex interactions among multiple genetic and environmental factors. Recent large-scale genetic association analysis for CAD identified 15 new loci. We examined the reproducibility of these previous association findings with 7990 cases and 6582 controls in a Japanese population. We found a convincing association of rs9319428 in *FLT1*, encoding fms-related tyrosine kinase 1 ($P = 5.98 \times 10^{-8}$). Fine mapping using tag single-nucleotide polymorphisms (SNPs) at *FLT1* locus revealed that another SNP (rs74412485) showed more profound genetic effect for CAD ($P = 2.85 \times 10^{-12}$). The SNP, located in intron 1 in *FLT1*, enhanced the transcriptional level of *FLT1*. RNA interference experiment against *FLT1* showed that the suppression of *FLT1* resulted in decreased expression of inflammatory adhesion molecules. Expression of FLT1 was observed in endothelial cells of human coronary artery. Our results indicate that the genetically coded increased expression of *FLT1* by a functional SNP implicates activation in an inflammatory cascade that might eventually lead to CAD.**
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## INTRODUCTION

Coronary artery disease (CAD) including myocardial infarction, one of the leading causes of death in many countries, results from complex interactions among multiple genetic and environmental factors. Although recent advances in the treatment and diagnosis related to some coronary risk factors, including type 2 diabetes mellitus, hypercholesterolemia, hypertension, obesity and inflammation, have improved quality of life for CAD patients, its mortality is still high.[1] Genetic risk factors might serve as a powerful tool for improving its mortality because large fraction of heritability (40–50%) is estimated by means of genetic epidemiological studies of family history and twin studies.[2] To date, multiple genome-wide association studies (GWAS) have collectively identified many genomic loci associated with CAD.[3,4] It is largely unknown and under investigation how these loci are implicated in the etiology of CAD; however, these findings showed the potent power of GWAS, which is free from hypothesis, to identify unexpected anchors to further understand the disease pathway and to explore novel therapeutic measures in the future.

Here, we report the identification of a novel functional single-nucleotide polymorphism (SNP) in *FLT1* associated with the risk of CAD through fine mapping at this loci that followed replicative study of previous findings. We also found that the increased expression of *FLT1* contributes to the pathogenesis of CAD by *in vitro* experiments.

## MATERIALS AND METHODS

### Study population

Characteristics of the study subjects were summarized in Table 1. For 7990 CAD case samples, myocardial infarctions were obtained from Osaka acute coronary insufficiency study group. The diagnosis of myocardial infarction has been described previously.[5] Angina pectoris including stable and unstable angina samples were from the BioBank Japan project (http://biobankjp.org/). We included 6582 control subjects consisting of healthy volunteers (1870 individuals) recruited from the Osaka-Midosuji Rotary Club, Osaka, Japan and Health Science Research Resources Bank, Osaka, Japan, and individuals registered in the BioBank Japan as subjects with other diseases (4712 individuals). From the results of principal component analysis, we found that there were no obvious deviation of genetic background between 'disease mix' and 'healthy control' samples (Supplementary Figure 1). All study subjects

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**Table 1 Characteristics of the study population**

| *Characteristics* | *CAD* | *Control*[a] |
|---|---|---|
| Age ± s.d. | 66.9 ± 10.5 | 58.0 ± 13.8 |
| Gender (% male) | 73.8 | 61.6 |
| BMI ± s.d. | 23.8 ± 3.3 | 22.4 ± 3.7 |
| Smoking (%) | 62.6 | 61.9 |
| Hypertension (%) | 77.1 | 46.4 |
| Diabetes (%) | 34.8 | 13.7 |
| Dyslipidemia (%) | 51.9 | 14.9 |

Abbreviations: BMI, body mass index; CAD, coronary artery disease.
[a]Clinical characteristics excluding age and gender for DNA samples recruited from Health Science Research Resources Bank, Osaka, Japan were not available.

provided written informed consent to participation in this study, or if they were under 20 years old, their parents gave consent. The protocol was approved by the Ethical Committee at the Center for Genomic Medicine, the Institute of Physical and Chemical Research (RIKEN), Yokohama and of each participating institution.

### *In silico* replication study

Genotypes were obtained using Illumina OmniExpress BeadChip (Illumina, Inc., San Diego, CA, USA). For quality control of the Illumina genotyping, the results were compared with those of the Invader assay;[6] we examined 376 samples for each SNP and found no inconsistency between the results of the Invader assay and Illumina genotyping, except for one genotyping data of rs4252120, indicating its accuracy rate $>0.999$.

### Statistical analysis

The association of the 15 SNPs was assessed by Cochrane–Armitage trend test. We also calculated the corrected *P*-values by multiplying the *P*-values with 15 (the number of the examined SNPs). Hardy–Weinberg equilibrium was assessed by $\chi^2$ test with 1 degree of freedom and the statistical *P*-value for the deviation was considered $<0.0033$ (0.05/15). Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) was used to estimate the power of the replication study for previous GWAS[3] under a prevalence of 0.2, type 1 error rate of 0.0033 (0.05/15) and each value of genotype relative risk for previous study on the basis of additive model. We conducted principal component analysis to assess population stratification using the genotyping data obtained from Illumina OmniExpress BeadChip.[7] Relationships between clinical profiles and genotype information of the cases were examined by one-way analysis of variance and $\chi^2$ test. The SNP–SNP epistasis and logistic regression analyses were examined by using the PLINK 1.07 software (http://pngu.mgh.harvard.edu/purcell/plink/).[8]

### Fine mapping for *FLT1* locus

We chose 74 tag SNPs with minor allele frequency of $>0.05$ on the *FLT1* genomic region (NCBI build 37 chromosome position; 28 869 587–29 074 234) based on the data of EAS (East Asian Ancestry) from 1000 Genomes Project (http://www.1000genomes.org/) using Haploview software.[9] In these selected SNPs, we failed to design primers to amplify 10 SNP loci for PCR-invader assay because of their repetitive sequences. At the first stage, we genotyped 64 tag SNPs with 2861 cases and 2066 controls (same as a part of samples for replication study) using multiplex-PCR-invader assay[6] or Illumina OmniExpress BeadChip. We could not obtain signals for 10 SNP loci, and three SNPs were monomorphic in our panel. Totally, 51 SNPs were successfully genotyped and analyzed. We chose 15 SNPs with *P*-value less than that of rs9319428 originally associated with CAD, to genotype all the samples. Statistical analysis was performed Cochrane–Armitage trend test.

### Luciferase assay

The H3K27Ac sequences of *FLT1* promoter and intron 1 enhancer region on chromosome 13q32 were searched by UCSC genome browser (http://genome.ucsc.edu) and the genomic fragment corresponding to the H3K27Ac sequences (NCBI build 37 chromosome position; 29 067 667–29 070 000), which does not include genomic sequence of rs74412485 SNP region was inserted into multiple cloning sites of the pGL3 basic vector (Promega Corporation, Madison, WI, USA). Promoter activity was confirmed in human umbilical vein endothelial cells (HUVECs; ScienCell Research Laboratories, Carlsbad, CA, USA). Then, 25 bp double-stranded oligonucleotide for rs74412485 (sense strand: 5′-TCAC ACGCTTACRTGACATTCGACA-3′; antisense strand: 5′-TGTCGAATGTCA YGTAAGCGTGTGA-3′) was cloned into the *FLT1* promoter-enhancer inserted pGL3 basic vector. Transfection was performed using the Nucleofector system (Amaxa, LONZA, Basel, Switzerland). Forty hours after transfection, we stimulated the cells with phorbol 12-myristate 13-acetate (4 ng ml$^{-1}$) and ionomycin (1 ng ml$^{-1}$) for 4 h and measured the luciferase activity using the dual-luciferase reporter assay system according to the manufacturer's protocol (Promega) and luminometer (Centro LB960; Berthold Technologies GmbH, Bad Wildbad, Germany). Each experiment was independently performed four times and each sample was studied in duplicate. Student's *t*-test was conducted to estimate statistical difference of non-risk allele and risk allele activity.

### RNA interference experiments

Double-strand stealth RNA interference oligonucleotides (HSS103744, HSS103745 and HSS103746 for *FLT1* and 12935-300 for negative control) were purchased from Invitrogen (Carlsbad, CA, USA). The oligonucleotides were transfected in human aortic endothelial cells (HAECs; ScienCell Research Laboratories) using the nucleofector system (Amaxa). Forty hours after transfection, HAECs were stimulated with phorbol 12-myristate 13-acetate (4 pg/ml) and ionomycin (0.5 ng ml$^{-1}$) for 5 h. RNA isolation, cDNA synthesis and mRNA quantification were carried out as described previously[5] using the following primer sets: 5′-TCAGCTACAATTCTTCCTGCTC-3′ and 5′-CACCT CACAGAGCCATTCTGA-3′ for *SELE* (selectin-E); 5′-CAATGTGCAAGAAG ATAGCCA-3′ and 5′-CAGCGTAGGGTAAGGTTCTTG-3′ for *ICAM1* (intercellular adhesion molecule 1); 5′-CTGTGCACAGCAACTTGTGA-3′ and 5′-TGGGCACAGAATCCATTTCAT-3′ for *VCAM* (vascular cell adhesion molecule 1); and 5′-GAAGCTCTGATGATGTCAGATACG-3′ and 5′-TTACTC TCAAGTCAATCTTGAGCG-3′ for *FLT1*. Each experiment was independently repeated three times and each sample was studied in duplicate. Statistical analyses were carried out using Student's *t*-test.

### Immunohistochemistry

Coronary artery segments were harvested at autopsy with written informed consent. The coronary arteries were dissected from the epicardial surface and a 5 mm slice from each segment was snap frozen and stored at −80 °C. The snap-frozen samples were sectioned at 3 μm thickness and fixed in acetone. The sections were stained with hematoxylin–eosin and used for detection of FLT1 and CD31 using immunohistochemistry. Anti-human FLT1 mouse monoclonal antibody (Flt-1 (D03)) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The antibody tested had no crossreactivity to the other proteins by western blot analyses using protein extracts of HUVECs. We purchased anti-human CD31 mouse monoclonal antibody (M0823) from Dako (Glostrup, Denmark). Single and double immunostaining was carried out as described previously.[10] As the negative controls, we stained the specimens with phosphate-buffered saline instead of each primary antibody, Flt-1 and CD31.

## RESULTS

### Replication study of 15 loci in a Japanese population

We obtained genotypes of 7990 cases and 6582 controls in a Japanese population to validate the association of CAD susceptibility and 15 SNPs loci recently identified by the CARDIoGRAMplusCAD consortium.[3] As we and the other already reported replications for CAD SNPs identified previous GWAS other than 15 SNPs elsewhere,[11,12] we focused on these novel ones. Hardy–Weinberg equilibrium for all SNPs was not deviated statistically in both cases and controls. The association results were summarized in Table 2. We found convincing association for one SNP, rs9319428 in intron 6 of *FLT1* (trend $P=5.98\times10^{-8}$, corrected $P=8.97\times10^{-7}$, odds ratio (OR) = 1.14), with CAD susceptibility in the Japanese population (Table 2). We also found nominal associations with statistical

**Table 2 Validation results of 15 SNP loci for CAD susceptibility with 7990 cases and 6582 controls in the Japanese population**

| SNP | Chr. | Gene | Minor allele | Risk allele | Reported risk allele[a] | Genotype frequencies | | RAF | Sample number | OR | 95% CI | Trend P | Corrected P |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Case | Control | (case/control) | (case/control) | | | | |
| Rs4845625 | 1 | *IL6R* | C | T | T | 0.266/0.489/0.245 | 0.257/0.499/0.244 | 0.510/0.507 | 7989/6577 | 1 | 0.87–1.06 | 5.39E−01 | NS |
| Rs515135 | 2 | *APOB* | A | A | G | 0.784/0.202/0.014 | 0.795/0.192/0.013 | 0.115 /0.109 | 7989/6581 | 1.1 | 0.87–1.01 | 8.61E−02 | NS |
| Rs2252641 | 2 | *ZEB2-AC074093.1* | T | T | C | 0.628/0.323/0.048 | 0.634/0.320/0.046 | 0.210/0.206 | 7988/6581 | 1 | 0.92–1.04 | 4.38E−01 | NS |
| Rs1561198 | 2 | *VAMP5-VAMP8* | A | A | A | 0.383/0.469/0.148 | 0.398/0.463/0.139 | 0.383/0.370 | 7979/6579 | 1.1 | 1.00–1.11 | 3.38E−02 | NS |
| Rs6544713 | 2 | *ABCG5-ABCG8* | T | T | T | 0.9993/0.0008/0.000 | 0.9995/0.0005/0.000 | 0.0004/0.0002 | 7985/6582 | 1.7 | 0.41–6.59 | 4.75E−01 | NS |
| Rs7692387 | 4 | *GUCY1A3* | A | G | G | 0.601/0.350/0.049 | 0.589/0.357/0.054 | 0.776/0.768 | 7989/6582 | 1.1 | 1.00–1.11 | 7.04E−02 | NS |
| Rs6842241 | 4 | *EDNRA* | A | A | A | 0.458/0.433/0.108 | 0.480/0.428/0.092 | 0.326 /0.306 | 7988/6581 | 1.1 | 1.05–1.16 | 2.07E−04 | 3.11E−03 |
| Rs17689550[b] | 5 | *SLC22A4-SLC22A5* | T | C | T | 0.964/0.035/0.001 | 0.693/0.036/0.001 | 0.982/0.981 | 7990/6545 | 1 | 0.87–1.23 | 7.58E−01 | NS |
| Rs10947789 | 6 | *KCNK5* | C | T | T | 0.605/0.344/0.052 | 0.589/0.359/0.052 | 0.777 /0.769 | 7984/6580 | 1.1 | 0.99–1.10 | 1.18E−01 | NS |
| Rs4252120 | 6 | *PLG* | C | NA | T | 1.000/0.000/0.000 | 1.000/0.000/0.000 | < 0.0001/0 | 6042/6580 | NA | NA | NA | NA |
| Rs2023938 | 7 | *HDAC9* | G | NA | G | 1.000/0.000/0.000 | 1.000/0.000/0.000 | < 0.0001/0 | 7977/6578 | NA | NA | NA | NA |
| Rs264 | 8 | *LPL* | T | C | C | 0.630/0.330/0.040 | 0.624/0.335/0.041 | 0.795/0.791 | 7987/6582 | 1 | 0.97–1.08 | 4.48E−01 | NS |
| Rs2954029 | 8 | *TRIB1* | A | T | A | 0.261/0.497/0.242 | 0.250/0.498/0.252 | 0.509 /0.499 | 7982/6577 | 1 | 1.00–1.09 | 8.26E−02 | NS |
| Rs9319428 | 13 | *FLT1* | T | T | T | 0.364/0.480/0.156 | 0.402/0.466/0.132 | 0.396/0.365 | 7990/6580 | 1.1 | 1.09–1.20 | 5.98E−08 | 8.97E−07 |
| Rs17514846 | 15 | *FURIN* | T | T | T | 0.717/0.259/0.024 | 0.740/0.239/0.021 | 0.154/0.142 | 7988/6576 | 1.1 | 1.04–1.18 | 2.08E−03 | 3.12E−02 |

Abbreviations: CAD, coronary artery disease; Chr., chromosome; CI, confidence interval; N/A, not assessed; NS, not significant; OR; odds ratio; RAF, risk allele frequency; SNP, single-nucleotide polymorphism.
[a]Reported in the CARDIoGRAMplusC4D Consortium.[3]
[b]A proxy ($r^2 = 1$) for rs2739093.

**Table 3 Association test for *FLT1* tag SNPs**

| SNP_ID | Location | Position | Minor allele | Risk allele | Genotype frequencies | | RAF (case/control) | Sample number (case/control) | OR | 95%CI | Trend P |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Cases | Controls | | | | | |
| Rs3794399 | Intron 24 | 28 318 755 | T | T | 0.754/0.231/0.015 | 0.759/0.225/0.015 | 0.131/0.128 | 7923/6542 | 1.03 | 0.96–1.10 | 4.87E−01 |
| Rs2296189 | Exon 24 | 28 319 505 | C | C | 0.742/0.237/0.021 | 0.739/0.243/0.018 | 0.139/0.139 | 7927/6539 | 1 | 0.94–1.07 | 9.81E−01 |
| Rs9513078 | Intron 23 | 28 321 036 | G | G | 0.474/0.429/0.096 | 0.509/0.408/0.083 | 0.311/0.287 | 7933/6425 | 1.12 | 1.07–1.18 | 9.85E−06 |
| Rs2387632 | Intron 16 | 28 342 206 | T | T | 0.643/0.315/0.042 | 0.685/0.285/0.030 | 0.199/0.172 | 7924/6547 | 1.2 | 1.13–1.27 | 4.84E−09 |
| Rs3936415 | Intron 16 | 28 345 193 | A | A | 0.702/0.270/0.028 | 0.733/0.245/0.022 | 0.163/0.144 | 7928/6546 | 1.16 | 1.08–1.23 | 1.21E−05 |
| Rs1324057 | Intron 15 | 28 351 298 | T | T | 0.471/0.428/0.101 | 0.520/0.394/0.086 | 0.315/0.283 | 7929/6546 | 1.17 | 1.11–1.23 | 3.37E−09 |
| Rs3751397 | Intron 13 | 28 386 456 | T | T | 0.318/0.491/0.191 | 0.355/0.473/0.172 | 0.437/0.409 | 7877/6257 | 1.12 | 1.07–1.18 | 2.03E−06 |
| Rs17086617 | Intron 13 | 28 388 549 | G | G | 0.329/0.490/0.181 | 0.361/0.482/0.157 | 0.426/0.398 | 7877/6546 | 1.12 | 1.07–1.18 | 1.16E−06 |
| Rs9554327 | Intron 11 | 28 405 034 | A | A | 0.341/0.488/0.171 | 0.387/0.466/0.147 | 0.415/0.380 | 7924/6350 | 1.16 | 1.10–1.22 | 1.70E−09 |
| Rs34961350 | Intron 10 | 28 417 765 | G | C | 0.309/0.491/0.199 | 0.273/0.500/0.228 | 0.555/0.523 | 7931/6439 | 1.14 | 1.09–1.19 | 4.75E−08 |
| Rs9513112 | Intron 10 | 28 423 565 | A | A | 0.355/0.481/0.165 | 0.384/0.479/0.138 | 0.405/0.377 | 7925/6546 | 1.12 | 1.07–1.18 | 1.22E−06 |
| Rs74972153 | Intron 3 | 28 440 267 | C | C | 0.727/0.250/0.023 | 0.759/0.220/0.021 | 0.148/0.131 | 7017/6506 | 1.15 | 1.18–1.23 | 4.81E−05 |
| Rs75419986 | Intron 3 | 28 457 861 | C | A | 0.888/0.109/0.003 | 0.860/0.135/0.005 | 0.943/0.928 | 7937/6427 | 1.29 | 1.17–1.42 | 1.48E−07 |
| Rs74412485 | Intron 1 | 28 468 295 | A | G | 0.929/0.070/0.001 | 0.897/0.099/0.004 | 0.964/0.947 | 7929/6512 | 1.5 | 1.34–1.68 | 2.85E−12 |
| Rs718273 | Intron 1 | 28 469 004 | A | G | 0.680/0.290/0.030 | 0.649/0.308/0.043 | 0.825/0.803 | 7917/6478 | 1.16 | 1.09–1.23 | 2.56E−06 |

Abbreviations: CI, confidence interval; ID, identifier; OR, odds ratio; RAF, risk allele frequency; SNP, single-nucleotide polymorphism.

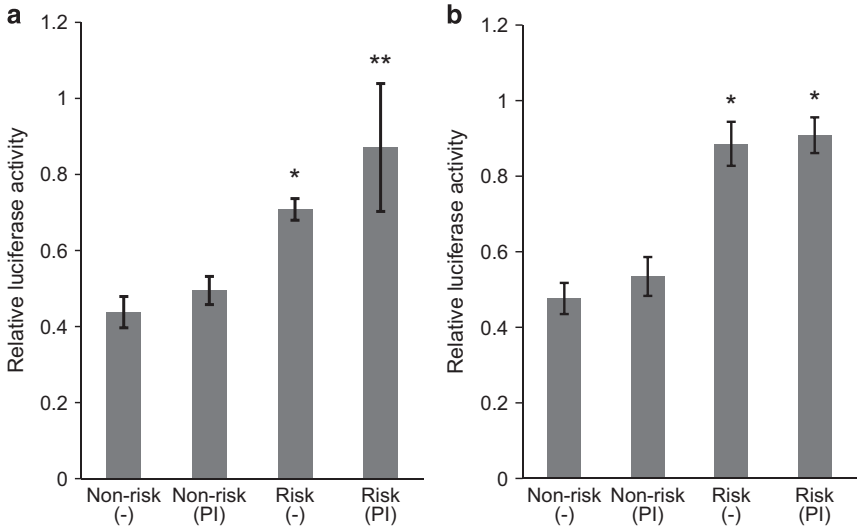


**Figure 1** Results of luciferase assay for rs74412485 in human umbilical venous endothelial cells (HUVECs) (**a**) and human aortic endothelial cells (HAECs) (**b**). −, Non-stimulated; PI, PMA (phorbol 12-myristate 13-acetate) and ionomycin stimulated. * and ** represents $P<0.01$ and $P<0.05$ when compared with non-risk allele, respectively. Each experiment was repeated four times and each sample was studied in duplicate.

significance after Bonferroni's correction for rs6842241 in 5′-flanking region of *EDNRA* and rs17514846 in intron 1 of *FURIN* with CAD (trend $P=0.000207$, corrected $P=0.00311$; OR = 1.10 and trend $P=0.00208$, corrected $P=0.0312$; OR = 1.11, respectively; Table 2). We found similar association results for these SNPs when compared between 7990 CAD cases and 1870 healthy controls (Supplementary Table 1). Other two SNPs (rs4252120 and rs2023938) were almost monomorphic, which were consistent with HapMap Japanese data, and we were not able to evaluate these SNPs. For the 10 remaining SNP loci, we did not detect any association for CAD and SNPs after Bonferroni's correction. However, we could not exclude the possibility of type 2 error, because this study did not have a sufficient power to detect modest OR of 1.06–1.11[3] ($1-\beta<0.28$). We also explored the possibility of confounding effect by age, sex and classical risk factors including diabetes, hypertension, smoking or hyperlipidemia within patient group using one-way analysis of variance and $\chi^2$ test for significant SNPs, and found no obvious relation between genotype and these factors ($P>0.05$), which are concordant with the previous study,[3] indicating that the significant SNPs are also independent risk factors for CAD in the Japanese population. We further examined the SNP–SNP epistasis for all SNPs and did not detect any statistical evidence for the SNPs interaction in the Japanese population (Supplementary Table 2).

### Fine mapping at *FLT1* locus

Since the SNP rs9319428 in *FLT1* associated with convincing statistical value with the risk of CAD in Japanese population, we further investigated implications between *FLT1* and CAD susceptibility through fine mapping followed by *in vitro* functional analyses and immunohistochemistry. To narrow down the *FLT1* locus, we looked up the SNPs on ~200 kb *FLT1* genomic region using 1000 genomes data, and genotyped 51 tag SNPs that represent this locus for association test with 2861 cases and 2066 controls, and found that 15 SNPs showed stronger association than the original SNP, rs9319428 (Supplementary Table 3). We further genotyped these loci with an additional 5076 cases and 4481 controls and found the strongest association with the increased risk of CAD at rs74412485 (OR = 1.50, $P=2.85\times10^{-12}$; Table 3). We could find no SNP in absolute LD with rs74412485 in the 1000 genomes database. LD map of 14 CAD-associated tag SNPs (original SNP, rs9319428 and 13 newly associated SNPs) is shown Supplementary Figure 2. In these tag SNPs, we found that three groups tagged by rs74412485 (rs75419986 and rs718273), rs9319428 (rs3751397, rs17086617, rs9554327, rs34961350, rs9513112 and rs74972153) and rs2387632 (rs9513078, rs3936415 and rs1324057) independently associated with the risk of CAD by means of conditioned analyses (Supplementary Table 4). These results indicate that the locus is architecturally complex and harbors at least three CAD-susceptible signals in Japanese populations. To confirm the association of *FLT1* SNPs does not depend on conventional risk factors including gender, age, hypertension, dyslipidemia, diabetes and smoking, we performed logistic regression analysis, except for individuals from Health Science Research Bank, and found similar association results for theses SNPs and CAD (Supplementary Table 5).

### *In vitro* functional analysis for the rs74412485 SNP

Since the most associated SNP rs74412485 was located in intron 1 of *FLT1*, we investigated whether the SNP would affect the gene expression of *FLT1* quantitatively by means of luciferase reporter assay in cultured HUVECs. We chose HUVECs for the assay because we observed abundant expression of *FLT1* in the cells by quantitative reverse transcription-PCR experiment (Supplementary Figure 3). As shown in Figures 1a, a clone containing risk allele showed ~1.7-fold greater transcriptional activity than a clone containing the non-risk allele. As shown in Figure 1b, this result was also confirmed in HAECs. These results indicate that the SNP affected the transcription level of *FLT1*. We could not find any candidate proteins predicted to bind to this DNA segment with high score (similarity >0.9) using TFBIND (http://tfbind.hgc.jp/),[13] a prediction software for searching transcription factor binding sites, based on the TRANSFAC database.[14]

### Knocking down experiment of *FLT1*

FLT1 is implicated in several inflammatory diseases, such as rheumatoid arthritis, cancer metastasis or atherosclerosis.[15–17] It is well known that several adhesion molecules from vascular endothelial cells play the critical roles in the inflammatory pathway.[18] Thus,

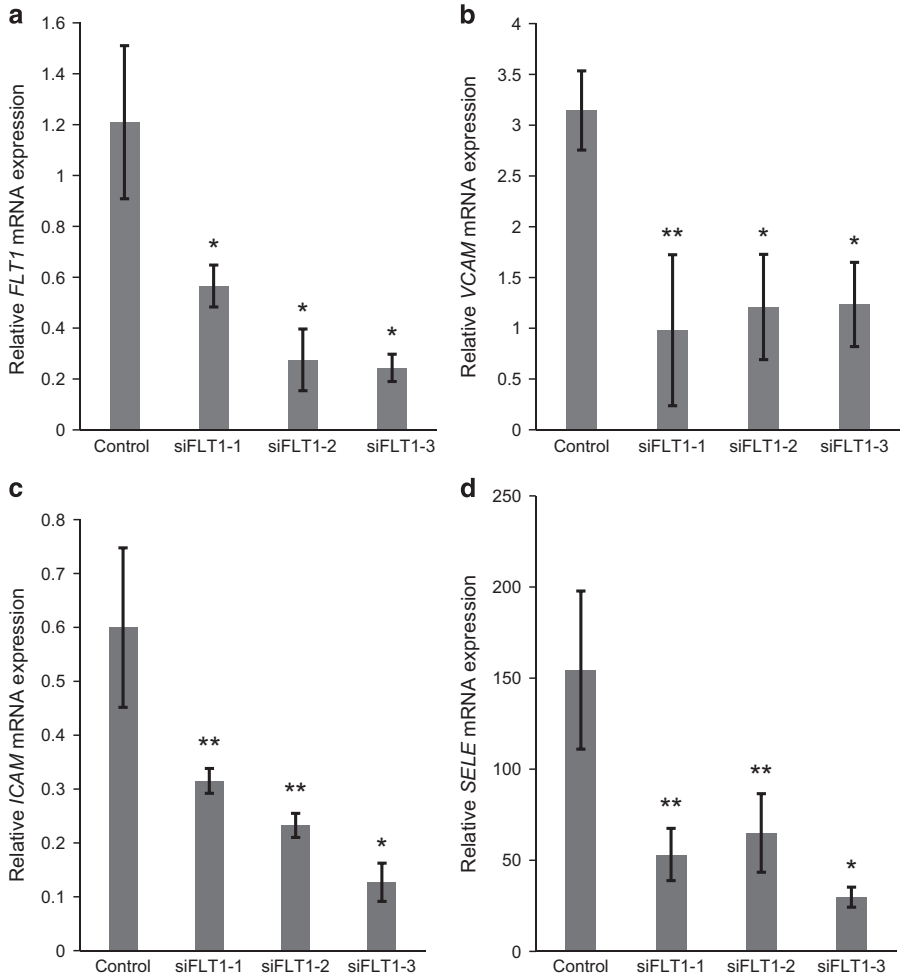


**Figure 2** Knocking down of *FLT1* suppresses mRNA expression of inflammatory adhesion molecules in human aortic endothelial cells (HAECs). (**a**) *FLT1* level in control or three kinds of *FLT1* small interfering RNA (siRNA)-treated HAECs, (**b**) *VCAM1*, (**c**) *ICAM1* and (**d**) *SELE* levels. * and **$P<0.01$ and $P<0.05$ when compared with control siRNA, respectively. Each experiment was repeated three times and each sample was studied in duplicate.

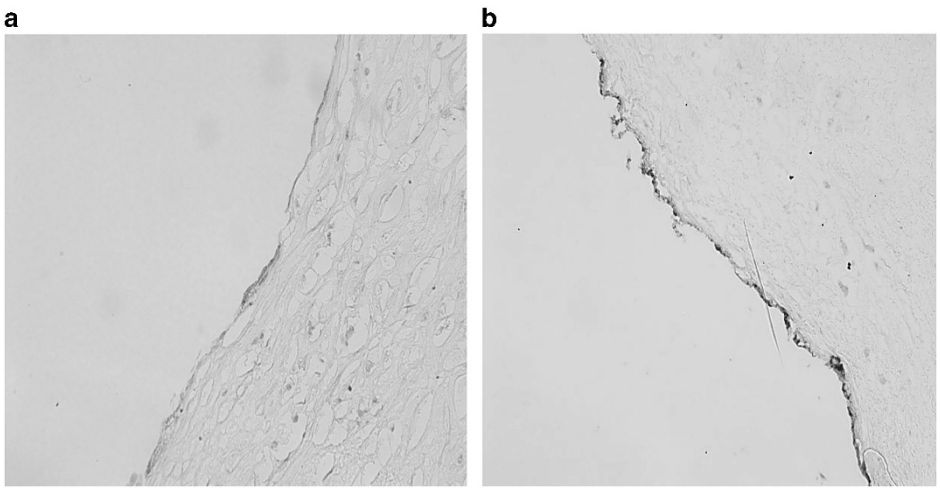


**Figure 3** Immunohistochemistry of the coronary artery. (**a**) Expression of FLT1 in endothelial cells (ECs), single staining. Magnification ×400, (**b**) colocalization of FLT1 (brown) and CD31 (blue) in ECs, double staining. Magnification x400 in **a** and **b**. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

we examined whether the cellular level of FLT1 could influence some inflammation-related cell adhesion molecules including SELE, ICAM1 and VCAM1. As shown in Figure 2, three kinds of small interfering RNA against *FLT1* significantly suppressed *FLT1* mRNA levels, and resulted in inhibition of expression of these adhesion molecules in HAECs.

**Immunohistochemistry for FLT1 with coronary artery segments**

To explore whether FLT1 protein is, in fact, expressed in the human coronary artery, we performed immunohistochemical staining with anti-FLT1 monoclonal antibody. We detected immunoreactivities of FLT1 in the endothelial cells (ECs) of coronary artery (Figure 3a). Coexpression of FLT1 and CD31 (a marker molecule for ECs) was also observed in the ECs by the immunodouble staining, although there were some EC-denudated areas (Figure 3b).

## DISCUSSION

In our replication study, *FLT1* locus was strongly associated with the risk of CAD in a Japanese population. A recent GWAS in a Korean population also showed a suggestive association between an *FLT1* SNP and CAD,[19] indicating that the locus is a common risk factor for CAD across multiple ethnic populations. A functional SNP rs74412485 was located in intron 1 of *FLT1*, encoding a member of vascular endothelial growth factor (VEGF) receptor family. VEGF is a multifactorial cytokine highly expressed in vascular endothelial cells and has pivotal roles in neonatal and postnatal vascular formation. It is suggested that VEGF possibly contributes to atherosclerosis through angiogenesis, endothelial regression and inflammation in the vascular wall, via the activation of two tyrosine kinase receptors, FLT1 and FLK1.[15,16] Soluble form of FLT1 is a nonfunctional decoy receptor for VEGF and functions as a negative regulator for VEGF/FLK1 signaling for angiogenesis.[15,16] FLT1 expression was also increased in several vascular cells by stimulation of angiotensin II. Previous report indicated the involvement between the several inflammatory diseases including rheumatoid arthritis, cancer and atherosclerosis and FLT1.[15–17] In our study, FLT1 was expressed in ECs of human coronary artery, and knocking down of *FLT1* led to reduced expression of inflammatory adhesion molecules, SELE, ICAM1 and VCAM1. Furthermore, previous observational studies have implicated the importance of adhesion molecules in cardiovascular diseases because they appear to be consistently expressed in atherosclerotic plaques and elevated levels of circulating adhesion molecules have been observed in atherosclerosis and CAD.[18,20–25] Hence, elevated expression of adhesion molecules such as E-selectin, ICAM1 and VCAM by increased expression of *FLT1* from CAD-susceptible allele could contribute to the pathogenesis of CAD, although relationship of FLT1 with activation of coronary artery endothelial cells involved in plaque instability and/or rupture remains to be clarified.

FLT1 localizes on the cell surface, suggesting a target for new drugs including humanized monoclonal antibody that impairs FLT1 function. Further investigation of FLT1 function involved in the inflammatory pathway may provide useful information for developing novel therapy using pharmaceutical approaches.

One of the two nominally replicated loci with statistical significance in a Japanese population was located in 5′-flanking region of endothelin receptor type A, *EDNRA*, on chromosome 4q31.33. *EDNRA* encodes a receptor for endothelin-1, a peptide that has a role in potent and long-lasting vasoconstriction[26] and proinflammatory effect.[27] Endothelin 1 mediates activation of vascular smooth muscle cells and was increased in human atherosclerotic lesions,[28,29] indicating that endothelin-1 contributes to the pathogenesis of chronic inflammation associated with atherosclerosis. Quantitative/qualitative change of the gene products affected by genetic polymorphisms might modify the homeostasis of inflammatory pathway and have an important role in the etiology of CAD.

We also found a modest association with statistical significance for an SNP, rs17514846 in intron 1 of *FURIN*. *FURIN* encodes a calcium-dependent serine endoprotease that belongs to proprotein convertase subtilisin/kexin (PCSK) enzyme family that cleaves latent precursor proteins into biologically active products.[30] The variant in *PCSK9*, encoding a serine protease enzyme protein belonging to the same family with FURIN, was also strongly associated with CAD.[31] PCSK9 interacts with hepatic low-density lipoprotein cholesterol receptor and inactivate the receptor by its degradation, and recent phase 1 clinical study demonstrated that the monoclonal antibody against PCSK9 markedly reduce circulating low-density lipoprotein cholesterol level in humans.[32] Taken together, these observations suggest that FURIN might also be a druggable molecule that targets CAD.

The remaining loci did not reach the statistical significance in our study, possibly because of insufficient statistical power. Future studies with much larger sample size are required to assess the association of these loci.

Finally, we believe that the knowledge of genetic architecture and their molecular pathway contributes to a better understanding of the pathogenesis of CAD and provides a useful clue for future investigation of biological and pharmaceutical approach to develop novel diagnostic methods, treatments and preventive measures for this common but serious disorder.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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