Original Article

CDKN2B Methylation and Aortic Arch Calcification in Patients with Ischemic Stroke

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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 (\( \beta = 0.275 \pm 0.116, \ P = 0.018 \)).

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 stroke1, 2). Aortic arch calcification (AAC), a surrogate measure for atherosclerosis, can be readily detected with chest radiography3, 4). AAC was proposed as a satisfactory index for measuring systemic atherosclerosis burden5, 6) and was associated with cardiovascular events2, 7, 8).

Growing evidence indicated that genetic factors may affect the initiation and development of artery calcification9, 10). The human chromosome 9p21 (Chr9p21), for example, has been associated with both atherosclerosis and arterial calcification in several genome-wide association studies11-13). 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 atherosclerosis14, 15). Studies also confirmed that ANRIL could bind and recruit epigenetic modifiers to CDKN2A/2B and induce DNA methylation16, 17).
Aim

Considering CDKN2A/2B involved in the development of atherosclerotic diseases, and DNA methylation of CDKN2A/2B has been frequently reported in many conditions, 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) 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. 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. For each calcified lesion, the Agatston score was calculated as the product of the area (mm²) 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.

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/µL. Rs4977574 at Chr9p21, which is significantly associated with calcification in the aorta based on the validation of a previous study, 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). Sequencing primers were CATGCTTTCTGAAACACCG (forward) and TAATGGAGGTGTGGTCAGCA (reverse). Reproducibility of genotyping was confirmed by randomly

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, aortic arch calcification; HTN, hypertension; DM, diabetes mellitus; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
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) \( \geq 50\% \) GC content; (3) \( \geq 0.60 \) ratio of observed/expected dinucleotides CpG. 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. 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. Categor-}

| Variants           | With (\( n = 248 \)) | Without (\( n = 74 \)) | \( P \) value |
|--------------------|-----------------------|-------------------------|--------------|
| 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        |

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 participants was 49.0 (43.8-58.3) years, with 58 (78.4%) males. The prevalence of hypertension was 83.1%, diabetes was 51.6%, dyslipidemia was 34.7%, and smoking was 39.1%. The median total cholesterol (TC) was 4.27 (3.55-5.01) mmol/L, triglycerides (TG) was 1.37 (1.06-1.76) mmol/L, high-density lipoprotein cholesterol (HDL-c) was 1.00 (0.83-1.16) mmol/L, low-density lipoprotein cholesterol (LDL-c) was 2.63 (1.93-3.20) mmol/L, and glucose was 5.3 (4.7-6.7) mmol/L. The median age of the patients with AAC was 64.0 (58.0-72.0) years, and the prevalence of hypertension, diabetes, dyslipidemia, smoking, and drinking was 69.0%, 51.6%, 59.5%, 34.7%, and 39.1%, respectively. The median age of the patients without AAC was 49.0 (43.8-58.3) years, and the prevalence of hypertension, diabetes, dyslipidemia, smoking, and drinking was 83.1%, 51.6%, 59.5%, 32.4%, and 28.6%, respectively. The median age of the patients with AAC was significantly higher than that of the patients without AAC (\( P < 0.001 \)). The prevalence of diabetes, dyslipidemia, and smoking was significantly lower in the patients with AAC than in the patients without AAC (\( P = 0.047 \), \( P = 0.048 \), and \( P = 0.048 \), respectively). The median TC, TG, HDL-c, LDL-c, and glucose were significantly higher in the patients with AAC than in the patients without AAC (\( P = 0.064 \), \( P = 0.048 \), \( P = 0.995 \), \( P = 0.064 \), and \( P = 0.064 \), respectively). The median age, male, hypertension, diabetes, dyslipidemia, smoking, and drinking were not significantly different between the patients with and without AAC (\( P = 0.144 \), \( P = 0.781 \), \( P = 0.781 \), \( P = 0.756 \), \( P = 0.756 \), and \( P = 0.995 \), respectively).
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, \( P^{\prime} < 0.001 \)), had a higher prevalence of HTN (83.1% vs 59.5%, \( P^{\prime} < 0.001 \)), lower prevalence of dyslipidemia (51.6% vs 64.9%, \( P^{\prime} = 0.047 \)), and higher HDL-c levels (1.00 vs 0.91, \( P^{\prime} = 0.048 \) (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 3. Differences of methylation levels between patients with and without AAC.

| Gene   | Position | With    | Without  | \( P \) value |
|--------|----------|---------|----------|--------------|
| 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    |
| …      | …        | …       | …        |              |
| 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   |
| …      | …        | …       | …        |              |

**Note:** \( P \) values indicate statistical significance. All differences between patients with and without AAC were statistically significant (\( P < 0.05 \)).
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 (β=0.275±0.116, 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=0.03).

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 (β=0.414±0.171, 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 (β=0.292±0.115, P=0.011) (Table 5).

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

| Model | β     | SE    | P value |
|-------|-------|-------|---------|
| 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   |

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. The p15INK4b protein encoded by CDKN2B can specifically bind to CDKN4/6 and result in G1 phase arrest and cell proliferation interruption. Methylation in CpG islands around promoter regions can generally reduce gene expression. Evidence that CDKN2B methylation represses expression and leads to unlimited cell proliferation has been confirmed in a spectrum of cancers.

Both inflammatory responses and migration of proliferating vascular smooth muscle cells (VSMCs) are considered essential for the development of atherosclerosis. Chronic vascular inflammation arising from atherosclerosis also contributes to arterial calcification. 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. As the expression of CDKN2B is repressed, Rb proteins may lose control and result in increased proliferation of macrophages and VSMCs. The association of CDKN2B methylation and coronary artery disease (CAD) has been previously observed. 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. 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. 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 convicible.

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.

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**Conflict of Interest**

None.

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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                        |
|------|--------------|------------|----------|-------------------------------|
|      |              | -1477      | -1197    | forward GGGATATGGAGGGAGGAGAT |
|      |              | -1047      | -838     | reverse CTTCTTCCTTTTCTCTCTCCC |
|      |              | -859       | -574     | forward GGGAAGAGGAAGGAAGAAGAG |
|      |              | -399       | -212     | reverse ATAAAATAAGGGGAATAGGGGAG |
|      |              | +70        | +335     | forward TGAGGAGTTAGYGTGTTAGGTAGT |
|      |              | +308       | +531     | reverse TCAATATACTACRAAACCACATAATCTAAAATC |
|      |              | -7         | +248     | forward GAGGGTAATGAAGTTTAGTTAGGTT |
|      |              | +223       | +455     | reverse CTATCRCACCTCTCCAACATCC |
|      |              | +430       | +650     | forward TGTTTTTTAGTTTATAGGGGTAGG |
|      |              |            |          | reverse CCAACCTAACCAAATAAAAAACC |

Zhou et al.
### 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   | -0.1* | 0.1   | -0.1  | 0.0   | -0.1  | -0.1  | -0.1  | 0.0   | -0.1* | 0.0   | 0.1   | 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   | -0.1  | -0.1  | 0.1   | -0.1  | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.1   | 0.1   | 0.3*  |
| 3        | 1.0   | 0.4*  | 0.1   | 0.0   | 0.0   | 0.0   | 0.1*  | 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   | 0.1   | 0.1   | 0.1   | 0.2*  |
| 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   | 0.1   | 0.2*  |
| 5        | 1.0   | 0.2*  | 0.3*  | 0.2*  | 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   | 0.1   | 0.2*  |
| 6        | 1.0   | 0.3*  | 0.3*  | 0.2*  | 0.1   | 0.1   | 0.1   | 0.1*  | 0.1   | 0.1   | 0.2*  | 0.2*  | 0.1   | 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   | 0.0   | 0.1   | 0.1   | 0.2*  |
| 8        | 1.0   | 0.1   | 0.1   | 0.2*  | 0.2*  | 0.2*  | 0.1   | 0.2*  | 0.1*  | 0.1   | 0.2*  | 0.1   | 0.1   | 0.0   | 0.0   | 0.1*  | 0.1   | 0.1   | 0.1   | 0.2*  |
| 9        | 1.0   | 0.0   | 0.0   | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.1   | 0.0   | 0.0   | 0.1   | 0.0   | 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   | 0.0   | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.1   | 0.0   | 0.0   | 0.1   | 0.1   |
| 11       | 1.0   | 0.0   | 0.0   | 0.0   | 0.1*  | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.0   | 0.1   | 0.2*  | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |
| 12       | 1.0   | 0.1   | 0.0   | 0.0   | 0.1*  | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |
| 13       | 1.0   | 0.1*  | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1   | 0.1*  | 0.0   | 0.0   | 0.1*  | 0.1   | 0.0   | 0.0   | 0.0   |
| 14       | 1.0   | 0.1   | 0.0   | 0.0   | 0.1   | 0.1   | 0.2*  | 0.1   | 0.1   | 0.0   | 0.0   | 0.1   | 0.0   | 0.1   | 0.1   | 0.2*  |
| 15       | 1.0   | 0.1   | 0.0   | 0.0   | 0.1   | 0.1   | 0.0   | 0.1   | 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   | 0.0   | 0.1   | 0.0   | 0.1   | 0.1   | 0.2*  |
| 17       | 1.0   | 0.2*  | 0.0   | 0.3*  | 0.2*  | 0.1*  | 0.2*  | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.1   | 0.0   | 0.1   | 0.1   | 0.1   | 0.2*  |
| 18       | 1.0   | 0.2*  | 0.0   | 0.3*  | 0.2*  | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.1   | 0.1   | 0.0   | 0.1   | 0.2*  |
| 19       | 1.0   | 0.1   | 0.0   | 0.1*  | 0.1   | 0.0   | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |
| 20       | 1.0   | 0.1   | 0.2*  | 0.1*  | 0.0   | 0.1   | 0.1   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   | 0.0   |
| 21       | 1.0   | 0.2*  | 0.3*  |
| 22       | 1.0   | 0.2*  | 0.3*  |
| 23       | 1.0   | 0.2*  |
| 24       | 1.0   | 0.2*  |

*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  | 0.4  | 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  | 0.3  | 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  | 0.5  |      |      |      |      |      |
| 8        | 1.0  | 0.3  | 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