**Association between DNA methylation in cord blood and maternal smoking: The Hokkaido Study on Environment and Children’s Health**

Kunio Miyake1,\*, Akio Kawaguchi2,\*, Ryu Miura3, Sachiko Kobayashi3, Nguyen Quoc Vuong Tran1, Sumitaka Kobayashi3, Chihiro Miyashita3, Atsuko Araki3, Takeo Kubota4, Zentaro Yamagata1 & Reiko Kishi3

1Department of Health Sciences, Graduate School of Interdisciplinary Research, University of Yamanashi, Yamanashi, Japan

2Yamanashi Community Medicine Support Center, University of Yamanashi Hospital, Yamanashi, Japan

3Center for Environmental and Health Sciences, Hokkaido University, Hokkaido, Japan

4Faculty of Child Studies, Seitoku University, Iwase 550, Chiba, Japan

\*Kunio Miyake and Akio Kawaguchi contributed equally to this work.

Correspondence and requests for materials should be addressed to K.M. (email: kmiyake@yamanashi.ac.jp).

****

**Figure S1.** Box plot ofnext generation sequencing analysis of *AHRR* cg21161138, *SHANK2* cg05780228, and *EVC2* cg01290904 before removal of outliners, supplement to Figure 4 and Table 3. Black arrows indicate the outliers.

** Figure S2.** Correlation between HumanMethylation450K array (HM450K) and next generation sequencing (NGS) data analysis for the DNA methylation status of *AHRR* cg21161138, *SHANK2* cg05780228, and *EVC2* cg01290904 before removal of outliers, supplement to Figure 5. Values of Spearmen correlation coefficient (ρ) are showed.Black arrows indicate the outliers.

****

**Figure S**3. Box plots for DNA methylation ratio in other CpG sites covered by primers of next generation sequencing, supplement to Figure 4 and Table 3.

|  |
| --- |
| **Table S1.** Differential methylation in cord blood DNA in the comparison between never-smokers (Ne-S) and sustained-smokers (Su-S).  |
| **CpG site** | **Chr.** | **Gene** | **Relation to gene** | **P-value** | **FDR** | **Beta** |
| cg05575921 | 5 | AHRR | Body | 9.65E-25 | 4.12E-19 | -0.083  |
| cg04063345 | 6 | ESR1 | Body | 7.18E-06 | 0.035  | -0.065  |
| cg12876356 | 1 | GFI1 | Body | 9.67E-08 | 0.003  | -0.063  |
| cg09935388 | 1 | GFI1 | Body | 4.82E-06 | 0.029  | -0.062  |
| cg18146737 | 1 | GFI1 | Body | 3.95E-07 | 0.009  | -0.057  |
| cg15626350\* | 6 | ESR1 | Body | 1.29E-05 | 0.048  | -0.054  |
| cg09662411 | 1 | GFI1 | Body | 8.19E-07 | 0.014  | -0.035  |
| cg21161138\* | 5 | AHRR | Body | 8.22E-12 | 1.17E-06 | -0.033  |
| cg25189904 | 1 | GNG12 | TSS1500 | 2.66E-07 | 0.007  | -0.033  |
| cg06338710 | 1 | GFI1 | Body | 4.93E-08 | 0.002  | -0.032  |
| cg20732787\* | 19 | NCLN | Body | 1.16E-05 | 0.046  | -0.024  |
| cg05780228\* | 11 | SHANK2 | Body | 8.23E-07 | 0.014  | -0.024  |
| cg11778756 | 11 | DDX10 | Body | 1.30E-08 | 0.001  | -0.023  |
| cg17710804 | 1 | C1orf113 | TSS1500;TSS200 | 1.25E-06 | 0.016  | -0.022  |
| cg18316974\* | 1 | GFI1 | Body | 8.76E-06 | 0.039  | -0.022  |
| cg22242148\* | 15 |  |  | 9.10E-07 | 0.014  | -0.022  |
| cg26098871\* | 17 | ENGASE | 3'UTR | 2.86E-06 | 0.022  | -0.021  |
| cg09068004\* | 5 | MARCH3 | TSS1500 | 2.34E-08 | 0.001  | 0.020  |
| cg09321403 | 6 |  |  | 3.49E-08 | 0.002  | 0.020  |
| cg11898431 | 6 |  |  | 1.11E-05 | 0.045  | 0.021  |
| cg15787636\* | 17 | TBCD | Body | 5.31E-06 | 0.030  | 0.021  |
| cg04284530 | 5 | MARCH3 | TSS1500 | 1.89E-06 | 0.020  | 0.022  |
| cg12098750 | 12 | ANKS1B | TSS1500;Body | 2.82E-06 | 0.022  | 0.022  |
| cg06612130 | 4 |  |  | 1.49E-06 | 0.016  | 0.023  |
| cg01290904 | 4 | EVC2 | 5'UTR;Body | 5.90E-06 | 0.031  | 0.023  |
| cg17924476 | 5 | AHRR | Body | 1.85E-07 | 0.006  | 0.024  |
| cg13492331 | 10 | FAM107B | Body | 5.06E-06 | 0.030  | 0.026  |
| cg04333296\* | 17 | LOC653653 | TSS200 | 5.44E-06 | 0.030  | 0.026  |
| cg23635789 | 20 | SRC | Body | 2.26E-06 | 0.021  | 0.026  |
| cg13932501 | 9 | AUH | Body | 7.71E-06 | 0.036  | 0.027  |
| cg07469926 | 5 | TRIM36 | Body | 3.64E-06 | 0.025  | 0.027  |
| cg15554505 | 19 |  |  | 9.54E-06 | 0.041  | 0.029  |
| cg04584103 | 14 | ASB2 | Body | 1.24E-06 | 0.016  | 0.029  |
| cg00394823 | 16 | ACSM3 | TSS200 | 5.12E-06 | 0.030  | 0.030  |
| cg05150608 | 14 |  |  | 7.49E-06 | 0.036  | 0.030  |
| cg05549655 | 15 | CYP1A1 | TSS1500 | 2.63E-07 | 0.007  | 0.031  |
| cg10078415 | 16 | ACSM3 | TSS1500 | 7.09E-06 | 0.035  | 0.035  |
| cg27434149 | 10 |  |  | 2.35E-06 | 0.021  | 0.043  |
| cg04180046 | 7 | MYO1G | Body | 2.41E-12 | 5.13E-07 | 0.044  |
| cg06478823\* | 16 | ACSM3 | TSS1500 | 2.69E-07 | 0.007  | 0.048  |
| cg07340025 | 5 |  |  | 5.48E-06 | 0.030  | 0.049  |
| cg26493188 | 16 | FLYWCH1 | 5'UTR | 6.37E-06 | 0.033  | 0.054  |
| cg01215511 | 16 | FLYWCH1 | 5'UTR | 4.96E-06 | 0.030  | 0.062  |
| cg24228532 | 18 |  |  | 4.69E-06 | 0.029  | 0.063  |
| cg12803068 | 7 | MYO1G | Body | 7.46E-09 | 0.001  | 0.066  |
| cg22549041\* | 15 | CYP1A1 | TSS1500 | 4.02E-06 | 0.027  | 0.070  |
| FDR < 0.05. |β value| > 0.02 |  |  |  |  |
| \*Probes marked as low-quality by Naeem et al.24 which may contain multiple SNPs/INDELs or contain SNP at CpG site or have absolute beta difference between whole-genome bisulfite sequencing (WGBS) and Illumina HumanMethylation450 (HM450K) bead array is greater than 0.3 |

|  |
| --- |
| **Table S2.** Differential methylation in cord blood DNA in the comparison between never-smokers (Ne-S) and stopped-smokers (St-S).  |
| **CpG site** | **Chr.** | **Gene** | **Relation to gene** | **P-value** | **FDR** | **Beta** |
| cg14418756 | 10 | STK32C | Body | 2.47E-06 | 0.031  | -0.058  |
| cg05618934\* | 4 |  |  | 6.95E-07 | 0.019  | -0.042  |
| cg27173819 | 10 | STK32C | Body | 2.00E-06 | 0.031  | -0.041  |
| cg05225883 | 10 | STK32C | Body | 3.79E-06 | 0.036  | -0.035  |
| cg12594615 | 2 | TBC1D8 | Body | 4.50E-06 | 0.036  | -0.030  |
| cg24055029\* | 6 | TNXB | Body | 1.79E-06 | 0.031  | -0.026  |
| cg04574346\* | 12 |  |  | 2.25E-06 | 0.031  | -0.025  |
| cg19571540\* | 14 |  |  | 2.12E-07 | 0.011  | -0.022  |
| cg03389650 | 7 |  |  | 4.54E-06 | 0.036  | -0.022  |
| cg24586758 | 5 | PCDHB3 | TSS200 | 2.22E-06 | 0.031  | -0.021  |
| cg02427511 | 16 |  |  | 3.83E-06 | 0.036  | 0.020  |
| cg01290904 | 4 | EVC2 | 5'UTR;Body | 2.52E-06 | 0.031  | 0.021  |
| cg17484472 | 16 |  |  | 5.43E-06 | 0.038  | 0.027  |
| cg27560452 | 16 |  |  | 8.60E-06 | 0.043  | 0.031  |
| cg26601609\* | 8 |   |   | 6.32E-06 | 0.039  | 0.048  |
| FDR < 0.05. beta value > |0.02|\*Probes marked as low-quality by Naeem et al.24 which may contain multiple SNPs/INDELs or contain SNP at CpG site or have absolute beta difference between whole-genome bisulfite sequencing (WGBS) and Illumina HumanMethylation450 (HM450K) bead array is greater than 0.3 |

|  |
| --- |
| **Table S3.** Differential methylation in cord blood DNA in the comparison between stopped-smokers (St-S) and sustained-smokers (Su-S).  |
| **CpG site** | **Chr.** | **Gene** | **Relation to gene** | **P-value** | **FDR** | **Beta** |
| cg05575921 | 5 | AHRR | Body | 5.81E-15 | 2.48E-09 | -0.073  |
| cg12876356 | 1 | GFI | Body | 2.03E-06 | 0.021  | -0.058  |
| cg06531741\* | 11 | HTR3B | TSS200 | 2.34E-05 | 0.047  | -0.052  |
| cg06463313\* | 21 | PFKL | Body | 2.33E-07 | 0.007  | -0.051  |
| cg09662411 | 1 | GFI1 | Body | 1.91E-05 | 0.044  | -0.034  |
| cg24733560\* | 20 | TAF4 | Body | 3.76E-06 | 0.025  | -0.034  |
| cg21161138\* | 5 | AHRR | Body | 9.50E-08 | 0.006  | -0.029  |
| cg14817490\* | 5 | AHRR | Body | 5.30E-06 | 0.029  | -0.028  |
| cg06780726\* | 13 | C13orf16 | Body | 2.11E-05 | 0.045  | -0.028  |
| cg09681977\* | 2 | HDAC4 | Body | 1.99E-05 | 0.044  | -0.026  |
| cg20284239 | 1 | GPR161 | 5'UTR | 1.48E-05 | 0.039  | -0.024  |
| cg05780228\* | 11 | SHANK2 | Body | 1.38E-05 | 0.038  | -0.022  |
| cg15813594 | 5 | EGFLAM | TSS200;Body | 6.81E-07 | 0.013  | -0.022  |
| cg21526530 | 15 | ATP8B4 | TSS1500 | 2.30E-05 | 0.047  | -0.021  |
| cg03923789 | 6 | PARK2 | Body | 1.02E-06 | 0.016  | -0.021  |
| cg23067299\* | 5 | AHRR | Body | 1.28E-06 | 0.017  | 0.021  |
| cg09456465 | 1 |  |  | 1.90E-05 | 0.044  | 0.021  |
| cg12612445\* | 21 | C21orf67 | Body | 5.25E-07 | 0.012  | 0.021  |
| cg10440011 | 17 | DPH1 | TSS1500 | 1.43E-05 | 0.039  | 0.021  |
| cg00213123\* | 15 | CYP1A1 | TSS1500 | 3.87E-06 | 0.026  | 0.021  |
| cg12104266 | 20 | ZNF335 | TSS1500 | 1.11E-05 | 0.037  | 0.021  |
| cg00284551 | 15 | SNORD115-8 | TSS1500 | 8.31E-06 | 0.033  | 0.021  |
| cg10717610 | 2 |  |  | 9.99E-06 | 0.036  | 0.021  |
| cg06682024 | 2 | MERTK | Body | 1.51E-05 | 0.039  | 0.021  |
| cg21201401\* | 20 | LIME1 | TSS200 | 6.72E-06 | 0.032  | 0.022  |
| cg17126924 | 17 |  |  | 7.21E-06 | 0.033  | 0.022  |
| cg13060531 | 1 | TTLL7 | TSS1500 | 2.78E-07 | 0.007  | 0.022  |
| cg24590430\* | 10 |  |  | 6.95E-06 | 0.032  | 0.022  |
| cg10663316 | 12 |  |  | 2.06E-05 | 0.044  | 0.022  |
| cg16929104 | 19 | GDF15 | TSS200 | 5.62E-06 | 0.031  | 0.023  |
| cg18276112 | 7 | FOXK1 | Body | 1.28E-05 | 0.038  | 0.023  |
| cg23742314 | 12 |  |  | 4.74E-06 | 0.028  | 0.023  |
| cg19829353 | 6 | HECA | Body | 1.98E-05 | 0.044  | 0.023  |
| cg21809624 | 17 |  |  | 6.25E-06 | 0.032  | 0.024  |
| cg13630239 | 10 | RRP12 | 3'UTR | 7.37E-06 | 0.033  | 0.024  |
| cg11654024 | 1 |  |  | 9.00E-06 | 0.033  | 0.025  |
| cg02793451 | 16 | TOX3 | TSS1500 | 2.96E-06 | 0.023  | 0.026  |
| cg12537162 | 17 | ITGB3 | TSS1500 | 2.76E-06 | 0.022  | 0.026  |
| cg15345477 | 10 |  |  | 4.38E-06 | 0.028  | 0.026  |
| cg16449464 | 2 | MAP1D | TSS1500 | 1.07E-07 | 0.006  | 0.027  |
| cg12376422 | 9 |  |  | 2.48E-05 | 0.048  | 0.027  |
| cg03221914 | 6 | HIST1H2AJ | TSS1500 | 4.83E-06 | 0.029  | 0.027  |
| cg05549655 | 15 | CYP1A1 | TSS1500 | 9.95E-06 | 0.036  | 0.028  |
| cg05901543\* | 16 | CDH15 | Body | 1.40E-05 | 0.039  | 0.029  |
| cg18107006 | 2 | HNRPLL | TSS1500 | 6.10E-07 | 0.012  | 0.029  |
| cg02459818\* | 19 | VN1R4 | 1stExon | 2.48E-05 | 0.048  | 0.029  |
| cg01290565 | 12 | CUX2 | Body | 2.39E-05 | 0.048  | 0.029  |
| cg07469926 | 5 | TRIM36 | Body | 1.06E-06 | 0.016  | 0.029  |
| cg10817554\* | 2 |  |  | 6.57E-06 | 0.032  | 0.029  |
| cg07807395 | 1 |  |  | 1.23E-06 | 0.016  | 0.030  |
| cg12738008\* | 8 | MYOM2 | Body | 5.88E-06 | 0.032  | 0.030  |
| cg01624202 | 15 |  |  | 1.82E-05 | 0.043  | 0.030  |
| cg08720517 | 5 | LOC389333 | 1stExon | 7.71E-06 | 0.033  | 0.032  |
| cg25223623 | 5 |  |  | 1.90E-05 | 0.044  | 0.034  |
| cg05150608 | 14 |  |  | 2.56E-05 | 0.048  | 0.034  |
| cg06478823\* | 16 | ACSM3 | TSS1500 | 2.40E-05 | 0.048  | 0.041  |
| cg16512885 | 1 |  |  | 3.11E-08 | 0.003  | 0.042  |
| cg22066599\* | 11 |  |  | 6.05E-06 | 0.032  | 0.042  |
| cg23486701 | 2 | SPTBN1 | Body | 8.89E-06 | 0.033  | 0.042  |
| cg01590338 | 14 |  |  | 1.37E-05 | 0.038  | 0.044  |
| cg04717895 | 16 |  |  | 2.25E-06 | 0.021  | 0.045  |
| cg05225883 | 10 | STK32C | Body | 6.94E-06 | 0.032  | 0.046  |
| cg12101586 | 15 | CYP1A1 | TSS1500 | 1.25E-05 | 0.038  | 0.046  |
| cg08034867 | 17 | ABR | Body | 3.45E-06 | 0.024  | 0.053  |
| FDR < 0.05. beta value > |0.02|\*Probes marked as low-quality by Naeem et al.24 which may contain multiple SNPs/INDELs or contain SNP at CpG site or have absolute beta difference between whole-genome bisulfite sequencing (WGBS) and Illumina HumanMethylation450 (HM450K) bead array is greater than 0.3 |

|  |
| --- |
| **Table S4.** Single-nucleotide polymorphisms that may affect result of HM450K |
| **Gene** | **CpG site** | **SNPs** | **Distance to CpG site** | **Minor allele frequency** | **1000 Genomes Search** |
| Japanese (in Tokyo, Japan) | Caucasian (British in England and Scotland; Finish in Finland; Utah Residents with North and Western European Ancestry) |
| CTCNAP2  | cg25949550 | rs111557555 | 21 | 0.0018 | G=1.0000; C=0.0000 | G=1.0000; C=0.0000 |
| ACSM3 | cg06478823 | None |  |  |  |  |
|  | cg10078415 | None |  |  |  |  |
|   | cg00394823 | None |   |   |   |   |
| FLYWCH1 | cg01215511 | None |  |  |  |  |
|   | cg26493188 | None |   |   |   |   |
| MARCH3 | cg04284530 | None |  |  |  |  |
|   | cg09068004 | None |   |   |   |   |
| IGR | cg05150608 | rs77154738 | 51 | 0.0018 | C=0.9856; T=0.0144 | C=1.0000; T=0.0000 |
| SHANK2 | cg05780228 | rs142581709 | 0 | 0.0009 | C=1.0000; T=0.0000 | C=1.0000; T=0.0000 |
| rs58493305 | 16 | 0.017811 | G=1.0000; A=0.0000 | G=1.0000; A=0.0000 |
| rs146007445 | 41 | 0.0005 | G=1.0000; A=0.0000 | G=1.0000; A=0.0000 |
| TRIM36 | cg07469926 | None |   |   |   |   |
| EVC2 | cg01290904 | rs78804781 | 26 | 0.005213 | C=1.0000; T=0.0000 | C=1.0000; T=0.0000 |

|  |
| --- |
| **Table S5.** List of bisulphite PCR primers. |
| **Name** | **Forward** | **Reverse** | **Length (bp)** | **Annealing (℃)** |
| ACSM3 (cg06478823) | TGTGGATTAGAGGATTTTGTGTTAG | CAATACTTCTTTTTCCACTCCAAATA | 91 | 59 |
| AHRR (cg05575921) | TATTTTTGAGAGGGTAGTTTTGTTT | AACCACTCCCAAAACCCAC | 120 | 59 |
| AHRR (cg21161138) | ATTTTAGTTTTAGGGTTTTTGAGGT | CTAATAATTAAAAAACCACCCCTA | 105 | 59 |
| CYP1A1 (cg05549655) | GTTATGTTAAATGGTATTGGGGTTT | AAAAAAAACAACCTACATATATCC | 81 | 59 |
| IGR (cg05150608) | TTGAATTTTTTTAAATTGGAGAAAT | CCTACACTAATTACAACTTCACCTAC | 103 | 57 |
| SHANK2 (cg05780228) | AGGTTGTGAAGGTATTTTTGGTTTA | CAACAAACAACTATATCCACTACTAAAAAA | 107 | 59 |
| TRIM36 (cg07469926) | AGAGGTGATGGTATAAAGGAGTATT | TAATAAAATATAAAACTACAAAATTTCAAA | 113 | 57 |
| EVC2 (cg01290904) | TTATTTTAGTTGTTGATGGGATTAG | AAACACACATAACCAAAACTCTTTC | 104 | 55 |