

A panel of DNA methylation signature from peripheral blood may predict colorectal cancer susceptibility

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Abstract

Background Differential DNA methylation panel derived from peripheral blood could serve as biomarkers of CRC susceptibility. However, most of the previous studies utilized post-diagnostic blood DNA which may be markers of disease rather than susceptibility. In addition, to date no study has evaluated the predictive potential of such markers in a prospective cohort and on a genome-wide basis. The aim of this study was to identify potential panel of DNA methylation biomarkers in peripheral blood that are associated with CRC risk and therefore serve as epigenetic biomarkers of disease susceptibility.

Methods DNA methylation profile of a nested case-control study with 166 CRC and 424 healthy normal subjects were obtained from Gene Expression Omnibus (GEO) database. The differentially methylated markers were identified by moderated t-statistics. The DNA methylation panel were constructed by stepwise logistic regression and least absolute shrinkage and selection operator in training dataset. A methylation risk score (MRS) model was constructed and the association between MRS and CRC risk assessed.

Results We identified 48 differentially methylated CpGs sites, of which 33 were hypomethylated. Of these, sixteen-CpG based MRS that was associated with CRC risk (OR = 2.68, 95% CI: 2.13, 3.38, $P < 0.0001$) was constructed. This association is confirmed in the testing dataset (OR = 2.02, 95% CI: 1.48, 2.74, $P < 0.0001$) and persisted in both males and females, younger and older subjects, short and long time-to-diagnosis. The MRS also predicted CRC with AUC 0.82 (95% CI: 0.76, 0.88), indicating high accuracy.

Conclusions Our study has identified a novel DNA methylation panel that is associated with CRC and could be-useful for the prediction of CRC risk in the future.

Introduction

Colorectal cancer (CRC) poses a great public health concern globally. It is the third most common cancer diagnosed among men and the second most common among women (1) and was responsible for estimated 1.8 million new cases and 881,000 deaths in 2018 (2). In the United States of America, CRC is the third most common cancer diagnosed with about 140,250 new cases and 50,630 deaths in

2017 (3). In addition to environmental factors, there is now proven evidence that CRC results from the accumulation of genetic and epigenetic changes, which changes colonic epithelial cells into adenocarcinoma cells (4).

Epigenetic alterations such as DNA methylation has been associated with many human diseases including cancer and has also been reported to occur early in the development of colorectal tumors (4) by playing a role in gene expression and genomic stability. DNA methylation markers show great potential in the detection and diagnosis of cancer (5) and a panel of differential DNA methylation could be a possible biomarker of CRC susceptibility.

Peripheral blood is an easily accessible source of genomic DNA that can be used to estimate DNA methylation profiles and could serve as useful non-invasive and informative biomarkers for cancer risk (6). Several studies have investigated peripheral blood DNA methylation biomarkers in different cancer types including head and neck, urothelial, breast, lung, bladder, gastric cancer, prostate and ovarian cancers (7-17). Some epidemiologic studies have assessed peripheral blood DNA methylation biomarkers in CRC. However, most of the studies used post-diagnostic blood DNA which may imply that DNA methylation alterations could be an early response of the hematologic system to the presence of CRC cells (18, 19). The few studies that utilized pre-diagnostic DNA focused on genomic methylation of leukocyte DNA (20, 21) while other studies involved candidate genes (22-24) and methylation at repetitive elements (25). There is no genome-wide DNA methylation study that has evaluated the association of pre-diagnostic peripheral blood DNA with CRC risk.

In order to identify potential panel of DNA methylation biomarkers in peripheral blood that are associated with CRC risk and therefore serve as epigenetic biomarkers of disease susceptibility, we performed epigenome-wide analysis of a nested case-control study using peripheral blood Illumina HumanMethylation450 bead-array DNA methylation data. We repurposed a data previously analysed by Cordero et al who focused on probes associated to genes encoding for miRNAs (26). We analysed the data using two methods including epigenome-wide methylation profiling to identify differentially methylated CpGs as well as machine learning algorithm to construct a sixteen-CpG based methylation risk score predictive of CRC risk.

Methods

Data source

The Illumina Human Methylation 450 Beadchip data of the Italian arm of the European Prospective Investigation into Cancer and Nutrition (EPIC-Italy) were obtained from Gene Expression Omnibus (GEO) with the accession number GSE51032. The EPIC is a multicenter prospective study aimed at investigating the complex relationships between nutrition and various lifestyle factors and the etiology of cancer and other chronic diseases (27). The EPIC-Italy cohort that was produced in Turin, Italy, is a sub-cohort that comprised of 46,857 volunteers, recruited from five different centers within Italy (Varese, Turin, Florence, Naples and Ragusa) with standardized lifestyle and personal history questionnaires, anthropometric data as well as blood samples collected for DNA extraction. At the last follow-up (2010), 424 participants remained cancer-free, 166 had developed primary colorectal cancer. We extracted the data containing the DNA methylation status of 485,512 CpG sites in the 166 participants who had developed primary colorectal cancer and the 424 matched cancer-free participants.

DNA methylation profiling in CRC and healthy normal subjects

The differential methylation analysis was conducted using the workflow by Maksimovic et al (28). Briefly, we pre-processed and normalized the data using R package minfi. The quality control, pre-filtering were conducted with the minfi package and the Functional Normalization (FunNorm) function was used for normalization (29, 30). Quality control was performed and probes with detection P-value > 0.01 in at least one sample were filtered out. After normalization, all probes containing single nucleotide polymorphism (SNPs) and probes mapped to sex chromosomes were filtered out to prevent bias due to unknown genetic background and mixed gender of samples, respectively. Cross-reactive probes, which refers to probes that have shown to map to several positions in the genome (31) were also filtered out. After normalization and quality control, the probes yielded were used for further analysis.

Hierarchical clustering

We conducted Hierarchical clustering using complete linkage with a Euclidian distance in the R

package pheatmap.

Functional analysis

In order to examine main biological functions that were controlled by DNA methylation, we used DMPs for Gene ontology (GO) analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) based on the gometh function in the R package missMethyl.

Selection of differentially methylated markers for risk model

The methylation level of all the probes were indicated as beta (β) values, which is the proportion of the methylated probe intensity to the total probe intensity (sum of methylated and un-methylated probe intensities plus constant α , where $\alpha = 100$). The beta values for CRC and healthy normal subjects were log transformed to obtain the M-values and used for further analysis, with the beta values used for visualization while the M-values were used for statistical analysis which is in conformity with Du et al. (32). The linear models for microarray data (LIMMA) package was used to identify differentially methylated genes between CRC cases and healthy normal subjects (33).

Moderated t-test and mean methylation value differences (delta (Δ) beta) were generated and we corrected P values of individual probe for multiple testing using the Benjamini-Hochberg method. A CpG site between CRC and healthy normal subjects was considered significant with a false discovery rate (FDR) < 0.05 and $\Delta\beta \geq 5\%$ and denoted differentially methylated positions (DMPs).

In addition, DMPs were used to build a risk score model. The entire sample of 590 were randomly split into 70% training and 30% testing sets stratified by case-control status. The stratification was to guarantee an equal distribution of CRC and healthy normal subjects between sets, prevent over fitting the data and allow for validation of the model. The stepwise logistic regression and least absolute shrinkage and selection operator (LASSO) (34) methods were then applied on the training set to select the best markers for CRC prediction using R packages MASS and glmnet respectively. For the LASSO selection analysis, we used 10-fold cross-validation to identify the tuning parameter and chose the minimum lambda, which is the value of lambda with the smallest mean cross-validated error. Nineteen CpGs were identified by using the stepwise regression method and twenty two CpGs were identified by using the LASSO analysis. In these two approaches, sixteen overlapping markers were

identified between the two methods.

Construction of methylation risk score

Logistics regression models were fitted on the training dataset using these sixteen markers and MRS for each patient was calculated. The calculation was carried out by multiplying the methylation level for each CpG site with the corresponding regression coefficient and summed over all CpG sites as follows:

$$\text{MRS} = \beta_1x_1 + \beta_2x_2 + \beta_3x_3 + \dots + \beta_kx_k$$

Where β represents the estimated regression coefficient of the CpG site k derived from the logistic regression analysis, and x represent the methylation level of the CpG site k .

Furthermore, we determined whether our finding could be validated in the testing dataset. The MRS was constructed on the training set and validated on the testing set by fitting a logistic regression model to determine the association of the MRS with CRC, with the MRS added into the model as a continuous variable.

Subgroup analyses

To assess the robustness of our findings, we determined whether the association between MRS and CRC risk differed by gender, age and time-to-diagnosis by conducting subgroup analyses according to these variables both in the training and testing datasets. We took advantage of the prospective design of this study and explored the effect of time-to-diagnosis. We categorized the CRC subjects into short (less than 6 years) and long (above 6 years) time-to-diagnosis using the median as cut-off. In addition, we conducted a case-only analysis and assessed whether methylation levels of the CpGs were correlated with time-to-diagnosis (the time interval between blood draw and diagnosis of CRC).

Statistical analysis

The distribution of the demographic characteristics in the study group was compared between CRC and healthy normal subjects using Chi-square and Kruskal-Wallis tests for categorical and continuous data respectively. To estimate the difference in methylation level between CRC and healthy normal, two-sample t-tests (moderated t-tests) with Bonferroni correction was performed for each CpG.

Univariate and multivariate logistic regression were used to estimate odds ratios (ORs) and

corresponding 95% confidence intervals (CI) for DNA methylation and MRS between CRC and healthy normal subjects, as well as subgroup analysis. The ROC curves were plotted with R package pROC, to estimate discriminatory power of the MRS. The area under the ROC curve (AUC) was calculated and the DeLong method was used to calculate the 95% confident internal (CI) for AUC. Correlation was performed using Pearson's method. The significance level used for all tests was two-tailed $P < 0.05$. All statistical analyses were carried out using R language software version 3.5.1 (<https://cran.r-project.org/bin/windows/base/old/3.5.1/>).

Results

Identification of differentially methylated markers

The workflow showing the step-by-step procedure for this analysis and the demographic characteristics of participants are presented in Fig. 1 and Table 1 respectively. We analysed the microarray methylation profile of 166 (87 males and 79 females) CRC and 424 (84 males and 340 females) healthy normal subjects. The average age of CRC subjects was 55 years old whereas, the normal subjects had a mean age of 53. The average time-to-diagnosis for cases was 6.2 years (range = 0-14.3). The Illumina Human Methylation 450 Beadchip contained DNA methylation status of 485,512 CpG sites. Pre-processing and quality control were performed and the poor performing probes were filtered out. A total of 399,934 CpG sites (Additional file 1: Figure S1) were yielded, and their methylation data were used for further analysis. A total of 49,299 CpGs (corresponding with 11,786 unique genes) were differentially methylated ($FDR < 0.05$) between the CRC and healthy normal subjects.

Table 1

Characteristics of Training and Testing Dataset of Nested Case Control Study Based on EPIC-Italy Cohort

Characteristics	Entire Dataset		Training Dataset		Testing Dataset	
	Cases	Control	Cases	Control	Cases	Control
Total	166	424	117	297	49	127
Age, Mean (SD)	55.07 (6.73)	53.23 (7.19)	55.94 (6.73)	53.08 (7.20)	55.25 (6.62)	53.56 (7.20)
< 60	128 (26.9)	348 (73.1)	89 (26.4)	245 (73.4)	10 (27.5)	103 (72.5)
≥ 60	38 (33.3)	76 (66.7)	28 (35.0)	52 (65.0)	39 (29.4)	24 (70.6)
Gender						
Male	87 (52.4)	84 (19.8)	55 (47.4)	61 (52.6)	32 (58.2)	23 (41.8)
Female	79 (47.6)	340 (80.2)	62 (20.8)	236 (79.2)	17 (14.0)	104 (86.2)
Time-to-diagnosis (years) Mean (SD)						
< 6	80 (48.2)	NA	56 (47.9)	NA	24 (49.0)	NA
≥ 6	86 (51.8)	NA	61 (52.1)	NA	25 (51.0)	NA

Abbreviations: NA, not applicable; SD, standard deviation

Gene Ontology (GO) terms and KEGG pathway enrichment analysis for genes associated with the 49,299 differentially methylated CpGs were performed. The GO analysis showed the molecular functions, cellular components and biological functions of differentially methylated genes under the criterion FDR < 0.05 (Additional file 2: Table S1). In the KEGG pathway genes showed enrichments in the metabolic pathway (FDR = 1.19e-03), cancer- pathways (FDR = 6.58e-03), human papillomavirus infection (FDR = 1.61e-02), Rap1 signaling pathway (FDR = 4.36e-04) and Axon guidance (FDR = 2.12e-03) (Additional file 3: Table S2).

Of the 49,299 CpGs differentially methylated, 48 CpGs (corresponding with 29 unique genes) which had absolute mean β -value difference ($|\Delta\beta| \geq 0.05$) were selected and denoted DMPs (Additional file 4: Table S3). Among the DMPs, a total of 15 CpGs (corresponding with 8 unique genes) were hypermethylated and 33 CpGs (corresponding with 21 unique genes) were hypomethylated. Hierarchical clustering was implemented to determine whether the identified DMPs could distinguish CRC from healthy normal subjects. The results shows that these two conditions in the CRC and healthy normal subjects exhibited distinctive DNA methylation patterns (Fig. 2). More so, it was

observed that the samples were classified into two distinct clusters by these DMPs.

Methylation risk score construction

The entire sample of 590 was randomly split into training (117 CRC subjects and 297 healthy normal subjects) and testing (49 CRC subjects and 127 healthy normal subjects) sets (Table 1). Differentially methylated markers associated with CRC risk were screened on the training dataset using LASSO selection and stepwise logistic regression analysis. The sixteen markers mapped to nine genes including *LGR6*, *PTPN12*, *PPFIA3*, *LOC399959*, *PCDHGA1*, *RNF39*, *ESYT3*, *MRGPRG* and *ATHL1* overlapping between the two methods were selected (Additional file 5: Figure S2). The associations of the sixteen individual markers with CRC by univariate and multivariate logistic regression analysis are presented in Additional file 6: Table S4 and Table 2 respectively.

Furthermore, using the sixteen-CpG panel we calculated a methylation risk score (MRS) for each subject on the training dataset using the formula:

$$\begin{aligned} \text{MRS} = & (-0.4100 * \text{cg06551493}) + (0.4332 * \text{cg01419670}) + (0.2895 * \text{cg16530981}) \\ & + (-0.5172 * \text{cg18022036}) + (-0.3915 * \text{cg12691488}) + (-0.3246 * \text{cg17292758}) \\ & + (-0.2886 * \text{cg16170495}) + (0.2451 * \text{cg11240062}) + (-0.5651 * \text{cg21585512}) + \\ & (0.3615 * \text{cg24702253}) + (-0.2445 * \text{cg17187762}) + (-0.3951 * \text{cg05983326}) \\ & + (-0.5089 * \text{cg06825163}) + (-0.2504 * \text{cg11885357}) + (- \\ & 0.2357 * \text{cg08829299}) + (-0.3607 * \text{cg07044115}). \end{aligned}$$

The methylation levels of 5 CpG sites were hypermethylated, and 11 CpG sites were hypomethylated.

The MRS (range, -5.59 to 4.35) was significantly higher for CRC subjects than in healthy normal subjects ($P < 0.000$), with a median MRS of 1.68 (IQR, 1.43) in CRC subjects and -0.430 (IQR, 2.89) in healthy normal subjects (Additional file 7: Figure S3a) in the training dataset. The MRS was associated with a 2.68-fold increased risk of CRC (OR = 2.68, 95% CI: 2.13, 3.38, $P < 0.0001$) Table 2. The MRS showed good predictive ability for discriminating between CRC and healthy normal subjects (AUC, 0.85; 95% CI: 0.81, 0.89) Figure 3a.

Validation of the sixteen-CpG panel MRS for CRC prediction in the testing dataset.

In order to validate the predictive performance of the sixteen-CpG panel MRS for the prediction of

CRC risk, the predictive model was applied to the testing dataset. The MRS (range, -5.73 to 3.89) was also significantly higher for CRC subjects than in healthy normal subjects ($P < 0.0001$), with median MRS of 1.83 (IQR, 1.80) in CRC subjects and -0.45 (IQR, 2.64) in healthy normal subjects (Additional file 7: Figure S3b). Consistent with the training dataset, the MRS was associated with a 2.02-fold increased risk of CRC (OR = 2.02, 95% CI: 1.48, 2.74, $P < 0.0001$) Table 2. Similar to the training dataset, the MRS showed good predictive ability for discriminating between CRC and healthy normal subjects (AUC, 0.82; 95% C: 0.76, 0.88) Figure 3b.

Subgroup analysis for the association between MRS and CRC risk

When the study subjects were stratified according gender, age and time-to-diagnosis, the MRS still demonstrated an increased risk of CRC among both male and female subjects, younger (< 60 years) and the older (≥ 60 years) subjects as well as short and long time to diagnosis in the training and testing datasets (Table 3). Also, the case-only analysis demonstrated no correlation between methylation levels time-to-diagnosis (Additional file 8: Table S5).

Table 2 Multivariate Analysis on the Associations of DNA Methylation Marker, MRS and Risk of CRC of Nested Case Control Study Based on EPIC-Italy Cohort

CpG ID	Gene Name	Entire Dataset			Training Dataset		
		OR	95% CI	P-value	OR	95% CI	P-value
cg06551493	PTPN12	0.62	0.49, 0.78	6.58e-05	0.71	0.54, 0.91	6.58e-05
cg01419670	NA	2.12	1.62, 2.85	1.47e-07	2.36	1.71, 3.361	1.47e-07
cg16530981	NA	1.96	1.52, 2.57	5.01e-07	2.15	1.60, 2.98	5.01e-07
cg18022036	NA	0.56	0.45, 0.70	5.47e-07	0.54	0.41, 0.69	5.47e-07
cg12691488	NA	0.73	0.47, 1.10	0.14	0.67	0.40, 1.08	0.14
cg17292758	PPFIA3	0.79	0.64, 0.98	0.04	0.79	0.61, 1.02	0.04
cg16170495	RNF39	0.66	0.54, 0.80	3.17e-05	0.68	0.54, 0.85	3.17e-05
cg11240062	NA	1.25	1.00, 1.57	0.06	1.30	1.00, 1.69	0.06
cg21585512	LOC399959	0.68	0.55, 0.83	1.61e-04	0.58	0.45, 0.74	1.61e-04
cg24702253	MRGPRG	1.74	1.28, 2.58	0.002	1.78	1.24, 2.81	0.002
cg17187762	NA	0.76	0.63, 0.93	0.006	0.78	0.62, 0.97	0.006
cg05983326	PCDHGA1	0.69	0.57, 0.84	2.68e-04	0.73	0.57, 0.91	2.68e-04
cg06825163	LGR6	0.70	0.57, 0.86	5.47e-04	0.67	0.52, 0.84	5.47e-04
cg11885357	ESYT3	0.89	0.73, 1.08	0.23	0.83	0.65, 1.04	0.23
cg08829299	ATHL1	0.86	0.70, 1.04	0.13	0.84	0.66, 1.05	0.13
cg07044115	NA	0.77	0.63, 0.93	0.008	0.82	0.66, 1.03	0.008
MRS		2.41	2.02, 2.90	0.02	2.68	2.13, 3.38	0.02

Abbreviations: CI, confidence interval; CRC: colorectal cancer; MRS, methylation risk score; ORs adjusted for age and gender; P values < 0.05 are in bold

Table 3 Associations of MRS and Risk of CRC According to Age, Gender and Time-To-Diagnosis of Nested Case Control Study Based on EPIC-Italy Cohort

Characteristics	Entire Dataset			Training Dataset			OR
	OR	95% CI	P-value	OR	95% CI	P-value	
Age							
< 60	2.35	1.95, 2.90	< 0.0001	2.62	2.06, 3.44	< 0.0001	1.9
≥ 60	2.60	1.77, 4.17	< 0.0001	2.87	1.77, 5.35	0.0002	2.2
Gender							
Male	1.97	1.47, 2.71	< 0.0001	2.10	1.44, 3.21	0.0002	1.9
Female	2.64	2.12, 3.37	< 0.0001	2.96	2.26, 4.08	< 0.0001	2.0
Time-diagnosis							
< 6 years	2.21	1.80, 2.77	< 0.0001	2.40	1.86, 3.20	< 0.0001	1.9
≥ 6 years	2.51	2.01, 3.23	< 0.0001	2.88	2.17, 4.01	< 0.0001	1.9

Abbreviations: CI, confidence interval; CRC: colorectal cancer; MRS, methylation risk score; OR, odds ratios adjusted for age and gender

Discussion

In this study, we repurposed a microarray peripheral blood DNA methylation data of CRC and healthy normal subjects obtained from GEO database. First, we identified differentially methylated CpGs between CRC and healthy normal subjects for CRC-specific methylation panel. Second, we divided the data into two sets and identified a panel of sixteen CpGs associated with CRC by logistic regression in the training dataset. Third, we constructed a predictive model- MRS, to predict risk of CRC based on the linear combination of methylation levels of the sixteen CpGs. The MRS was tested first on the training dataset and was associated with risk of CRC, the prediction evaluation when conducted by ROC analysis attained an AUC of 0.85. Subgroup analyses demonstrated that these significant associations persisted in both males and females, younger and older subjects as well as long and

short time-to-diagnosis. The MRS, when validated on the testing dataset attained an AUC of 0.82 indicating that the risk predictive value of the MRS panel is replicable for predicting CRC risk. Our findings shows a panel of peripheral blood DNA methylation that is a potential biomarker for CRC susceptibility.

Previous studies have developed multiple gene methylation-based panels to predict individual's susceptibility to CRC. For example, Liu et al. and Luo et al. both reported DNA methylation-based panels in blood leukocyte that were associated with 6.51-fold (95% CI, 3.77–11.27) and 1.54-fold (95% CI: 1.15–2.05) increased risk of CRC respectively and this is similar to our result. However, since both studies involved post-diagnostic DNA samples based on case-control studies. The association detected may have resulted from a response to CRC cells rather than CRC susceptibility.

Although the mechanisms underlying the aberrations in the methylation of peripheral blood DNA among individuals who are susceptible to CRC are not clear, our analysis used pre-diagnostic peripheral blood DNA, which indicates that methylation aberrations in peripheral blood DNA could possibly be a long-term CRC predisposition risk markers or a far early response to CRC cells before the cancer could be detected by techniques used before now such as endoscopy and cytology. In addition, there was no correlation between DNA methylation and time-to-diagnosis in case-only analysis, which also supports the suggestion that peripheral blood DNA could be a long-term event. Contrary to our result, the previous studies that utilized pre-diagnostic blood DNA found no association between pre-diagnostic genomic DNA methylation status and CRC risk (20, 21). This difference may be because of the heterogeneous methodology and assays. The two studies evaluated genomic leukocyte genomic DNA methylation levels by liquid chromatography/tandem mass spectrometry, which only considers DNA hypomethylation and not regional hypermethylation that can also contribute to increased risk of CRC.

The presence of specific single nucleotide polymorphisms (SNPs) have also been used to evaluate an individual's risk for CRC both by candidate and multiple genes (by a method called a genetic risk score (GRS)) as well as genome-wide association study (GWAS). Similar to the associations we found between MRS and CRC risk, GRS based on SNPs have been associated with CRC risk. For example,

Cho et al. (35), reported a higher GRS that was associated with CRC (OR, 2.57; 95% CI, 1.89, 3.49) using thirteen SNPs. In addition, Jung et al. (36) in a case-cohort study, demonstrated that participants in the highest quartiles of the genetic risk score had an increased risk of CRC (hazard ratio, 2.65; 95% CI, 1.43 to 4.91) compared with those in the lowest quartile using seven SNPs. Furthermore, a GWAS study found a SNPs developed polygenic risk scores (PRSs) that was associated with about 2-fold increased risk of CRC (37).

In the present study, the methylation-based markers for CRC included LGR6, PTPN12, PPFIA3, LOC399959, PCDHGA1, RNF39, ESYT3, MRGPRG and ATHL1, all of which were located in the promoter regions or first introns of nearby genes. There are limited epidemiological reports on the association between these markers and CRC risk. One of the genes, LGR6 (Leucine Rich Repeat Containing G Protein-Coupled Receptor 6) regulates the phosphoinositide 3-kinase/AKT signaling pathway and plays a tumor-promoting role in CRC development indicating that it might be a potential diagnostic and prognostic biomarker for CRC (38). Protein tyrosine phosphatase non-receptor type 12 (PTPN12) are signaling molecules that regulate a variety of cellular processes and has been found to be epigenetically regulated in triple-negative breast cancer and could play a role in triple-negative breast cancer tumorigenesis (39, 40). In addition, the methylation of PTPRF-interacting protein alpha 3 (PPFIA3) in serum has shown a potential for the detection of gastric cancer (41). More so, the other genes have been investigated in other diseases including LOC399959, a protein coding gene which has been expressed in the retina and has been found to be susceptible to pathological myopia (42, 43). The hypermethylation of RNF39 (RING finger protein 39) has been associated with multiple sclerosis (44). Protocadherin gamma subfamily A, 1 (PCDHGA1) has been associated with carotid artery intima media thickness (45) and ESYT3 (Extended Synaptotagmin 3) haplotype has been associated with the pathogenesis of coronary artery disease in females (46).

The pathway analysis demonstrated that metabolic pathway, cancer pathways, human papillomavirus infection, Rap1 signaling pathway and Axon guidance were associated with CRC. The biological processes involve cellular component organization or biogenesis, and cellular localization. The Rap1 signaling pathway has been implicated in previous genome-wide profile of colorectal cancer (47) and

has been known to play several important roles in tumor cell invasion and metastasis (48). The pathway and biological processes put together demonstrates that multiple pathways, which were affected by aberrant methylation were involved in CRC tumorigenesis.

To our knowledge, our analysis is the first to assess the potential link between genome-wide DNA methylation in peripheral blood and future risk of CRC. Our analysis has revealed that there is potential in the use of peripheral blood-based DNA methylation profiling for CRC risk prediction. We have shown, with a ROC indicating good performance, a MRS model consisting of sixteen CpG panel that has ability to differentiate CRC from healthy normal subjects.

One important strength of our study was its prospective design. The utilization of blood samples collected before diagnosis which indicated that the DNA methylation preceded development of CRC by up to 6 years, enabled us to assess genome-wide measures of DNA methylation as potential biomarkers of risk as compared to measures of DNA methylation in retrospective designs which may have resulted from molecular changes due to carcinogenesis and medication.

A limitation of our study is its lack of replication. To the best of our knowledge, there are currently no other pre-diagnostic blood DNA Illumina Human Methylation 450 data for CRC studies available. We recommend that other prospective cohorts studies assess associations between genome-wide DNA methylation and CRC risk.

Conclusion

Our study has identified a novel DNA methylation panel based on genome-wide analysis that is associated with CRC and suggest that differential peripheral blood DNA methylation panel may be an easily available biomarker for prediction of CRC risk in the future. Further studies with larger cohort data will be needed to confirm this pattern.

Abbreviations

AUC

area under the ROC curve; CI:confidence interval; CRC:colorectal cancer; DMPs:differentially methylated probes; ESYT3:Extended Synaptotagmin 3; EPIC:European Prospective Investigation into Cancer and Nutrition; FDR:false discovery rate; GEO:Gene Expression Omnibus; GO:Gene ontology; KEGG:Kyoto Encyclopedia of Genes and Genomes; LASSO:least absolute shrinkage and selection

operator; LIMMA:linear models for microarray data; LGR6:Leucine Rich Repeat Containing G Protein-Coupled Receptor 6; MRS:methylation risk score; OR:odds ratio; PCDHGA1:Protocadherin gamma subfamily A, 1 PPFIA3, PTPRF-interacting protein alpha 3; PTPN12:Protein tyrosine phosphatase non-receptor type 12; ROC:Receiver Operating Characteristic.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

The raw data sets used to support the conclusions of this article are available on NCBI's Gene Expression Omnibus (GEO) through GEO accession number GSE51032.

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51032>

Conflict of interest

Authors declare that they have no competing interests.

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Authors' contributions

JUO, DL and YZ: designed the study; JUO, DL, HH, YZ and YL: conducted data acquisition and analysis; JUO, YL and DL: drafted the manuscript; JUO, DL, JX and YZ: revised the manuscript. All authors read and approved the final version for publication.

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Figures

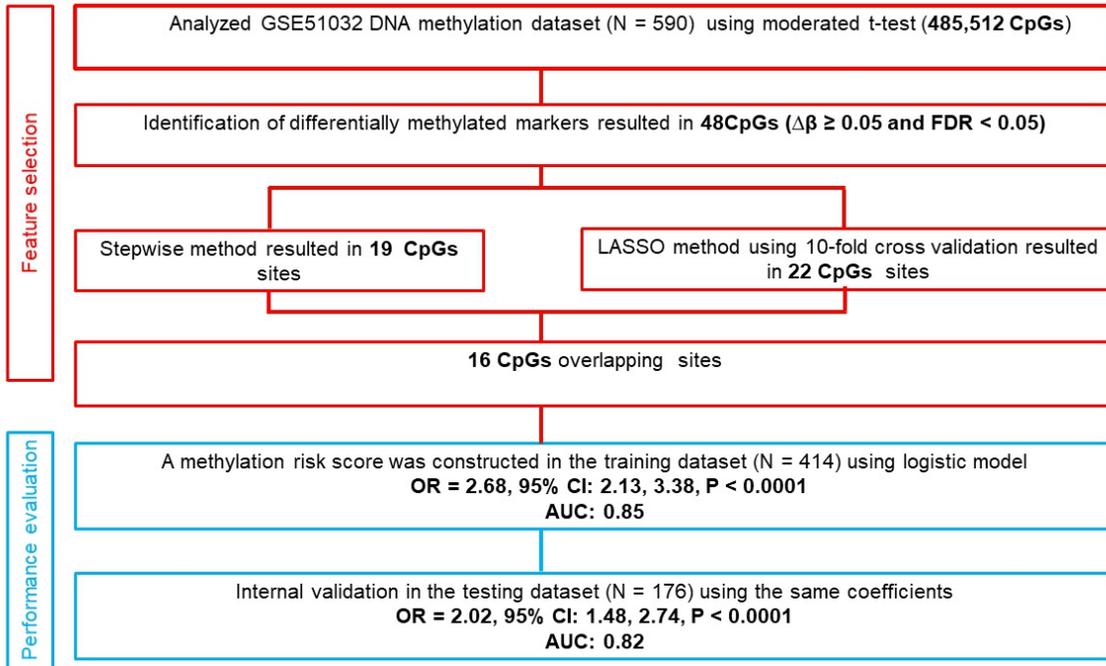


Figure 1

Overall workflow of the step-by-step analyses process of this study.

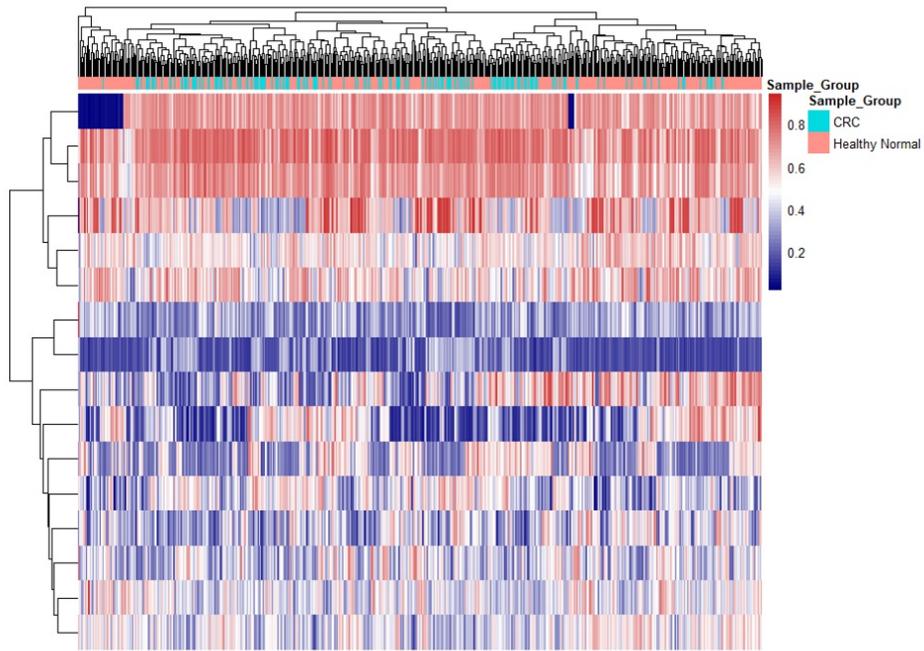


Figure 2

The heatmap showing the methylation levels of 48 CpG sites differentially methylated in the entire dataset. Row represents specific markers (N = 48). Column represents samples (N = 590)

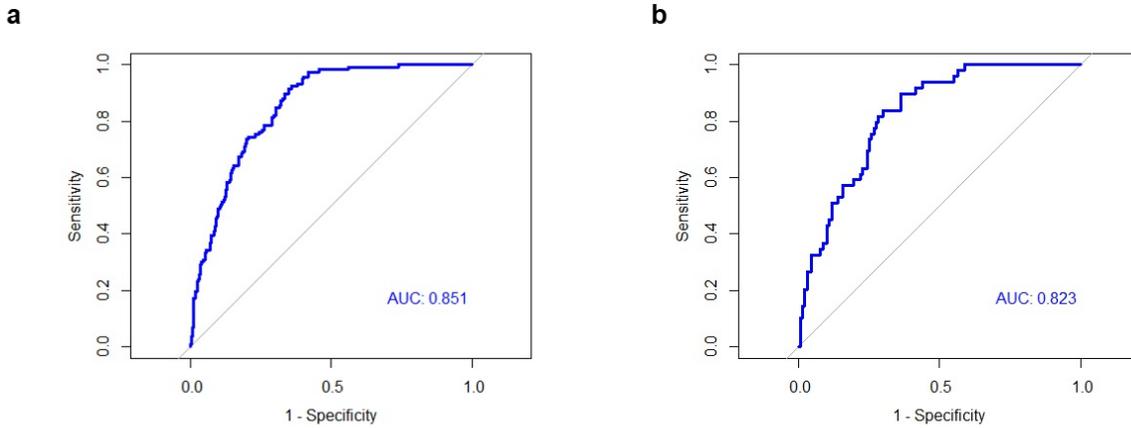


Figure 3

Classification performance of methylation risk score (MRS) for CRC risk (a) Receiver operating characteristic (ROC) curve for methylation risk score (MRS) prediction of CRC, with area-under the curve (AUC) of 0.85 (95% CI: 0.82-0.88) on training dataset. (b) Receiver operating characteristic (ROC) curve for methylation risk score (MRS) prediction of CRC, with area-under the curve (AUC) 0.82 (95% CI: 0.76-0.88) on testing dataset.

Supplementary Files

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