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Genetically Predicted Levels of DNA Methylation Biomarkers and Breast Cancer Risk: Data From 228 951 Women of European Descent

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Abstract

Background: DNA methylation plays a critical role in breast cancer development. Previous studies have identified DNA methylation marks in white blood cells as promising biomarkers for breast cancer. However, these studies were limited by low statistical power and potential biases. Using a new methodology, we investigated DNA methylation marks for their associations with breast cancer risk.

Methods: Statistical models were built to predict levels of DNA methylation marks using genetic data and DNA methylation data from HumanMethylation450 BeadChip from the Framingham Heart Study ($n = 1595$). The prediction models were validated using data from the Women's Health Initiative ($n = 883$). We applied these models to genomewide association study (GWAS) data of 122 977 breast cancer patients and 105 974 controls to evaluate if the genetically predicted DNA methylation levels at CpG sites (CpGs) are associated with breast cancer risk. All statistical tests were two-sided.

Results: Of the 62 938 CpG sites investigated, statistically significant associations with breast cancer risk were observed for 450 CpGs at a Bonferroni-corrected threshold of P less than 7.94×10^{-7} , including 45 CpGs residing in 18 genomic regions, that have not previously been associated with breast cancer risk. Of the remaining 405 CpGs located within 500 kilobase flanking regions of 70 GWAS-identified breast cancer risk variants, the associations for 11 CpGs were independent of GWAS-identified variants. Integrative analyses of genetic, DNA methylation, and gene expression data found that 38 CpGs may affect breast cancer risk through regulating expression of 21 genes.

Conclusion: Our new methodology can identify novel DNA methylation biomarkers for breast cancer risk and can be applied to other diseases.

Breast cancer is the most common cancer for women in the United States as well as many countries around the world (1). DNA methylation plays critical roles in cancer development, including breast cancer (2).

DNA methylation of several genes in white blood cells had been associated with breast cancer risk; however, inconsistent results were found (3–7). Most of these studies had a retrospective design. Two prospective studies found that overall DNA

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hypomethylation in white blood cells was associated with increased breast cancer risk (8,9). In addition, a panel of 250 CpGs sites (CpGs) in white blood cell DNA was identified to be predictive of breast cancer risk (10). However, none of these CpGs were consistently observed in a later study (9). These studies were limited by small sample size, lack of replication, and/or reverse causation. Furthermore, the repeatability of DNA methylation measurements at some CpGs using the HumanMethylation450 BeadChip (Illumina, San Diego, CA) was not optimal (11), which may have contributed to the inconsistency across studies.

A recent study indicated the epigenetic supersimilarity of monozygotic twin pairs (12). More recently, 24 heritable CpGs were associated with breast cancer risk (13). Multiple genetic variants had been identified as DNA methylation quantitative trait loci (meQTL) (14–16), suggesting that DNA methylation at some CpGs are genetically determined and thus can be predicted using genetic variants. Studies using *cis* (500 kilobase [kb] flanking regions) meQTL single-nucleotide polymorphisms (SNPs) had discovered novel CpGs for diseases (17,18). However, the proportion of variance explained by a single-meQTL SNP for most CpGs is typically small. Herein, we propose a new methodology to build statistical models to predict DNA methylation in white blood cells via multiple SNPs in a reference dataset and then apply the models to large genomewide association study (GWAS) datasets to evaluate genetically predicted DNA methylation in association with disease risk. We tested this methodology by investigating the association of genetically predicted DNA methylation with breast cancer risk using data from 122 977 breast cancer patients and 105 974 controls.

Methods

Building DNA Methylation Prediction Models and Evaluating Prediction Performance

Figure 1 presents the overall workflow. Genetic and DNA methylation data of white blood cell samples from 1595 unrelated European participants included in the Framingham Heart Study (FHS) were obtained from dbGaP (phs000342 and phs000724). The HumanMethylation450 BeadChip DNA methylation data were subjected to quality control and normalization using the “minfi” package (<https://bioconductor.org/packages/release/bioc/html/minfi.html>) (19) and were then regressed on age, sex, cell type composition, and top 10 principal components to get residuals. Genotyping was conducted using the Affymetrix 500K (Affymetrix, Santa Clara, CA) mapping array and data were imputed to the 1000 Genomes Phase I Version 3. SNPs with imputation quality no less than 0.80 and minor allele frequency no less than 0.05 were used. For each CpG, we built a statistical model using allelic dosage data of *cis*-SNPs to predict DNA methylation residuals, following the elastic net method ($\alpha = 0.50$) with tenfold cross-validation (20) (Supplementary Methods, available online). Genetic data and white blood cell DNA methylation data from the HumanMethylation450 BeadChip of 883 unrelated European women included in the Women’s Health Initiative (WHI) from dbGaP (phs000315, phs000675, and phs001335) were used for the external validation of models. Genotyping was conducted using the HumanOmni1-Quad_v1-0_B (Illumina), and the HumanOmniExpress array (Illumina) and data were imputed to the 1000 Genomes Phase I Version 3. DNA methylation and genotyping data were processed following a similar procedure used for the FHS data. For each CpG, the predicted DNA methylation level was estimated using its prediction model, and the correlation between predicted and measured DNA methylation level was evaluated using Spearman correlation. In total, prediction models for

63 000 CpGs built by using FHS data were externally validated by WHI data (Supplementary Methods, available online).

Statistical Analyses

We used MetaXcan (20,21) to investigate genetically predicted DNA methylation in association with breast cancer risk by applying prediction models to summary statistics of breast cancer GWAS from the Breast Cancer Association Consortium (BCAC) (22), including 122 977 patients and 105 974 controls of European descent. The BCAC includes three datasets: 46 785 patients and 42 892 controls genotyped on the iCOGS (Illumina), 61 282 patients and 45 494 controls on the OncoArray (Illumina), and 14 910 patients and 17 588 controls on varied GWAS arrays (22). Genotyping data were imputed to the 1000 Genomes Phase I Version 3. Among the 751 157 SNPs included in the predicting levels for 63 000 CpGs, summary statistics for associations between SNPs and breast cancer risk in the BCAC were available for 750 914 (>99.9%) SNPs, corresponding to 62 938 CpGs, which were included in the final analyses. This study was approved by the BCAC Data Access Coordination Committee. The association *z* score was estimated using the following formula:

$$z_m \approx \sum_{s \in \text{Model}_m} w_{sm} \frac{\hat{\sigma}_s}{\hat{\sigma}_m} \frac{\hat{\beta}_s}{\text{se}(\hat{\beta}_s)}.$$

In the formula, w_{sm} is the weight of SNP *s* on CpG *m*. $\hat{\sigma}_s$ and $\hat{\sigma}_m$ are the estimated variances of SNP *s* and CpG *m*, respectively. $\hat{\beta}_s$ and $\text{se}(\hat{\beta}_s)$ are the effect size and standard error of SNP *s* on breast cancer risk, respectively (Supplementary Methods, available online). Association *P* values were also calculated by MetaXcan, and a Bonferroni-corrected threshold of P less than 7.94×10^{-7} ($0.05/62\,938$) was used to determine statistically significant associations of genetically predicted levels of CpGs with breast cancer risk. All statistical tests were two-sided.

We then conducted a genomewide complex trait analysis-conditional and joint analysis (GCTA-COJO) (23) and MetaXcan (21) analyses to assess whether associations of predicted DNA methylation with breast cancer risk were independent of GWAS-identified breast cancer susceptibility variants. We also performed stratification analyses by datasets within the BCAC (ie, iCOGS, OncoArray, and GWAS) and by estrogen (ER) status. Heterogeneity across BCAC datasets and between ER status was estimated by Cochran *Q* test (Supplementary Methods, available online).

Functional Analyses

Functional annotation of CpGs were conducted using ANNOVAR (24). The enrichments of breast cancer-associated CpGs in putative functional elements, including DNase I hypersensitive sites and genomic regions overlapping with histone modification marks (eg, H3K27me3, H3K36me3, and H3K4me3), were evaluated by eFORGE (25) v1.2 using data from the Roadmap Epigenomics Project (26) (Supplementary Methods, available online).

Identifying Consistent Directions of Associations Across DNA Methylation, Gene Expression, and Breast Cancer Risk

For breast cancer-associated CpGs, we investigated DNA methylation in correlation with expression of their nearby genes annotated by ANNOVAR, using data of 1367 unrelated European participants from the FHS (Supplementary Methods, available online). For genes with expression levels statistically

Figure 1. Study design flowchart. BCAC = Breast Cancer Association Consortium; CpGs = CpG sites; ER = estrogen receptor; FHS = Framingham Heart Study; GWAS = genomewide association study; GTEx = genotype-tissue expression; meQTL = DNA methylation quantitative trait loci; SNP = single nucleotide polymorphism; WHI = Women's Health Initiative.

significantly correlated with DNA methylation levels at these CpGs, we built genetic models to predict their expression levels using data from 6124 different tissue samples of 369 participants of European ancestry from the Genotype-Tissue Expression (GTEx) project (27). The models were applied to the BCAC data to estimate associations between genetically predicted expression of these genes and breast cancer risk, using MetaXcan (20,21) (Supplementary Methods, available online). For both analyses, we used false discovery rate (FDR) less than 0.05 to determine statistically significant correlations and/or associations. To elucidate putative pathways through which DNA methylation affects breast cancer risk, association results across DNA methylation, gene expression, and breast cancer risk were integrated to assess the consistency of association directions (Supplementary Methods, available online).

Comparison Between Prediction Model Approach and Single meQTL SNP Approach

Of the 62 938 CpGs investigated, meQTLs had been identified for 24 845 CpGs (15). We compared the prediction performance of these 24 845 CpGs via prediction models or single-meQTL SNPs. We also investigated these 24 845 CpGs for their DNA methylation levels, predicted via single-meQTL SNPs, in association with breast cancer risk using the BCAC data, following the inverse-variance weighted method (28) (Supplementary Methods, available online). The association results were compared with those from the prediction model approach.

Results

DNA Methylation Prediction Models

A flow diagram describing the number of CpGs and SNPs during each analysis step is shown in Supplementary Figure 1

(available online). Genetic and white blood cell DNA methylation data from FHS were used to build DNA methylation prediction models. In total, 473 865 autosomal CpGs were assayed and 370 785 were retained after quality control. Statistical models were established to predict DNA methylation levels for 223 959 CpGs, 61 219 of which were within CpG islands. Of these 223 959 CpGs, the predicted and measured DNA methylation levels are positively correlated with a correlation coefficient of at least 0.10 (ie, $R_{FHS} \geq 0.10$ and $R_{FHS}^2 \geq 0.01$ for 81 361 CpGs). To validate these 81 361 models, we applied them to the WHI data and calculated the squared values of correlation coefficients between predicted and measured DNA methylation levels (ie, R_{WHI}^2). For these 81 361 models, a high correlation of R_{FHS}^2 and R_{WHI}^2 was observed (Pearson correlation $r=0.95$; Figure 2), indicating that CpGs predicted well in FHS were also predicted well in WHI. Notably, 70 269 of the 81 361 CpGs showed an R_{WHI}^2 no less than 0.01. For 7269 of these 70 269 CpGs, their corresponding probes on the HumanMethylation450 BeadChip overlap with genetic polymorphisms (based on dbSNP Build 151). Such overlapping could potentially affect the estimation of their DNA methylation levels (15), hence, these 7269 CpGs were excluded. In total, 63 000 CpGs were included in downstream analyses.

Associations of Genetically Predicted DNA Methylation With Breast Cancer Risk

We conducted MetaXcan analyses to estimate genetically predicted DNA methylation of the 63 000 CpGs in association with breast cancer risk. Among 751 157 SNPs included in prediction models of these 63 000 CpGs, data were available for 750 914 (>99.9%) SNPs in BCAC, corresponding to 62 938 CpGs. For most of these CpGs, a substantial majority of SNPs were used in association analyses. The Manhattan plot for the associations results is shown in Supplementary Figure 2 (available online).

Figure 2. Performances of DNA methylation prediction models in the prediction dataset and the validation dataset. A total of 81 361 models had a prediction performance in the FHS (R^2_{FHS}) greater than or equal to 0.01. This figure shows the performance of these models in the prediction dataset, FHS, and in the validation dataset, WHI. The x-axis represents the R^2_{FHS} (squared correlation coefficients of predicted and measured DNA methylation levels). We then apply these models to the genetic data in WHI to predict the DNA methylation levels of these 81 361 CpGs. The y-axis represents the performance of these models in the WHI (R^2_{WHI} , squared correlation coefficients of predicted and measured DNA methylation levels). The black line represents the identity line ($y=x$). CpGs = CpG sites; FHS = Framingham Heart Study; WHI = Women's Health Initiative.

Of the 62 938 CpGs, statistically significant associations were observed for 450 at P less than 7.94×10^{-7} , a Bonferroni-corrected threshold (Table 1 and 2; Supplementary Table 1, available online). For these 450 CpGs, 12 286 SNPs were included in their prediction models, with an average of 27 SNPs for each CpG. Of the 12 286 SNPs, genotypes of 10 099 (82.2%) were associated with DNA methylation levels of these CpGs ($P < .05$). Among these 450 CpGs, 45 reside in 18 genomic regions that are 500 kb away from GWAS-identified breast cancer risk variants. After adjusting for proximally located GWAS-identified risk variants, statistically significant associations ($P < 7.94 \times 10^{-7}$) were retained for 17 CpGs within 10 genomic loci (Table 1). Among the remaining 405 CpGs located within 500 kb flanking regions of 70 GWAS-identified breast cancer risk variants, statistically significant associations ($P < 7.94 \times 10^{-7}$) remained for 11 CpGs within seven genomic regions after adjusting for corresponding GWAS-identified risk variants (Table 2). The predicted DNA methylation levels of these 450 CpGs could explain 2.2% of the familial relative risk of breast cancer (Supplementary Methods, available online).

For these 450 CpGs, stratified analyses by ER status were conducted to evaluate the heterogeneity between ER-positive and ER-negative diseases. Most CpGs were associated with risks of both (Supplementary Tables 2 and 3, available online); nevertheless, 39 and eight CpGs were respectively more statistically significantly associated with ER-positive and ER-negative disease with Cochran P less than 1.11×10^{-4} (0.05/450) (Supplementary Table 2, available online). All 450 CpGs showed consistent associations across three BCAC subsets (Supplementary Table 4, available online).

To explore potential regulatory functions of the 450 CpGs, eFORGE was used to estimate the enrichments of them in putative

functional genomic regions. These 450 CpGs were enriched in genomic regions harboring H3K4me1 marks, indicative of enhancers, in human mammary epithelial cells as well as in 36 of 38 cell types and tissues assayed in the Roadmap Epigenomics Project (26) (Supplementary Figure 3, available online). Compared with all the 62 938 CpGs, these 450 CpGs were statistically significantly enriched in noncoding RNA-exonic regions with hypergeometric distribution test P less than 5.55×10^{-3} (0.05/9) (Supplementary Table 5, available online). In addition, of these 450 CpGs, 36, 37, and seven were respectively within or close to 10 kb metastable epialleles identified by three recent studies (29–31).

To determine whether these 450 CpGs are somawide, we rebuilt prediction models without adjusting for cell type composition. Totally, models for 411 CpGs were established with R^2_{FHS} no less than 0.01. For these 411 CpGs, a high correlation between R^2 based on models with or without cell type composition adjustment was observed (Spearman correlation $r = 0.95$; Supplementary Figure 4A, available online). All these 411 CpGs were associated with breast cancer risk at P less than 7.57×10^{-5} , and the association z scores were highly correlated with those based on prediction models with cell type composition adjustment (Spearman correlation $r = 0.98$; Supplementary Figure 4B, available online).

DNA Methylation Affecting Breast Cancer Risk Through Regulating Gene Expression

We investigated whether DNA methylation of the 450 CpGs could influence flanking gene expression using the FHS data. Among 342 CpGs and 158 genes with DNA methylation and gene expression data, statistically significant correlations were observed for 100 CpG gene pairs, including 100 CpGs and 62 genes, at FDR less than 0.05 (Supplementary Table 6, available online). In total, 60 of these 100 statistically significant correlations were negative. Especially, for 22 CpGs that reside in promoter regions, DNA methylation at 20 CpGs were negatively correlated with gene expression. We evaluated the associations between genetically predicted expression of these 62 genes and breast cancer risk using the GTEx and BCAC data. Gene expression prediction models were established for 45 genes, 32 of which were statistically significantly associated with breast cancer risk at FDR less than 0.05 (Supplementary Table 7, available online).

To explore whether DNA methylation at CpGs could breast cancer risk through regulating gene expression, we integrated all association results and identified consistent directions of associations across 38 CpGs, 21 genes, and breast cancer risk (Table 3; Supplementary Table 8, available online). Among these 38 CpGs, five reside in genomic regions not previously reported for breast cancer risk via GWAS, including cg22221025 and cg26668989 in GSTM4, cg16647868 and cg19040266 in SLC22A5, and cg25839482 in IMP3. Except for LRRC25, the associations between predicted expression of the other 20 genes and breast cancer risk attenuated substantially on adjusting for SNPs included in prediction models of their corresponding CpGs (Table 3; Supplementary Table 8, available online). The associations of these 20 genes with breast cancer risk may be modulated by DNA methylation.

Comparison of the Genetic Prediction Model Approach with the Single-meQTL SNP Approach

To evaluate the performance of DNA methylation prediction improved by prediction model approach, we compared the R^2

Table 1. Seventeen DNA methylation marks associated with breast cancer risk identified in genomic regions not yet reported for breast cancer risk

CpG	Chr	Position	Closest gene	Classification	Z score*	OR (95% CI)*	P*	R ² _{FHS} †	R ² _{WHI} †	Closest breast cancer risk SNP	Distance to risk SNP (kb)	P* adjusted for risk SNP
cg04794690	1	17 768 059	RCC2; ARHGEF10L	Intergenic	5.04	1.36 (1.20 to 1.53)	4.74×10^{-7}	0.01	0.04	rs2992756	-1039	2.61×10^{-8}
cg22221025	1	110 186 044	GSTM4	Upstream	5.36	1.15 (1.09 to 1.21)	8.53×10^{-8}	0.05	0.03	rs11552449	-4262	3.04×10^{-7}
cg26668989	1	110 186 163	GSTM4	Upstream	6.37	1.20 (1.13 to 1.27)	1.92×10^{-10}	0.04	0.04	rs11552449	-4262	1.23×10^{-9}
cg04411307	2	69 391 395	ANTXR1	Intronic	5.10	1.05 (1.03 to 1.07)	3.38×10^{-7}	0.31	0.22	rs6725517	44 262	3.19×10^{-7}
cg16190888	2	69 428 235	ANTXR1	Intronic	-5.20	0.87 (0.82 to 0.91)	1.99×10^{-7}	0.04	0.03	rs6725517	44 299	2.06×10^{-7}
cg03277049	3	156 534 076	LINC00886	ncRNA_intronic	-5.01	0.92 (0.90 to 0.95)	5.52×10^{-7}	0.10	0.10	rs58058861	-15 751	5.01×10^{-7}
cg11359771	5	131 558 794	P4HA2	5'UTR	5.00	1.21 (1.13 to 1.31)	5.59×10^{-7}	0.01	0.02	rs6596100	-848	1.70×10^{-7}
cg16647868	5	131 706 066	SLC22A5	Intronic	4.95	1.05 (1.03 to 1.08)	7.36×10^{-7}	0.20	0.27	rs6596100	-701	2.02×10^{-7}
cg19040266	5	131 723 239	SLC22A5	Intronic	-5.05	0.93 (0.91 to 0.96)	4.31×10^{-7}	0.13	0.10	rs6596100	-684	1.32×10^{-7}
cg11920449	6	36 645 608	CDKN1A	TSS1500	-5.04	0.97 (0.96 to 0.98)	4.56×10^{-7}	0.69	0.67	rs9257408	7719	7.78×10^{-7}
cg03714916	6	36 645 886	CDKN1A	TSS1500	-5.17	0.94 (0.92 to 0.96)	2.31×10^{-7}	0.17	0.16	rs9257408	7720	3.95×10^{-7}
cg03171419	8	37 700 802	GPR124	3'UTR	-5.26	0.87 (0.82 to 0.92)	1.46×10^{-7}	0.04	0.04	rs13365225	842	7.86×10^{-8}
cg07540652	8	81 805 956	ZNF704; PAG1	Intergenic	5.05	1.19 (1.11 to 1.27)	4.48×10^{-7}	0.03	0.02	rs2943559	5388	2.71×10^{-7}
cg25626611	12	115 102 065	TBX5-AS1; TBX3	Intergenic	6.01	1.12 (1.08 to 1.16)	1.91×10^{-9}	0.09	0.05	rs1292011	-734	1.09×10^{-7}
cg07211768	12	115 102 290	TBX5-AS1; TBX3	Intergenic	5.98	1.08 (1.06 to 1.11)	2.25×10^{-9}	0.16	0.14	rs1292011	-734	1.52×10^{-7}
cg25938347	15	75 639 163	NEIL1	TSS200	5.51	1.29 (1.18 to 1.42)	3.59×10^{-8}	0.01	0.02	rs151090251	8231	1.84×10^{-7}
cg25839482	15	75 931 953	IMP3	3'UTR	-5.57	0.94 (0.93 to 0.96)	2.59×10^{-8}	0.21	0.25	rs151090251	8524	1.57×10^{-7}

*MetaXcan was used to estimate association Z scores, ORs, 95% CIs, and P values. All statistical tests were two-sided. Chr = chromosome; CI = confidence interval; CpG = CpG sites; FHS = Framingham Heart Study; kb = kilobase; ncRNA = noncoding RNA; OR = odds ratio per SD increase in genetically predicted DNA methylation level (continuous variable); SNP = single-nucleotide polymorphisms; TSS = transcription start site; UTR = untranslated region; WHI = Women's Health Initiative.

†Correlation between predicted and measured DNA methylation levels.

Table 2. Eleven DNA methylation marks associated with breast cancer risk identified in genomic regions within 500 kb of known breast cancer risk variants but representing independent association signals

CpG	Chr	Position	Closest gene	Classification	z score*	OR (95% CI)*	P*	R ² _{FHS} †	R ² _{WHI} †	Breast cancer risk SNPs	Distance to risk SNPs (kb)	P* adjusted for risk SNPs
cg18789177	2	217 729 408	TNPI1, LOC105373876	Intergenic	5.73	1.28 (1.17 to 1.39)	1.01×10^{-8}	0.02	0.01	rs4442975; rs34005590	191; 233	7.44×10^{-8}
cg16971831	5	56 110 935	MAP3K1	5'UTR	-11.90	0.51 (0.46 to 0.57)	1.23×10^{-32}	0.01	0.06	rs16886397; rs16886113; rs2229882; rs7726354; rs16886034; rs16886181; rs12655019; rs889312	23; -115; 57; 145; -127; -81; 84; -79	7.42×10^{-8}
cg20580673	14	91 735 665	GPR68, CCDC38C	Intergenic	-6.16	0.76 (0.69 to 0.83)	7.26×10^{-10}	0.02	0.03	rs941764	105	8.03×10^{-9}
cg00787180	14	91 751 731	CCDC88C	Intronic	-5.57	0.88 (0.84 to 0.92)	2.54×10^{-8}	0.05	0.06	rs941764	89	6.72×10^{-7}
cg09032423	16	4015 231	ADCY9	3'UTR	-5.01	0.82 (0.77 to 0.89)	5.56×10^{-7}	0.02	0.01	rs11076805	91	1.02×10^{-7}
cg12776287	18	24 125 939	KCTD1	Intronic	-5.36	0.95 (0.93 to 0.97)	8.12×10^{-8}	0.27	0.21	rs1436904; rs527616	444; 211	1.45×10^{-8}
cg19738924	18	24 126 072	KCTD1	Intronic	-5.60	0.93 (0.91 to 0.96)	2.20×10^{-8}	0.14	0.14	rs1436904; rs527616	444; 211	2.91×10^{-8}
cg15073853	19	18 549 131	ISYNA1	TSS200	9.28	1.09 (1.07 to 1.11)	1.77×10^{-20}	0.26	0.21	rs4808801	22	9.24×10^{-26}
cg21962901	19	18 549 134	ISYNA1	TSS200	9.37	1.11 (1.09 to 1.13)	7.27×10^{-21}	0.19	0.15	rs4808801	22	9.24×10^{-26}
cg11102782	19	18 549 136	ISYNA1	TSS200	8.80	1.08 (1.06 to 1.10)	1.38×10^{-18}	0.32	0.26	rs4808801	22	9.24×10^{-26}
cg09232727	22	29 140 725	HSCB	Intronic	-6.23	0.76 (0.70 to 0.83)	4.60×10^{-10}	0.02	0.03	rs17879961; rs132390	-19; 480	2.12×10^{-8}

*MetaXcan was used to estimate association z scores, ORs, 95% CIs, and P values. All statistical tests were two-sided. Chr = chromosome; CI = confidence interval; CpG = CpG sites; FHS = Framingham Heart Study; kb = kilobase; ncRNA = noncoding RNA; OR = odds ratio per SD increase in genetically predicted DNA methylation level (continuous variable); SNP = single-nucleotide polymorphisms; TSS = transcription start site; UTR = untranslated region; WHI = Women's Health Initiative.

†Correlation between predicted and measured DNA methylation levels.

Table 3. Selected* consistent directions of associations across DNA methylation, gene expression, and breast cancer risk

CpG	Chr	Position	Gene	Classification	CpG vs breast cancer risk		CpG vs Gex		Gex vs breast cancer risk		Gex vs breast cancer risk adjusted for DNA methylation†	
					Dir	P‡	Dir	P§	Dir	P‡	Dir	P‡
cg26668989	1	110186163	GSTM4	Upstream	Positive	1.92×10^{-10}	Negative	5.37×10^{-5}	Negative	2.04×10^{-5}	Negative	.52
cg08614201	1	145715134	CD160	5'UTR	Positive	5.00×10^{-7}	Negative	3.26×10^{-51}	Negative	9.35×10^{-4}	Negative	.93
cg20311333	1	155197753	GBAP1	TSS1500	Positive	2.54×10^{-11}	Negative	4.55×10^{-12}	Negative	2.22×10^{-9}	Positive	.86
cg02834765	1	155214859	GBA	TSS1500	Negative	1.16×10^{-7}	Negative	2.44×10^{-5}	Negative	5.77×10^{-8}	Negative	.62
cg16030869	4	38867304	FAM114A1	Upstream	Positive	1.72×10^{-8}	Positive	6.47×10^{-5}	Positive	1.72×10^{-8}	Positive	.22
cg17942617	5	81327376	ATG10	Intronic	Negative	6.81×10^{-13}	Positive	9.66×10^{-11}	Negative	6.84×10^{-11}	Negative	.75
cg16647868	5	131706066	SLC22A5	Intronic	Positive	7.36×10^{-7}	Negative	9.84×10^{-9}	Negative	1.81×10^{-6}	Negative	.90
cg12078157	6	13612218	SIRT5	3'UTR	Negative	1.44×10^{-7}	Negative	1.62×10^{-5}	Positive	1.74×10^{-4}	Positive	.51
cg05216056	6	28887836	TRIM27	Exonic	Negative	3.71×10^{-7}	Negative	1.45×10^{-29}	Positive	7.34×10^{-4}	Negative	.99
cg14701867	10	64193068	ZNF365	Intronic	Negative	5.92×10^{-10}	Negative	1.15×10^{-6}	Positive	.02	Positive	.26
cg23754390	11	835074	CD151	Intronic	Positive	2.51×10^{-7}	Positive	9.14×10^{-22}	Positive	2.03×10^{-3}	Negative	.81
cg04111478	11	1991677	MRPL23	Downstream	Positive	2.20×10^{-8}	Positive	.008	Positive	3.63×10^{-6}	Positive	.57
cg15531562	11	65601754	SNX32	Intronic	Positive	1.41×10^{-13}	Positive	.002	Positive	6.15×10^{-8}	Positive	.74
cg06065225	11	65640137	EFEMP2	Intronic	Negative	2.37×10^{-9}	Negative	.009	Positive	1.93×10^{-11}	Positive	.42
cg23526087	14	68973466	RAD51B	Intronic	Negative	4.21×10^{-21}	Negative	5.62×10^{-13}	Positive	4.55×10^{-4}	Positive	1.00
cg25839482	15	75931953	IMP3	3'UTR	Negative	2.59×10^{-8}	Negative	1.27×10^{-4}	Positive	6.18×10^{-6}	Negative	.78
cg18878992	17	43974344	MAPT	TSS1500	Negative	7.07×10^{-10}	Negative	.003	Positive	8.78×10^{-6}	Positive	.09
cg21757127	19	18525886	LRR25	Downstream	Negative	1.33×10^{-15}	Negative	1.71×10^{-5}	Positive	4.25×10^{-16}	Positive	2.21×10^{-5}
cg09516349	19	18529339	SSBP4	TSS1500	Positive	4.95×10^{-25}	Positive	9.70×10^{-4}	Positive	2.55×10^{-23}	Positive	.49
cg22161383	19	18545441	ISYNA1	3'UTR	Positive	3.85×10^{-27}	Negative	.003	Negative	9.62×10^{-10}	Negative	.01
cg14066757	19	44285568	KCNN4	TSS200	Negative	3.55×10^{-16}	Negative	.01	Positive	6.12×10^{-15}	Positive	.23

*Selected from 38 consistent directions of associations across DNA methylation, gene expression, and breast cancer risk. Complete list is available in [Supplementary Table 8](#) (available online). Chr = chromosome; CpG = CpG sites; Dir = direction of association and/or correlation; Gex = gene expression; TSS = transcription start site; UTR = untranslated region.

†Adjusted for all predicting variants included in prediction models of corresponding CpGs.

‡P values were calculated using MetaXcan. All statistical tests were two-sided.

§P values were calculated using Spearman correlation test. All statistical tests were two-sided.

Figure 3. The performance of DNA methylation prediction using the prediction model approach and using the single-meQTL SNP approach. For a total of 24 845 CpGs, prediction models were built in the present study and meQTLs were identified in a previous study. This figure presents the prediction performances of models and meQTLs for these CpGs. The x-axis represents the performance (R^2) of DNA methylation prediction using the single-meQTL SNP approach (ie, squared correlation coefficients of predicted and measured DNA methylation levels in the meQTL data). The y-axis represents performance (R^2) of DNA methylation prediction using the prediction model approach (ie, squared correlation coefficients of predicted and measured DNA methylation levels in the FHS data). The black line represents the identity line ($y=x$). CpGs = CpG sites; FHS = Framingham Heart Study; meQTL = DNA methylation quantitative trait loci; SNP = single-nucleotide polymorphism.

from prediction models with those from meQTLs. Among the 24 845 CpGs having models as well as meQTLs, prediction performances of models (R^2_{FHS}) were statistically significantly higher than those of single-meQTL SNPs (R^2_{meQTL}) (Figure 3). Especially, 21 874 CpGs (84.1%) were predicted better ($R^2_{\text{FHS}} > R^2_{\text{meQTL}}$) using models.

To determine whether our prediction model approach could identify more breast cancer-associated CpGs than the meQTL approach, for the 24 845 CpGs having prediction models as well as meQTLs, we investigated their DNA methylation levels, predicted by single-meQTL SNPs, in association with breast cancer risk. For these 24 845 CpGs, there was a strong correlation (Pearson correlation $r=0.88$) between z scores from prediction model and single-meQTL SNP approaches. The P values from the prediction model approach were lower than those from the single-meQTL SNP approach (Supplementary Figure 5, available online). Of the 450 breast cancer-associated CpGs, meQTLs were identified for only 162 CpGs and 128 reached P less than 2.01×10^{-6} (Bonferroni correction; $0.05/24\,845$) based on the single-meQTL SNP approach (Supplementary Table 9, available online). Therefore, only 128 (28.4%) of the 450 breast cancer-associated CpGs could be identified using the single-meQTL SNP approach.

Discussion

Using breast cancer as an example, we tested a novel methodology to identify CpGs associated with disease risk. We identified 450 CpGs that were statistically significantly associated with breast cancer risk. Of these, 38 CpGs may affect breast cancer risk through regulating expression of 21 genes. We demonstrate

that our methodology is successful in identifying novel DNA methylation biomarkers for disease risk. Our findings provide substantial new information regarding the mechanistic relationship of genetics, epigenetics, and gene expression and their associations with breast cancer risk.

Traditional epidemiologic studies of DNA methylation were limited by small sample size, confounders, and reverse causation. Our study focused on genetically determined DNA methylation, which is immune from reverse causation and confounders. Compared with the approach using single-meQTL SNPs as genetic instruments, our prediction model approach statistically significantly improved prediction performances. More important more than half of the breast cancer-associated CpGs would be missed using the single-meQTL SNP approach. Although we focused on breast cancer, this methodology can be applied to other diseases.

We observed consistent directions of associations across 38 CpGs, 21 genes, and breast cancer risk. Among them, five CpGs in three genes, *GSTM4*, *SLC22A5*, and *IMP3*, are within genomic regions that had not been associated with breast cancer risk via GWAS. *GSTM4* overexpression could help maintain a reduced state of cytochrome c, which contributes to methotrexate resistance in breast cancer cells (32). A mutation in *SLC22A5* was reported to enhance cancer cell metastasis in breast tissues (33). The overexpression of *IMP3* was observed in BRCA-mutated invasive breast carcinomas (34). Three CpGs in *CD160* were associated with increased breast cancer risk by the downregulation of *CD160* expression. This gene was suggested to have anticancer activity (35). Another three CpGs were located in *MAPT*, that were associated with breast cancer metastasis (36). In ER-negative breast cancers, the knockdown of a natural antisense of *MAPT* (*MAPT-AS1*) resulted in inhibited cancer cell proliferation (37). Recently, a CpG in *GREB1*, cg18584561, was associated with breast cancer risk (13). In the present study, this CpG was removed because of low quality, hence, a comparison could not be made. Another study (38) reported that a rare variant, *BRCA1* c.-107A>T, could silence *BRCA1* and increase breast cancer risk via DNA methylation. However, this variant is very rare and not included in data of FHS, WHI, and BCAC; hence, we could not investigate this variant for its association with either *BRCA1* methylation or breast cancer risk.

One limitation of our study is that prediction models were built using data from white blood cells, not breast tissues. However, it is unfeasible to obtain breast tissues from healthy individuals. Although in The Cancer Genome Atlas, genotype as well as DNA methylation were profiled for histologically normal tissue samples from 115 breast cancer patients of European descent (39), the DNA methylation profiles of “histologically normal” tissue samples from breast cancer patients may differ from those of tissue samples from healthy women. Multiple studies have suggested that meQTLs could be consistently detected across different tissues (16,40–42), indicating that genetically determined DNA methylation at many CpGs have cross-tissue consistency. Therefore, the genetic models we built using data from white blood cells should capture DNA methylation of many CpGs in breast tissues.

The present study has multiple strengths. Our novel methodology overcomes limitations of traditional epidemiological studies and is more accurate and powerful than studies based on the single-meQTL approach. A large number of samples in the reference dataset were used for model building, and the performances of models were excellent as demonstrated by external validation. The BCAC GWAS data, with to date the largest sample size, provided strong statistical power to identify

associations between CpGs and breast cancer risk. By integrating multiomics data, we found consistent evidence to support that DNA methylation may affect breast cancer risk through regulating gene expression.

In summary, using a novel methodology, we identified multiple CpGs statistically significantly associated with breast cancer risk and proposed that several CpGs may affect breast cancer risk through regulating gene expression. Our study demonstrates the utility of integrative analyses of multiomics data in identifying novel biomarkers for risk of developing breast cancer and provides new insights into the etiology of this malignancy.

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