J Atheroscler Thromb, 2017; 24: 609-620.
http://doi.org/10.5551/jat.36897

Original Article

CDKN2B Methylation and Aortic Arch Calcification in Patients with Ischemic Stroke

Shuyu Zhou[1], Biyang Cai[1], Zhizhong Zhang[1], Yumeng Zhang[2], Li Wang[3], Keting Liu[4], Hao Zhang[1], Lingli Sun[1], Huan Cai[4], Guangming Lu[3], Xinfeng Liu[1] and Gelin Xu[1]

[1]Department of Neurology, Jinling Hospital, Medical School of Nanjing University, Nanjing, China
[2]Department of Gerontology, Nanjing Drum Tower Hospital, Medical School of Nanjing University, Nanjing, China
[3]Department of Medical Imaging, Jinling Hospital, Medical School of Nanjing University, Nanjing, China
[4]Department of Neurology, Jinling Hospital, Southern Medical University, Nanjing, China

***Aim*: *CDKN2A/2B* near chromosome 9p21 has been proposed as a potential genetic etiology for both atherosclerosis and arterial calcification. DNA methylation, which can change the expression of *CDKN2A/2B*, may be an underlying mechanism for this association. This study aimed to evaluate whether *CDKN2A/2B* methylation is related to aortic arch calcification (AAC) in patients with ischemic stroke.**

***Methods*: DNA methylation levels of *CDKN2A/2B* was measured using venous blood samples in 322 patients with ischemic stroke. A total of 36 CpG sites around promoter regions of *CDKN2A/2B* were examined. AAC was quantified with Agatston score based on results of computed tomography angiography.**

***Results*: There were 248 (77.0%) patients with and 74 (23.0%) patients without evident AAC. Compared with patients without AAC, patients with AAC had higher methylation levels of *CDKN2B* (5.72 vs 4.94, $P<0.001$). Using a generalized linear model, positive correlation between methylation levels and log-transformed calcification scores was detected at *CDKN2B* ($\beta=0.275\pm0.116$, $P=0.018$).**

***Conclusion*: Patients with higher levels of DNA methylation of *CDKN2B* may bear increased risk for AAC. Further studies to reveal the underlying mechanisms of this association are warranted for establishing a cause−effect relationship.**

Key words: Aortic arch calcification, *CDKN2A/2B*, DNA methylation, Ischemic stroke

Introduction

Atherosclerotic lesion of the aortic arch is a common etiology for ischemic stroke[1, 2]. Aortic arch calcification (AAC), a surrogate measure for atherosclerosis, can be readily detected with chest radiography[3, 4]. AAC was proposed as a satisfactory index for measuring systemic atherosclerosis burden[5, 6] and was associated with cardiovascular events[2, 7, 8].

Growing evidence indicated that genetic factors may affect the initiation and development of artery calcification[9, 10]. The human chromosome 9p21 (Chr9p21), for example, has been associated with both atherosclerosis and arterial calcification in several genome-wide association studies[11-13]. As a chromosome region devoid of protein-coding genes, Chr9p21 only transcribes a long non-coding RNA, namely antisense noncoding RNA in the INK4 locus (*ANRIL*). The closest protein-coding genes to Chr9p21 locus are two cyclin-dependent kinase inhibitors, *CDKN2A* and *CDKN2B*, both of which are involved in cell cycle regulation. Previous studies showed that variations in Chr9p21 may increase the levels of *ANRIL* transcription, which in turn downregulate *CDKN2A/2B* expression and enhance cell proliferation, and subsequently promote atherosclerosis[14, 15]. Studies also confirmed that *ANRIL* could bind and recruit epigenetic modifiers to *CDKN2A/2B* and induce DNA methylation[16, 17].

Address for correspondence: Gelin Xu, Department of Neurology, Jinling Hospital, Medical School of Nanjing University, 305 East Zhongshan Road, Nanjing, 210002, Jiangsu, China
E-mail: gelinxu@nju.edu.cn
Received: June 26, 2016
Accepted for publication: September 12, 2016

Aim

Considering *CDKN2A/2B* involved in the development of atherosclerotic diseases[18, 19], and DNA methylation of *CDKN2A/2B* has been frequently reported in many conditions[20, 21], we hypothesized that *CDKN2A/2B* methylation may increase the susceptibility of AAC. We tested this hypothesis in a group of Chinese patients with ischemic stroke who were at a high risk of developing atherosclerosis and calcification.

Methods

Study Population

This study was approved by the Ethical Review Board of Jinling Hospital. Informed consent was obtained from all enrolled patients. Consecutive patients with ischemic stroke aged ≥18 years were screened from Nanjing Stroke Registry Program (NSRP)[22] between July 2012 and September 2013. Patients with malignant neoplasm, severe liver or kidney diseases, autoimmune diseases, parathyroid gland diseases, or calcium−phosphorus metabolism disorders were excluded. As stents may influence the accuracy of calcification assessment, patients with a history of stenting treatment in aortic arch, brachiocephalic trunk, subclavian arteries, and common carotid arteries were also excluded. Finally, 324 patients were enrolled. Demographic characteristics and cardiovascular risk factors, which included age, sex, history of hypertension (HTN) and diabetes mellitus (DM), dyslipidemia, cigarette smoking, and alcohol drinking, were collected.

Artery Calcification Measurement

Each enrolled patient underwent neck computed tomography angiography (CTA) for AAC evaluation. CTA was performed with a dual-source 64 slice CT system (Siemens, Forchheim, Germany). Imaging was acquired by scanning from 4 cm below the aortic arch to the superior border of the orbit in craniocaudal direction. The aortic arch was recognized as a section from the initial segment to the first centimeter of the common carotid, vertebral, and subclavian arteries beyond the origin of the vertebral arteries. Details of scanning parameters have been reported elsewhere[23]. Calcification scores in the aortic arch were measured with the Syngo Calcium Scoring system (Siemens, Forchheim, Germany). A focus of ≥4 contiguous pixels accompanied with a CT density of ≥130 Hounsfield units (HU) was defined as calcification according to the method of Agatston score[24]. For each calcified lesion, the Agatston score was calculated as the product of the area (mm^2) and a factor assigned according to the maximum attenuation value of the lesion (HU=130−199 [1], 200−299 [2], 300−399 [3], >399 [4]). The total score of the aortic arch was calculated by adding up the scores of all lesions. Finally, patients with Agatston score of 0 or >0 were dichotomized into groups without or with AAC, respectively. Calcification scores were dual-assessed by two radiologists who were blinded to epi-genotyping results.

Table 1. Baseline characteristics of the study participants.

Variants	All (n=322)
Age, years	62.0 (55.0-70.0)
Male, n (%)	229 (71.1)
HTN, n (%)	250 (77.6)
DM, n (%)	110 (34.2)
Dyslipidemia, n (%)	176 (54.7)
TC, mmol/L	4.21 (3.58-5.00)
TG, mmol/L	1.40 (1.09-1.88)
HDL-c, mmol/L	0.98 (0.82-1.15)
LDL-c, mmol/L	2.61 (1.93-3.18)
Glucose, mmol/L	5.3 (4.6-6.6)
Smoking, n (%)	132 (41.0)
Drinking, n (%)	96 (29.8)
AAC, n (%)	248 (77.0%)
AAC score	221.5 (3.8-803.7)
Ln (AAC + 1)	5.40 (1.56-6.69)

Data are presented as number of individuals (%) or median (interquartile range).
AAC, artic arch calcification; HTN, hypertension; DM, diabetes mellitus; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

DNA Isolation and Genotyping

Venous blood samples were drawn in the morning after an overnight fasting for assaying biochemical parameters and epi-genotyping. Genomic DNA was extracted from whole blood using commercially available kits (TIANGEN Biotech, Beijing, China). DNA was quantified and then diluted to a working concentration of 10 ng/uL. Rs4977574 at Chr9p21, which is significantly associated with calcification in the aorta based on the validation of a previous study[25], was selected for genotyping. Single nucleotide polymorphism of rs4977574 (AA, AG, GG) was genotyped via polymerase chain reaction ligase detection reaction with an ABI Prism 377 Sequence Detection System (Applied Biosystems, CA, USA)[26]. Sequencing primers were CATGCTTTCTGAAACAACACG (forward) and TAATGGAGGTGTGGTCAGCA (reverse). Reproducibility of genotyping was confirmed by randomly

Table 2. Comparison of demographic characteristics of patients with and without AAC.

Variants	AAC		*P* value
	With ($n=248$)	Without ($n=74$)	
Age, years	64.0 (58.0-72.0)	49.0 (43.8-58.3)	**<0.001**
Male, n (%)	171 (69.0)	58 (78.4)	0.144
HTN, n (%)	206 (83.1)	44 (59.5)	**<0.001**
DM, n (%)	86 (34.7)	24 (32.4)	0.781
Dyslipidemia, n (%)	128 (51.6)	48 (64.9)	**0.047**
TC, mmol/L	4.27 (3.55-5.01)	4.16 (3.69-4.78)	0.756
TG, mmol/L	1.37 (1.06-1.76)	1.58 (1.15-2.03)	0.064
HDL-c, mmol/L	1.00 (0.83-1.16)	0.91 (0.79-1.06)	**0.048**
LDL-c, mmol/L	2.63 (1.93-3.20)	2.60 (1.93-3.09)	0.995
Glucose, mmol/L	5.3 (4.7-6.7)	5.3 (4.6-6.6)	0.670
Smoking, n (%)	97 (39.1)	35 (47.3)	0.227
Drinking, n (%)	71 (28.6)	25 (33.8)	0.390

selecting 10% of the samples, and the concordance was 100%.

DNA Methylation Analysis

CpG islands adjacent to promoter regions of *CDKN2A/2B* were selected for measurement according to the following criteria: (1) 200 bp minimum length; (2) ≥50% GC content; (3) ≥0.60 ratio of observed/expected dinucleotides CpG[27]. Six CpG regions from CpG islands of *CDKN2A* and three from those of *CDKN2B* were sequenced. Bisulfite conversion of 1 ug genomic DNA was performed using the EZ DNA Methylation™-GOLD Kit (ZYMO RESEARCH, CA, USA) according to the manufacturer's protocol. Sodium bisulfite can preferentially deaminate un-methylated cytosine residues to thymines, whereas methyl-cytosines remain unmodified. After PCR amplification (HotStarTaq polymerase kit, TAKARA, Tokyo, Japan) of target CpG regions and library construction, products were sequenced using Illumina MiSeq Benchtop Sequencer (CA, USA) in accordance with the method of BiSulfite Amplicon Sequencing[28]. Primer sequences used for PCR were shown in **Supplemental Table 1**. All samples achieved a mean coverage of >600 X. Methylation levels of 24 CpG sites in *CDKN2A* and 12 sites in *CDKN2B* were measured. Each tested CpG site was named as its relative distance (in bp) to transcriptional start site and listed in **Supplemental Table 2**. The methylation level of each CpG site was calculated as the percentage of the methylated cytosines over total tested cytosines. The average methylation level was calculated using methylation levels of all measured CpG sites within the gene.

Statistical Analysis

Normality of quantitative variables was assessed using Shapiro−Wilk test. As all continuous data in this study did not meet the normality assumption, they were described as median (interquartile range) and compared using Mann−Whitney *U* test. Categorical variables were compared using Fisher's exact test.

Spearman correlations were used to evaluate pairwise correlations of methylation levels between different CpG sites in the same gene. Considering the extremely left-skewed distribution of calcification scores, we added 1 to each calcification score, and the value was then log-transformed as the formula: Ln (calcification score + 1). This transformation may result in a less skewed distribution, as suggested in previous studies[13, 25]. Generalized linear model was used to explore the association between methylation levels and log-transformed calcification scores, adjusting for potential confounders including age, sex, HTN, DM, dyslipidemia, and smoking. For multiple testing, Bonferroni correction was performed. Kruskal−Wallis test was performed to compare methylation levels across the genotypes of rs4977574 (AA, AG, and GG).

Data were analyzed using IBM SPSS Statistics Version 22.0 (Armonk, NY: IBM Corp.). A two-tailed value of $P<0.05$ was considered statistically significant.

Results

Of the 324 enrolled participants, 2 (0.6%) failed in epi-genotyping. Finally, 322 (99.4%) patients were included for data analysis. Baseline characteristics were listed in **Table 1**. The median age of these 322

Table 3. Differences of methylation levels between patients with and without AAC.

Gene	Position	AAC		*P* value
		With	Without	
CDKN2A	1	4.20 (2.82-5.88)	4.33 (2.74-6.19)	0.571
	2	6.89 (5.30-8.90)	7.13 (5.51-8.55)	0.844
	3	8.09 (6.62-10.4)	8.02 (6.25-10.0)	0.379
	4	5.86 (4.25-7.81)	5.80 (4.38-7.87)	0.864
	5	4.80 (4.04-5.49)	5.08 (4.33-5.59)	0.132
	6	2.79 (2.34-3.36)	2.56 (2.09-3.07)	**0.038**
	7	2.33 (1.87-2.83)	2.27 (1.77-2.71)	0.174
	8	4.43 (3.78-5.08)	4.01 (3.56-4.67)	**0.037**
	9	4.30 (2.37-7.33)	5.26 (3.27-8.83)	0.130
	10	2.04 (0.96-3.23)	1.75 (1.00-2.56)	0.233
	11	3.57 (2.38-4.89)	3.70 (2.22-5.13)	0.885
	12	0.96 (0.57-1.34)	0.88 (0.56-1.25)	0.363
	13	1.20 (0.96-1.49)	1.34 (0.94-1.52)	0.441
	14	1.21 (1.03-1.46)	1.11 (0.88-1.41)	**0.039**
	15	2.05 (1.66-2.41)	1.93 (1.54-2.48)	0.630
	16	1.33 (1.02-1.66)	1.35 (1.08-1.62)	0.906
	17	3.23 (2.64-3.97)	3.01 (2.48-3.46)	**0.005**
	18	2.19 (1.71-2.61)	2.14 (1.74-2.53)	0.487
	19	2.49 (2.01-2.97)	2.48 (1.96-2.87)	0.827
	20	2.75 (2.18-3.27)	2.60 (2.10-3.07)	0.259
	21	15.5 (13.5-17.2)	15.6 (14.4-17.1)	0.313
	22	2.52 (2.08-3.15)	2.62 (2.07-3.20)	0.762
	23	4.27 (3.50-5.09)	4.32 (3.51-4.84)	0.853
	24	1.69 (1.26-2.46)	1.77 (1.31-2.48)	0.631
	Average	3.95 (3.58-4.27)	3.92 (3.67-4.23)	0.909
CDKN2B	1	5.43 (4.51-6.44)	4.97 (4.04-5.81)	**0.005**
	2	4.45 (3.48-5.34)	3.99 (3.06-5.06)	**0.014**
	3	3.89 (3.10-4.87)	3.86 (3.10-4.64)	0.700
	4	4.20 (3.53-5.11)	3.89 (3.14-4.41)	**0.007**
	5	7.66 (6.65-8.80)	6.65 (5.80-7.68)	**<0.001**
	6	6.95 (5.99-8.12)	6.00 (5.04-6.80)	**<0.001**
	7	8.12 (7.04-9.49)	7.10 (6.41-7.92)	**<0.001**
	8	3.52 (3.03-4.11)	3.07 (2.76-3.49)	**<0.001**
	9	3.94 (3.34-4.47)	3.41 (2.76-3.82)	**<0.001**
	10	6.08 (5.21-7.03)	5.11 (4.20-5.92)	**<0.001**
	11	7.53 (6.32-8.72)	6.56 (5.47-7.05)	**<0.001**
	12	5.74 (5.02-6.70)	5.01 (4.37-5.60)	**<0.001**
	Average	5.72 (5.03-6.34)	4.94 (4.48-5.47)	**<0.001**

patients was 62.0 (55.0−70.0) years, and 229 (71.1%) of them were males. Of these analyzed patients, 250 (77.6%) had a history of HTN and 110 (34.2%) had a history of DM.

Based on Agatston scores, there were 248 (77.0%) and 74 (23.0%) patients classified as with and without AAC, respectively. AAC scores presented a highly skewed distribution with a median (interquartile range) of 221.5 (3.8−803.7). Compared with patients without AAC, those with AAC were older (64.0 vs 49.0 years, $P<0.001$), had a higher prevalence of HTN (83.1% vs 59.5%, $P<0.001$), lower prevalence of dyslipidemia (51.6% vs 64.9%, $P=0.047$), and higher HDL-c levels (1.00 vs 0.91, $P=0.048$) (**Table 2**).

Methylation levels of 36 CpG sites were listed in **Supplemental Table 3**. Methylation levels of CpG sites measured within *CDKN2A* were not strongly

Table 4. Association of methylation levels and log-transformed calcification scores detected by generalized liner model.

	β	SE	*P* value
Model 1			
CDKN2A	−0.011	0.220	0.961
Age	0.159	0.011	**<0.001**
Sex	−0.065	0.297	0.827
HTN	0.600	0.299	**0.044**
DM	0.039	0.257	0.880
Dyslipidemia	−0.137	0.245	0.576
Smoking	−0.016	0.272	0.954
Model 2			
CDKN2B	0.275	0.116	**0.018**
Age	0.148	0.012	**<0.001**
Sex	−0.057	0.295	0.847
HTN	0.653	0.296	**0.027**
DM	0.042	0.253	0.869
Dyslipidemia	−0.077	0.244	0.751
Smoking	−0.049	0.269	0.854

Generalized liner model was adjusted for age, sex, HTN, DM, dyslipidemia and smoking.

correlated, whereas those within *CDKN2B* were well correlated (**Supplemental Table 4-5**). As shown in **Table 3**, univariate comparison of these 36 sites and the average methylation levels indicated that methylation levels of *CDKN2B* were higher in patients with AAC than in those without AAC (5.72 vs 4.94, $P<0.001$).

As shown in **Table 4**, generalized liner model detected a positive correlation between average methylation levels of *CDKN2B* and log-transformed calcification scores ($\beta=0.275\pm0.116$, $P=0.018$) after adjusting for age, sex, HTN, DM, dyslipidemia, and smoking. The association still remained after further correction for multiple comparison (corrected $P=0.036$).

Further, we assessed the association between rs4977574 and methylation of *CDKN2B*. After adjusting for potential risk factors, rs4977574 (G as coded allele) was associated with AAC in the study population ($\beta=0.414\pm0.171$, $P=0.015$). There were no differences in average methylation levels of *CDKN2B* among three genotypes of rs4977574 (AA vs AG vs GG: 5.56 vs 5.58 vs 5.46, $P=0.626$). When rs4977574 was further added into the generalized linear model, the average methylation levels of *CDKN2B* still correlated with log-transformed calcification scores ($\beta=0.292\pm0.115$, $P=0.011$) (**Table 5**).

Table 5. Association of *CDKN2B* methylation levels and AAC after adjustment of rs4977574 and other confounders.

Variants	β	SE	*P* value
CDKN2B	0.292	0.115	**0.011**
Age	0.148	0.012	**<0.001**
Sex	0.010	0.293	0.972
HTN	0.645	0.293	**0.028**
DM	0.112	0.252	0.656
Dyslipidemia	−0.072	0.242	0.766
Smoking	−0.125	0.268	0.641
Rs4977574	0.439	0.170	**0.010**

Discussion

This study observed that methylation levels of *CDKN2B* were relatively higher in patients with AAC than those in patients without AAC. A positive correlation between *CDKN2B* methylation and AAC load was detected. These results verified our hypothesis that DNA methylation in *CDKN2B* may increase the susceptibility of artery calcification.

CDKN2B is a well-characterized tumor suppressor gene which is involved in cell cycle regulation via retinoblastoma (Rb) pathway[29]. The $p15^{INK4b}$ protein encoded by *CDKN2B* can specifically bind to CDKN4/6 and result in G1 phase arrest and cell proliferation interruption[12]. Methylation in CpG islands around promoter regions can generally reduce gene expression[30]. Evidence that *CDKN2B* methylation represses expression and leads to unlimited cell proliferation has been confirmed in a spectrum of cancers[21, 31].

Both inflammatory responses and migration of proliferating vascular smooth muscle cells (VSMCs) are considered essential for the development of atherosclerosis[32]. Chronic vascular inflammation arising from atherosclerosis also contributes to arterial calcification[10]. Under certain circumstances, a subpopulation of VSMCs may be predisposed to differentiate into osteoblastic and proliferative phenotypes. They can acquire osteoblast-like characteristics and become calcifying vascular cells, participating in spontaneous mineral deposition[33-35]. As the expression of *CDKN2B* is repressed, Rb proteins may lose control and result in increased proliferation of macrophages and VSMCs[15, 19].

The association of *CDKN2B* methylation and coronary artery disease (CAD) has been previously observed[20]. Zhuang and colleagues found that the methylation levels of *CDKN2B* were significantly higher in CAD patients than in controls. Based on quantitative assessment of calcification, our study

observed similar results in the aortic arch. Therefore, the higher the methylation level, the more serious the artery calcification might be.

Methylation levels of *CDKN2B* were not directly linked to genotypes of rs4977574, which was associated with AAC in previous and in our studies. It was possible that genetic variants directly contribute to *ANRIL* expression rather than to *CDKN2B* methylation according to evidence from previous studies[12, 36]. *CDKN2B* methylation was likely to be modulated by *ANRIL* or other epigenetic changes.

There are several limitations to our study. First, the nature of the cross-sectional study limited us to reach a causal inference. We cannot determine if the observed associations is attributed to methylation effects on AAC or vice versa. Second, *CDKN2A/2B* expression was not tested in this study due to lack of fresh leukocytes, which prevented us from evaluating the interactions between methylation variation and *CDKN2A/2B* gene expression. Therefore, future studies need to be conducted to provide more functional evidence. Third, considering varied predisposition of DNA methylation in different tissues, methylation measured from white blood cells may not represent that of vessel walls, although the role of white blood cells in atherogenesis is well-defined[32]. Because of the difficulty in obtaining vascular tissues from human body via invasive therapy, methylation tests from peripheral blood is still a convenient and rational method for investigation. In addition, a larger sample size is favorable for confirmation and more reliable results. The study was conducted in subjects with ischemic stroke, which may lead to selection bias as the prevalence of AAC was higher than that in the general population. Therefore, further exploration in the population with health controls is more convincible.

In conclusion, *CDKN2B* methylation is independently associated with AAC. Patients with higher methylation levels in *CDKN2B* may have increased risk for AAC. Further studies on the underlying mechanisms of this association are warranted to establishing a cause−effect relationship.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (NSFC, # 81571143 to GX, # 81530038 to XL and # 81220108008 to XL). We thank all patients for participating in this study. We are also grateful to Center for Genetics & Genomic Analysis, Genesky Biotechnologies Inc. (Shanghai, 201203) for their technical support in sequencing.

Conflict of Interest

None.

References

1) Atherosclerotic disease of the aortic arch as a risk factor for recurrent ischemic stroke. The French Study of Aortic Plaques in Stroke Group, N Engl J Med, 1996; 334: 1216-1221
2) Iribarren C, Sidney S, Sternfeld B, Browner WS: Calcification of the aortic arch: risk factors and association with coronary heart disease, stroke, and peripheral vascular disease, Jama, 2000; 283: 2810-2815
3) Hyman JB and Epstein FH: A study of the correlation between roentgenographic and post-mortem calcification of the aorta, Am Heart J, 1954; 48: 540-543
4) Hashimoto H, Iijima K, Hashimoto M, Son BK, Ota H, Ogawa S, Eto M, Akishita M, Ouchi Y: Validity and usefulness of aortic arch calcification in chest X-ray, J Atheroscler Thromb, 2009; 16: 256-264
5) Bos D, Leening MJ, Kavousi M, Hofman A, Franco OH, van der Lugt A, Vernooij MW, Ikram MA: Comparison of Atherosclerotic Calcification in Major Vessel Beds on the Risk of All-Cause and Cause-Specific Mortality: The Rotterdam Study, Circ Cardiovasc Imaging, 2015; 8
6) Allison MA, Criqui MH and Wright CM: Patterns and risk factors for systemic calcified atherosclerosis, Arterioscler Thromb Vasc Biol, 2004; 24: 331-336
7) Li J, Galvin HK, Johnson SC, Langston CS, Sclamberg J, Preston CA: Aortic calcification on plain chest radiography increases risk for coronary artery disease, Chest, 2002; 121: 1468-1471
8) Iijima K, Hashimoto H, Hashimoto M, Son BK, Ota H, Ogawa S, Eto M, Akishita M, Ouchi Y: Aortic arch calcification detectable on chest X-ray is a strong independent predictor of cardiovascular events beyond traditional risk factors, Atherosclerosis, 2010; 210: 137-144
9) Rutsch F, Nitschke Y and Terkeltaub R: Genetics in arterial calcification: pieces of a puzzle and cogs in a wheel, Circ Res, 2011; 109: 578-592
10) Demer LL and Tintut Y: Inflammatory, Metabolic, and Genetic Mechanisms of Vascular Calcification, Arterioscler Thromb Vasc Biol, 2014; 34: 715-723
11) Ye S, Willeit J, Kronenberg F, Xu Q, Kiechl S: Association of genetic variation on chromosome 9p21 with susceptibility and progression of atherosclerosis: a population-based, prospective study, J Am Coll Cardiol, 2008; 52: 378-384
12) Holdt LM and Teupser D: Recent studies of the human chromosome 9p21 locus, which is associated with atherosclerosis in human populations, Arterioscler Thromb Vasc Biol, 2012; 32: 196-206
13) O'Donnell CJ, Kavousi M, Smith AV, Kardia SL,Feitosa MF, Hwang SJ, Sun YV, Province MA, Aspelund T, Dehghan A, Hoffmann U, Bielak LF, Zhang Q, Eiriksdottir G, van Duijn CM, Fox CS, de Andrade M, Kraja AT, Sigurdsson S, Elias-Smale SE, Murabito JM, Launer LJ, van der Lugt A, Kathiresan S, Krestin GP, Herrington DM, Howard TD, Liu Y, Post W, Mitchell BD, O'Connell JR,

Shen H, Shuldiner AR, Altshuler D, Elosua R, Salomaa V, Schwartz SM, Siscovick DS, Voight BF, Bis JC, Glazer NL, Psaty BM, Boerwinkle E, Heiss G, Blankenberg S, Zeller T, Wild PS, Schnabel RB, Schillert A, Ziegler A, Munzel TF, White CC, Rotter JI, Nalls M, Oudkerk M, Johnson AD, Newman AB, Uitterlinden AG, Massaro JM, Cunningham J, Harris TB, Hofman A, Peyser PA, Borecki IB, Cupples LA, Gudnason V, Witteman JC: Genome-wide association study for coronary artery calcification with follow-up in myocardial infarction, Circulation, 2011; 124: 2855-2864

14) Visel A, Zhu Y, May D, Afzal V, Gong E, Attanasio C, Blow MJ, Cohen JC, Rubin EM, Pennacchio LA: Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice, Nature, 2010; 464: 409-412
15) Motterle A, Pu X, Wood H, Xiao Q, Gor S, Ng FL, Chan K, Cross F, Shohreh B, Poston RN, Tucker AT, Caulfield MJ, Ye S: Functional analyses of coronary artery disease associated variation on chromosome 9p21 in vascular smooth muscle cells, Hum Mol Genet, 2012; 21: 4021-4029
16) Zaina S: Unraveling the DNA methylome of atherosclerosis, Curr Opin Lipidol, 2014; 25: 148-153
17) Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M, Xiong Y: Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene, Oncogene, 2011; 30: 1956-1962
18) Jarinova O, Stewart AF, Roberts R, Wells G, Lau P, Naing T, Buerki C, McLean BW, Cook RC, Parker JS, McPherson R: Functional analysis of the chromosome 9p21. 3 coronary artery disease risk locus, Arterioscler Thromb Vasc Biol, 2009; 29: 1671-1677
19) Hannou SA, Wouters K, Paumelle R, Staels B: Functional genomics of the CDKN2A/B locus in cardiovascular and metabolic disease: what have we learned from GWASs?, Trends Endocrinol Metab, 2015; 26: 176-184
20) Zhuang J, Peng W, Li H, Wang W, Wei Y, Li W, Xu Y: Methylation of p15INK4b and expression of ANRIL on chromosome 9p21 are associated with coronary artery disease, PloS one, 2012; 7: e47193
21) Esteller M: CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future, Oncogene, 2002; 21: 5427-5440
22) Liu X, Xu G, Wu W, Zhang R, Yin Q, Zhu W: Subtypes and one-year survival of first-ever stroke in Chinese patients: The Nanjing Stroke Registry, Cerebrovasc Dis, 2006; 22: 130-136
23) Lu L, Zhang LJ, Poon CS, Wu SY, Zhou CS, Luo S, Wang M, Lu GM: Digital subtraction CT angiography for detection of intracranial aneurysms: comparison with three-dimensional digital subtraction angiography, Radiology, 2012; 262: 605-612
24) Pham PH, Rao DS, Vasunilashorn F, Fishbein MC, Goldin JG: Computed tomography calcium quantification as a measure of atherosclerotic plaque morphology and stability, Invest Radiol, 2006; 41: 674-680
25) van Setten J, Isgum I, Smolonska J, Ripke S, de Jong PA, Oudkerk M, de Koning H, Lammers JW, Zanen P, Groen HJ, Boezen HM, Postma DS, Wijmenga C, Viergever MA, Mali WP, de Bakker PI: Genome-wide association study of coronary and aortic calcification implicates risk loci for coronary artery disease and myocardial infarction, Atherosclerosis, 2013; 228: 400-405
26) Xiao Z, Xiao J, Jiang Y, Zhang S, Yu M, Zhao J, Wei D, Cao H: A novel method based on ligase detection reaction for low abundant YIDD mutants detection in hepatitis B virus, Hepatol Res, 2006; 34: 150-155
27) Gardiner-Garden M and Frommer M: CpG islands in vertebrate genomes, J Mol Biol, 1987; 196: 261-282
28) Masser DR, Berg AS and Freeman WM: Focused, high accuracy 5-methylcytosine quantitation with base resolution by benchtop next-generation sequencing, Epigenetics Chromatin, 2013; 6: 33
29) Gil J and Peters G: Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all, Nat Rev Mol Cell Biol, 2006; 7: 667-677
30) Portela A and Esteller M: Epigenetic modifications and human disease, Nat Biotechnol, 2010; 28: 1057-1068
31) Herman JG and Baylin SB: Gene silencing in cancer in association with promoter hypermethylation, N Engl J Med, 2003; 349: 2042-2054
32) Wierda RJ, Geutskens SB, Jukema JW, Quax Paul HA, van den Elsen PJ: Epigenetics in atherosclerosis and inflammation, J Cell Mol Med, 2010; 14: 1225-1240
33) Doherty TM, Asotra K, Fitzpatrick LA, Qiao JH, Wilkin DJ, Detrano RC, Dunstan CR, Shah PK, Rajavashisth TB: Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads, Proc Natl Acad Sci U S A, 2003; 100: 11201-11206
34) Johnson RC, Leopold JA and Loscalzo J: Vascular calcification: pathobiological mechanisms and clinical implications, Circ Res, 2006; 99: 1044-1059
35) Zhu D, Mackenzie NC, Farquharson C, Macrae VE: Mechanisms and clinical consequences of vascular calcification, Front Endocrinol (Lausanne), 2012; 3: 95
36) Holdt LM, Beutner F, Scholz M, Gielen S, Gäbel G, Bergert H, Schuler G, Thiery J, Teupser D: ANRIL expression is associated with atherosclerosis risk at chromosome 9p21, Arterioscler Thromb Vasc Biol, 2010; 30: 620-627

Supplemental Table 1.
Primer sequences for *CDKN2A/2B* genes (start and end site were named as its relative distance to transcriptional start site)

Gene	PCR size (bp)	Start site	End site	Primer	
CDKN2A	282	− 1477	− 1197	forward	GGGATATGGAGGGGGAGAT
				reverse	CTTCTTCCTCTTTCCTCTTCCC
	211	− 1047	− 838	forward	GGGAAGAGGAAAGAGGAAGAAG
				reverse	ATTAAACTAAACCRCTACACRCCTCTAAC
	286	− 859	− 574	forward	AATAAAATAAGGGGAATAGGGGAG
				reverse	CCATCTTCCCACCCTCAA
	188	− 399	− 212	forward	GTAGTTAAGGGGGTAGGAGTGG
				reverse	ACTACTACCCTAAACRCTAACTCCTCAA
	266	+ 70	+ 335	forward	TTGAGGAGTTAGYGTTTAGGGTAGTAGT
				reverse	TCAATAATACTACRAAAACCACATATCTAAATC
	224	+ 308	+ 531	forward	GTYGGTTGGTTTTTTATTTTGTTAGAG
				reverse	AACCTAAACTCAACTTCATTACCCTC
CDKN2B	255	− 7	+ 248	forward	GAGGGTAATGAAGTTGAGTTTAGGTT
				reverse	CTATCRCACCTTCTCCACTAATCC
	234	+ 223	+ 455	forward	GGGGATTAGTGGAGAAGGTG
				reverse	TAAAATACACACCTCCRACCAAC
	221	+ 430	+ 650	forward	TGTTTTTTAAGTTTTTATAGGGTGAGG
				reverse	CCAACCTAACCAAAATAATAAAAACC

Supplemental Table 2. Methylated CpG sites measured in this study.

Gene	Position	Genomic location*	Relative to TSS, bp
CDKN2A	1	Chr9: 21995909	−1419
	2	Chr9: 21995896	−1406
	3	Chr9: 21995867	−1377
	4	Chr9: 21995713	−1223
	5	Chr9: 21995470	−980
	6	Chr9: 21995457	−967
	7	Chr9: 21995455	−965
	8	Chr9: 21995354	−864
	9	Chr9: 21995314	−824
	10	Chr9: 21995312	−822
	11	Chr9: 21995305	−815
	12	Chr9: 21995108	−618
	13	Chr9: 21994859	−369
	14	Chr9: 21994782	−292
	15	Chr9: 21994734	−244
	16	Chr9: 21994727	−237
	17	Chr9: 21994286	+205
	18	Chr9: 21994215	+276
	19	Chr9: 21994211	+280
	20	Chr9: 21994208	+283
	21	Chr9: 21994155	+336
	22	Chr9: 21994109	+382
	23	Chr9: 21994076	+415
	24	Chr9: 21993993	+498
CDKN2B	1	Chr9: 22009259	+54
	2	Chr9: 22009179	+134
	3	Chr9: 22009165	+148
	4	Chr9: 22009134	+179
	5	Chr9: 22009000	+313
	6	Chr9: 22008981	+332
	7	Chr9: 22008956	+357
	8	Chr9: 22008890	+423
	9	Chr9: 22008845	+468
	10	Chr9: 22008830	+483
	11	Chr9: 22008815	+498
	12	Chr9: 22008804	+509

*The chromosomal location of each CpG site according to assembly GRCh37/hg19.

Supplemental Table 3. Distribution of methylation levels (%) of 36 CpG sites in *CDKN2A/2B* genes.

Gene	Position	Min	Q1	Median	Q3	Max
CDKN2A	1	0.00	2.81	4.26	5.94	17.19
	2	0.00	5.31	6.98	8.82	19.69
	3	0.00	6.53	8.08	10.17	21.40
	4	0.00	4.27	5.83	7.85	18.60
	5	0.00	4.08	4.89	5.49	9.66
	6	0.00	2.29	2.71	3.29	9.23
	7	0.00	1.84	2.33	2.79	7.69
	8	1.79	3.69	4.37	5.03	10.44
	9	0.00	2.44	4.42	7.90	23.53
	10	0.00	0.98	1.97	3.14	8.82
	11	0.00	2.38	3.61	4.93	13.33
	12	0.00	0.57	0.93	1.33	2.85
	13	0.00	0.95	1.21	1.50	2.60
	14	0.00	0.97	1.18	1.44	2.94
	15	0.00	1.64	2.05	2.43	6.86
	16	0.00	1.03	1.34	1.65	3.37
	17	0.00	2.59	3.17	3.83	25.26
	18	0.49	1.72	2.18	2.58	7.49
	19	0.00	2.01	2.49	2.95	7.49
	20	0.44	2.17	2.72	3.24	8.85
	21	4.86	13.74	15.51	17.20	34.15
	22	0.71	2.07	2.57	3.18	8.62
	23	0.00	3.50	4.27	5.05	10.98
	24	0.00	1.26	1.70	2.46	6.17
	Average	2.39	3.61	3.94	4.25	6.15
CDKN2B	1	1.83	4.42	5.35	6.24	12.21
	2	0.00	3.42	4.37	5.24	10.88
	3	0.00	3.10	3.89	4.82	9.32
	4	0.00	3.34	4.10	4.99	18.82
	5	4.42	6.44	7.40	8.61	16.37
	6	3.44	5.54	6.66	7.78	13.05
	7	4.06	6.84	7.86	9.06	18.45
	8	0.86	2.94	3.41	3.96	12.05
	9	0.00	3.23	3.74	4.36	17.09
	10	0.00	4.95	5.85	6.74	18.04
	11	3.70	6.18	7.13	8.48	27.93
	12	2.43	4.80	5.48	6.45	23.90
	Average	3.45	4.83	5.54	6.15	11.08

Q1: 1st quartile (25th percentile), Q3: 3rd quartile (75th percentile).

Supplemental Table 4. Spearman pairwise correlations for CpG sites of *CDKN2A*.

Position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	1.0	0.5*	0.4*	0.4*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	−0.1*	0.1	−0.1	0.0	−0.1	−0.1	−0.1	0.0	−0.1*	0.0	0.1	0.1	0.2*
2		1.0	0.5*	0.4*	0.0	0.0	0.0	0.1*	0.0	0.0	0.0	0.0	−0.1	−0.1	0.1	−0.1*	0.0	0.0	0.0	0.0	0.1	0.1	0.1*	0.3*
3			1.0	0.4*	0.1	0.0	0.0	0.1*	0.0	0.0	0.0	0.0	0.0	0.0	0.1	−0.1	0.1	0.1	0.1	0.1	0.1	0.1*	0.2*	0.3*
4				1.0	0.1	0.1	0.0	0.1	0.1	0.0	0.0	−0.1	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.2*
5					1.0	0.2*	0.3*	0.2*	0.0	0.0	0.0	0.1	0.2*	0.1	0.1	0.2*	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1*
6						1.0	0.3*	0.3*	−0.1	0.1	0.1	0.1*	0.1	0.2*	0.2*	0.2*	0.1	0.1*	0.1	0.0	0.0	0.1*	0.1	0.2*
7							1.0	0.2*	0.0	0.1	0.0	0.1	0.0	0.1	0.2*	0.2*	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.1
8								1.0	0.1	0.1	0.1	0.2*	0.2*	0.1	0.3*	0.2*	0.1*	0.2*	0.1	0.1*	0.0	0.1*	0.1	0.1
9									1.0	0.0	−0.1	0.0	0.0	0.0	0.0	0.0	0.0	−0.1	−0.1	−0.1*	−0.1*	0.0	0.0	0.0
10										1.0	0.1	0.0	−0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	−0.1	0.0	0.1
11											1.0	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.2*	0.0	0.0
12												1.0	0.0	0.0	0.1*	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.0	−0.1
13													1.0	0.1	0.0	0.1*	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14														1.0	0.1*	0.1	0.1	0.1	0.1	0.1*	0.0	0.1*	0.1*	0.0
15															1.0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.2*
16																1.0	0.1	0.0	0.2*	0.1	0.1	0.0	0.0	0.1
17																	1.0	0.1*	0.2*	0.3*	0.2*	0.1*	0.2*	0.0
18																		1.0	0.2*	0.3*	0.2*	0.1	0.0	0.1
19																			1.0	0.3*	0.1*	0.0	0.1*	0.1
20																				1.0	0.2*	0.1	0.1*	0.0
21																					1.0	0.1*	0.1	0.1
22																						1.0	0.2*	0.3*
23																							1.0	0.2*
24																								1.0

*$p < 0.05$

Supplemental Table 5. Spearman pairwise correlations for CpG sites of *CDKN2B*.

Position	1	2	3	4	5	6	7	8	9	10	11	12
1	1.0	0.5	0.5	0.4	0.6	0.6	0.6	0.3	0.3	0.4	0.5	0.4
2		1.0	0.5	0.5	0.5	0.5	0.5	0.3	0.3	0.4	0.3	0.3
3			1.0	0.4	0.5	0.5	0.5	0.3	0.3	0.3	0.3	0.3
4				1.0	0.5	0.5	0.5	0.3	0.3	0.3	0.4	0.4
5					1.0	0.8	0.7	0.4	0.4	0.6	0.5	0.5
6						1.0	0.8	0.4	0.4	0.6	0.5	0.6
7							1.0	0.3	0.4	0.5	0.5	0.5
8								1.0	0.3	0.3	0.3	0.3
9									1.0	0.6	0.6	0.5
10										1.0	0.8	0.7
11											1.0	0.7
12												1.0

All $p < 0.001$