**Genome-Wide DNA Methylation Profiles of Low- and High-Grade Adenoma Reveals A Potential Biomarkers for Early Detection of Colorectal Carcinoma**

Jian Fan1,4 #, Jun Li2 #, Shicheng Guo3 # ,Chengcheng Tao1, Haikun Zhang1,4, Wenjing Wang2, Ying Zhang1, Dake Zhang1\*, Shigang Ding2\*, Changqing Zeng1\*.

1，Key Laboratory of Genomic and Precision Medicine, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China

2，Department of Gastroenterology, Peking University Third Hospital, Beijing 100191, China

3，Center for Precision Medicine Research, Marshfield Clinic Research Institute, Marshfield, WI 54449, USA

4，University of Chinese Academy of Sciences, Beijing 100049, China

# These authors contributed equally to this work; \* Corresponding Author

## **Abstract**

**Background:** Abnormal DNA methylation is a hallmark of human cancers and may be a promising biomarker for early diagnosis of human cancers. However, the majority of DNA methylation biomarkers that have been identified are based on the hypothesis that early differential methylation regions (DMRs) are maintained throughout carcinogenesis and could be detected at all stages of cancer. In this study, we identified potential early biomarkers of colorectal cancer (CRC) development by genome-wide DNA methylation assay (Illumina infinium450, 450K) to normal (N=20) and pre-colorectal cancer samples including 18 low-grade adenoma (LGA) and 22 high-grade adenoma (HGA), integrating with GEO and ArrayExpress datasets (N=833).

**Results:** We identified 209 and 8,692 CpG sites that were significantly hyper-methylated in LGA and HGA, respectively. Pathway analysis identified nervous system-related methylation changes that are significantly associated with early adenoma development. Together with GEO and ArrayExpress datasets, integration analysis revealed that DNA methylation in the promoter region of *ADHFE1* has the most potential for being an early diagnostic biomarker for colorectal adenoma and cancer (sensitivity=0.96, specificity=0.95, area under the curve=0.97).

**Conclusions:** Overall, we demonstrated LGA and HGA provided an important proxy for early methylation biomarker identification and the gene could be a promising methylation biomarker for colorectal cancer.

**Key words:**

DNA methylation, Low-grade adenoma, High-grade adenoma, Colorectal cancer, Biomarker

## **Background**

Colorectal cancer (CRC) is the third leading cause of cancer-related deaths worldwide [1, 2]. Current evidence indicates that genetic mutations and epigenetic alterations progressively accumulate in the tumor genome during carcinogenesis, and these alterations may serve as primary biomarkers for early detection and treatment of cancer [3]. Abnormal alterations in methylation status specifically hyper-methylation or hypo-methylation has also been associated with abnormal tissue differentiation. Altered methylation has been observed in the promoter regions of tumor suppressor genes and miRNA have been observed in almost all cancer types [4, 5]. Over the past decades, detection of altered DNA methylation has been widely studied to develop cancer biomarkers [6] and the majority have been identified are based on the hypothesis that early differential methylation regions (DMRs) are maintained throughout carcinogenesis and could be detected at all stages of cancer. For example, altered methylation patterns have been detected with hepatic disease progression in the context of hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) [7, 8]. Moreover, recent evidence demonstrated that cell-free DNA (cfDNA) methylation can be used for early cancer diagnosis and tissue-of-origin mapping for metastatic cancer [4].

Abnormal alterations of DNA methylation have been recognized as an important event in cancer development[9]. Global hypo-methylation arises early in carcinogenesis and has been linked to chromosomal instability and loss of imprinting [10, 11]. Generally during cancer development, hundreds of genes are [silenced or activated](https://en.wikipedia.org/wiki/Regulation_of_transcription_in_cancer" \l "Transcription_silencing/activation_in_cancers" \o "Regulation of transcription in cancer) [12-14]. Although silencing of some genes in cancers occurs by mutation, a large proportion of carcinogenic gene silencing is a result of altered DNA methylation [15]. DNA methylation-based silencing in cancer typically occurs at multiple CpG sites in the [CpG islands](https://en.wikipedia.org/wiki/CpG_site" \l "CpG_island" \o "CpG site) present in the promoters of protein-coding genes [16]. Although extensive epigenetic alterations have been defined over the past years, CRC is still not well understood at the molecular level[3]. Against a background of whole genome hypo-methylation, gene-specific promoter hyper-methylation has been found to promote CRC by down-regulating the expression of key tumor suppressor genes such as *CDKN2A*, *MLH1*, and *CDH1* [17-19]. CRC is a heterogeneous disease that typically originates from a [benign tumor](https://en.wikipedia.org/wiki/Adenoma" \o "Adenoma), often in the form of an adenoma, eventually progressing to a malignant cancer within a temporal window that may exceed 10 years [20]. Because CRC exceeds many other cancers in both incidence and mortality, capacity to detect and monitor molecular changes during the colorectal adenoma (CA) stage provides an excellent opportunity to prevent cancer progression and improve survival outcomes [21]. While a large number of studies have focused on CRC, a subset of them have focused specifically on the adenoma as an intermediate stage which required more specific molecular definition. For instance, a ten-gene methylation signature in adenoma was found to change with pathologic progress [9]. Notably, colorectal adenoma has two pathologic stages: low-grade adenoma (LGA) and high-grade adenoma (HGA) [22]. our research compared and defined differences of Genome wide profiling of DNA methylation, especially changes across these two precancerous stages had not been reported [23]. We hypothesized that these alterations in LGA methylation represented candidates as potential early diagnostic biomarkers. We further posit that comprehensive understanding to the genome-wide DNA methylation profile for the early stage pre-cancerous lesions (LGA and HGA) will provide important resources early diagnosis and candidate biomarkers for potential oncogenic progression.

In this study, we conducted a series of genome-wide DNA methylation array of 18 LGA and 22 HGA and compared the frequency, location, and pattern of methylation status to 20 normal tissue samples. Dynamic DNA methylation changes were identified for LGA and HGA, and we found that methylation changes appeared in LGA were increased or maintained in HGA and cancer. Enrichment analyses of differential methylation regions (DMRs) were performed to further investigate the potential influence of DNA methylation on functional difference in adenoma initiation and development. Moreover, we separated different methylation sites (DMSs) between LGA and normal into hyper-DMS and hypo-DMS and evaluated their respective performance for CA and CRC prediction. To validate our findings, we compared them to genome-wide DNA methylation profiles of 833 samples from public database. Finally, we describe the identification and analysis of one functional methylation signature at the promotor region of *ADHFE1* as a potential biomarker for early CRC development.

## **Results**

#### Landscape of DNA methylation of pre-cancerous benign lesions

We profiled DNA methylation at the single-base level for 18 LGA, 22 HGA, and 20 normal tissues. We found significant genome-wide DNA methylation differences among normal, low-, and high-grade adenoma (**Figure 1A** and **1B**). Compared to normal tissue, LGA had genome wide hypo-methylation (P = 5.2x10-5, rank sum test) which was even lower in HGA (P = 3.7x10-6, compared with normal, rank sum test, **Figure 1C**). Methylation levels of all target sites in the array demonstrated the known bimodal distribution in normal, LGA, and HGA (**Figure 1D**), and the amount of fully methylated sites of lesions decreased with increasing degree of malignancy (right peak, **Figure 1D**; **Figure 1E**). Almost all DMSs in LGA compared to normal tissues kept at least an equivalent methylation level if not higher than in HGA and cancer (**Figure S1**). The 209 significantly hyper-methylated sites in LGA were further hyper-methylated in 22 HGA and 504 cancer samples collected from public databases (**Figure 1F and S2, Table S1**), and hypo-DMSs had a diametric tendency (**Figure S3**) suggesting that DNA demethylation may occur very early in precancerous lesions. Over 60% of DMRs that were observed in both LGA (71.4%, 314/440) and HGA (61.9%, 4,213/6,805) were hypo-methylated compared to normal tissues (**Figure 1G, Table S2-3**). However, with LGA as the reference, most DMRs observed in HGA were hyper-methylated (76.0%, 660/868) (**Figure 1G, Table S4**). In addition, there were limited overlaps between genes with DMRs in LGA compared to normal tissues and those compared to HGA, suggesting different epigenetic process(**Figure 1H**) [24].

#### Nervous system processes were associated with adenoma development

Enrichment analysis of 603 DMRs between HGA and LGA located on, and most highly enriched functional terms included the nervous system and those associated with signal transduction (**Figure 2A**), specifically dopaminergic synapse and serotonergic synapse pathways, which play a role in the gut-brain axis model of signaling cross-talk between organ systems [25]. These results correspond to gene methylation findings in **Figure 1G** where HGA vs Normal includes almost all genes that are listed in LGA vs Normal and HGA vs LGA DMRs. To figure out the potential function changes from LGA to HGA, Gene Ontology (GO) enrichment was performed for 275 genes that were significantly different in methylation status between LGA vs Normal and HGA vs Normal without considering the differences in methylation status between HGA vs LGA. 571 significantly different methylated genes were highlighted in HGA vs LGA and HGA vs Normal without LGA vs Normal (**Figure 2B**). For the 275 genes with significantly different methylation patterns in only the LGA vs Normal and HGA vs Normal comparisons, GO analysis selected the top enriched terms of proteolysis as well as extracellular matrix disassembly, inorganic anion transport, and cobalamin metabolic processes. Cell adhesion, positive regulation of positive chemotaxis, and neuropeptide signaling pathway were term hits on the overlapping part between LGA vs Normal and HGA vs LGA. Genes that were significantly different in methylation status between LGA and HGA were enriched for chemical synaptic transmission, transmission of nerve impulse, calcium ion transmembrane transport, and similar neural processing terms. Like the DMR enrichment analysis, terms related to the nervous system were selected yet exhibited different term patterns between HGA vs LGA compared to LGA vs Normal.

**Hyper-methylated CpG sites exhibited better discrimination between normal, pre-cancerous, and cancerous tissues than the hypo-methylated pattern for CRC**

To distinguish the discriminatory ability of DNA methylation patterns for normal tissue, CA, and CRC, we collected 833 genome-wide DNA methylation datasets from GEO and ArrayExpress, public datasets which included 278 normal tissue samples, 51 adenoma samples, and 504 cancer samples. We separated DMSs of LGA vs Normal into two groups including hyper-DMSs (209 sites) and hypo-DMSs (441 sites). We found both hyper-DMSs and hypo-DMSs could effectively distinguish methylation pattern differences between disease (adenoma and cancer) and normal samples (**Figure 3A** and **Figure 3B**). Meanwhile, we also conducted two machine learning-based predictions with the DMSs identified in our dataset and observed that hyper-methylated sites can better distinguish between normal samples and disease samples via random forest and neural network methods (**Table 1**). For hyper-methylated sites, the area under the curve (AUC) of receiver operating characteristic (ROC) curves were 0.91 and 0.85, respectively. For hypo-methylated sites, AUC of ROC curves were lower at 0.72 and 0.76, respectively (**Figure 3C** and **Figure 3D**). Unsupervised tSNE cluster analysis produced the same result (**Figure 3E** and **Figure 3F**). To avoid inconsistent results caused by unstable methylation based on single CpG sites, we compared the mean beta value (mBV) of these sites. We found that hyper-methylated mBVs were significantly different between normal tissue and CRC(P<2.2x10-16); however, there was no significant difference between the adenoma and cancer (P= 0.29, **Figure 3G**) in which the average mBV of the normal tissue, adenoma, and cancer are 0.22, 0.54, and 0.57, respectively. We observed similar results for hypo-methylation sites in which the average mBV of the normal tissue, adenoma, and cancer were 0.70, 0.44, and 0.50, respectively (**Figure 3G**). Finally, we found the AUC of ROC curves with hyper-mBV and hypo-mBV were 0.98 and 0.95, respectively. Permutation analysis based on a bootstrap strategy indicated that the model based on hyper-methylated sites had better discriminatory power than the model of hypo-methylated sites (P<2.2x10-8, **Figure 3H**).

#### The promoter of ADHFE1 may be a potential biomarker for colorectal adenoma and cancer

## Next, we grouped the DMRs of normal tissue and LGA into hyper- and hypo-DMRs and performed enrichment analysis by Ingenuity Pathway Analysis (IPA). The top enriched functional term for hyper DMRs was ethanol degradation II (P=5.4x10-3) which was mostly contributed by methylation sites on two genes, *ADHFE1* and *ACSS3*, which can facilitate the conversion from ethanol to acetaldehyde and from acetic acid to acetyl-CoA, respectively (**Figure 4A**). The expression of both genes were down-regulated in colonic and rectal cancer tissue compared with normal tissue (P<0.01), a result consistent with the DNA methylation changes between LGA and HGA (R2= -0.49 and -0.59, **Figure 4B** and **Figure 4C**). We found that the average methylation level of CpG sites located in CpG islands within the promoter regions of *ADHFE1* and *ACSS3* were significantly increased in cancer samples compared to normal samples (mBVs=0.2 and 0.18, respectively). We further analyzed the promoter region within the CpG island of the two genes to distinguish between normal and disease tissues. When setting the cutoff at 0.25 for the *ADHFE1* promoter, the minimal error rate was only 4.68% (39/833, **Figure 4D**); the heatmap of sites within the region reflected the same result (**Figure 4E**). ROC curve analysis of mBV of the *ADHFE1* promoter for all 833 samples produced an AUC of 0.97 with specificity and sensitivity at 0.95 and 0.96 (**Figure 4F**). For cancer samples, an AUC as high as 0.98 was determined (**Figure S4**). For *ACSS3*, the minimal error rate of its promoter was 16.68% (139/833) with a cutoff set at 0.42 (**Figure 4G**) which performed inferiorly to *ADHFE1* in terms of discrimination power. Meanwhile, we also compared *ADHFE1* with *SEPT9*, an FDA-approved methylation-based biomarker for CRC screening. The correlation of the two genes was 0.77 and we determined that *ADHFE1* had a better prediction power than *SEPT9* (**Figure 5A and S5**) [26]. Furthermore, we observed *ADHFE1* to have a much better separation boundary compared to *SEPT9* (**Figure 5B**). In view of most detected cfDNA being actually the fragments from white cells, we checked DNA methylation status of *ADHFE1* promoter in 656 whole blood cases from public data. As expected, all sites in the promoter were consistently at low methylation level (Figure S6). **Discussion**

Whole-genome DNA hypo-methylation and hyper-methylation analysis of the promoter regions of cancer-related genes are regarded as a common method of characterizing diverse cancers [27]. In our study, we found that whole-genome DNA hypo-methylation may start at the benign adenoma stage (LGA) and lead to further hypo-methylation at HGA and CRC (**Figure 1C**). As many previous studies have reported, a bimodal distribution can characterize DNA methylation pattern, and we noted that the hyper-methylated peak can clearly reflect progressive hypo-methylation (**Figure 1D** and **Figure 1E**)[28]. We identified 440 and 6,805 DMRs in low- and hyper-grade adenoma respectively, and of these DMRs, 314(71.4%) in LGA and 4,213 (61.9%) in HGA were hypo-methylated compared to normal tissues. On the contrary, most DMRs (660/868, 76.0%) differences between HGA and LGA were hyper-methylated. Aside from a little overlap between HGA genes, significantly distinct DMRs were located between LGA vs Normal and HGA vs LGA which indicates that LGA vs Normal and HGA vs LGA are possibly not the same process with a degree difference but two different epigenetic processes. These genome-wide demethylation patterns may indicate that though hypo-methylation dominates the carcinogenesis of CRC, hyper-methylation sites may contribute more to the distinct malignancy of these lesions.

To find functional differences between differing methylation patterns in normal, pre-cancerous, and cancerous tissues, enrichment analysis was applied to 603 genes with DMRs between HGA and LGA which determined that the most enriched terms were related to nervous system and signal transduction (**Figure 2A**). The term gut–brain-axis describes an integrative physiology concept that incorporates all, including afferent and efferent neural, endocrine, nutrient, and immunological signals, cross-talk between the central nervous system and the gastrointestinal system that may be dysregulated during carcinogenesis [25]. Our KEGG enrichment analysis further highlighted the significance of dopaminergic synapse and serotonergic synapse to CRC development. Serotonin (5-hydroxytryptamine, 5-HT) is popularized as a contributor to feelings of well-being and happiness though its actual biological function is complex and multifaceted with roles in modulating cognition, reward, learning, memory, and numerous physiological processes[29]. Brain 5-HT gets much more respect, and certainly more press and research, than the vastly larger store of 5-HT in the gut though both are important for physiological functions [30]. Dopamine (3,4-dihydroxyphenethylamine, DA) is an organic chemical of the catecholamine and phenethylamine families that functions both as a hormone and a neurotransmitter and plays several important roles in the brain and body [31]. In the brain, dopamine functions as a neurotransmitter to send signals to other nerve cells [31]. Outside the central nervous system, dopamine functions primarily as a local paracrine messenger to reduce gastrointestinal motility and protect the intestinal mucosa [31]. The interaction of tumor and the nervous system has also been found in gastric cancer and liver cancer [32, 33]. Our study suggests that the gut–brain-axis and related molecules may be important contributors to the development and progression of CRC even at the benign adenoma stage.

DNA methylation has always been considered as a potential biomarker for many diseases due to its tissue specificity and stability [34]. Here, we analyzed DNA methylation patterns as a mechanism to distinguish disease samples (including adenoma and cancer) from normal samples during CRC development. We identified 209 hyper-methylated sites and 441 hypo-methylated sites between LGA and normal samples and noted that both hyper- and hypo-methylated sites could effectively distinguish between normal and CRC tissues. Further validation with random forest and neural network analyses confirmed our observations. Specifically, AUCs of ROC curves for our prediction model using hyper-methylated sites were larger than those using hypo-methylated sites, despite the observation that hypo-methylated sites were more than twice the number of hyper-methylated ones. Since tumors are known to have whole-genome hypo-methylation, we speculate that gene hyper-methylation at several key sites and/or global hypo-methylation during early CA may be the driver events for CRC. To reduce bias caused by unstable methylation on single CpG sites, we compared mBV of these sites among tissue groups. We found that hyper-methylated mBVs were significantly different between normal tissue and cancers (P<2.2x10-16), while no significance was found between the adenoma and CRC (P= 0.288, **Figure 3G**). Permutation analysis based on bootstrap strategy suggest that the model based on hyper-methylated sites has better discrimination power than the model of hypo-methylated sites (P<2.2x10-8, **Figure 3H**) which may lend support to the theory that hyper-methylation at several key sites may trigger widespread hypo-methylation throughout the genome during cancer development.

Colorectal adenoma is considered the middle stage between normal status and cancer; therefore, our study focused on identifying and comparing the differences in DNA methylation patterns among normal, pre-cancerous, and cancerous colorectal tissues. IPA enrichment analysis of hyper-DMRs identified in very early stage cancers selected Ethanol degradation II as the top term for functional impact, in which *ADHFE1* and *ACSS3* were hit. Intense early changes in DNA methylation patterns at the promotor region of these genes supports their potential use as adenoma biomarker. It is known that *ADHFE1* encodes for hydroxyacid-oxoacid transhydrogenase which is responsible for the oxidation of 4-hydroxybutyrate in mammalian tissues [35]. Some studies have also reported that the gene is associated with cell proliferation and differentiation [35-37]. In CRC tissue, *ADHFE1* is hyper-methylated in the promoter region corresponding to down-regulation of expression that may facilitate tumor growth [37]. Our results suggest that the DNA methylation of the *ADHFE1* promoter is a potential biomarker for distinguishing colorectal adenoma and cancer from normal tissue.

As the only FDA-approved liquid biopsy marker for DNA methylation, *SEPT9* has been applied for colon cancers screening [38]. Actually the detection signal of *SEPT9* has been shown to be more distinguishable in tissues than at cfDNA samples [39]. The better performance of *ADHFE1* than *SEPT9* at tissue level made it a promising liquid biopsy biomarker for CRC. Further efforts with a larger, more diverse sample population is needed to validate the predictive efficacy of this biomarker at cfDNA.

In addition, a recent study found a promising biomarker cg10673833 which distinguished tumor patients from healthy people by cfDNA [40]. However, the methylation level of this marker showed only a slight upward trend from normal tissues to adenoma and cancer, in our samples as well as in public data. In view of the very low methylation of cg10673833 in blood, most likely its detection of cancer was mainly due to largely increased metabolism of the tumor tissue that caused increased shedding of ctDNA. Comparing with cg10673833, the better discrimination of normal to adenoma and cancer by *ADHFE1* raises a great potential for this candidate as a methylation marker to indicate pathological changes.

sides *ADHFE1,* we obtained a group of in oursamples. For their potential being candidates of methylation markers, we examined these sites ole from GEO.As shown in the of ,207 out of 209 sites showed their low methylation level as < 0.3 in average, implying the deserving further investigation for is.

## **Conclusions**

Adenoma samples are perfect proxy for colorectal carcinoma early biomarker identification. Our study focused on early benign adenoma, in order to get the earliest clue to detect colorectal disease. DNA methylation is a promising biomarker for cancer diagnosis and surveillance for its tissue specificity and robustness. We established the DNA methylation landscape of LGA and HGA and noted the hyper-methylated peak has a regular decrease companied with disease procession. Furthermore, we found the development of adenoma is associated with functions of nervous system, while the initiation of the adenoma is more associated with cell biological functions. Another relatively independent work was based on the precious finding in LGA, in which we found *ADHFE1* is a potential early diagnosis biomarker of colorectal carcinoma and adenoma. 833 samples from the public database strongly support the gene is a promising biomarker.

## **Methods**

**Sample collection and pathological confirmation**

In the Department of Gastroenterology of Peking University Third hospital from March 2015 to June 2016, we collected 18 LGA and 22 HGA specimens from patients who underwent endoscopic treatment for CA removal, and obtained adjacent normal tissue specimens from 20 patients with adenoma during the treatment. Tissue specimens were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and confirmed by pathologist by light microscopy. All the patients were drug treatment naive (no any treatment before their surgeries), except for one with chemo- and radio-therapies 8 months prior to his surgery. Clinical information of patients, and sample information and loading quantity are provided on **Table S5 and S6.**

**DNA isolation and bisulfite conversion**

DNA was isolated using QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s protocol. Bisulfite conversion was performed using the EZ DNA Methylation-Gold Kit according to the instruction manual (Zymo Research, Irvine, CA, USA).

**Methylation data processing**

Epigenome-wide DNA methylation assessment for this study was performed using the Illumina Infinium Human Methylation 450 BeadChip (Illumina, San Diego, CA, USA), which simultaneously profiles the methylation status for >485,000 CpG sites at single-nucleotide resolution and covers 96% of CpG islands with additional coverage of island shores (<2 Kb from CpG Islands), island shelves (2–4 Kb from CpG islands), and regions flanking them. The raw data from the array was processed using The GenomeStudio Methylation (version 1.8, Illumina) module which calculated methylation levels. The GenomeStudio is the official software for array data processing of Illumina, which integrates data normalization, background adjustment. And mehthylation calculation. Normalization was performed by comparing with control probes when set the option as controls, and background adjustment was performed automatically by the software selecting Subtract Background. Probes located on sex chromosomes or failed detection P value testing of at least one sample or SNP (single nucleotide polymorphism), were removed from analysis using R package IMA (vision 3.1.2)[41]. DMRs were defined as rank sum test following false discovery rate (FDR) adjusted P value<0.05 and |β|>0.15, and DMSs were defined as rank sum test following FDR adjusted P value<0.05 and |β|>0.20. Promoter regions were defined as 5′UTR, TSS200, TSS1500 and first exons.

**Public data collection and processing**

To ensure consistency of data processing, we only compared our samples with publically accessible samples with raw *idat* files. GSE68060, GSE68838, GSE77954, GSE77965, GSE81211, GSE101764, GSE107352 and GSE75546 were collected from GEO while E-MTAB-6450 was collected from ArrayExpress [42-47] (**Table S6**). Some cell line samples and metastatic cancer samples were removed upon further study. In total, we collected 278 normal samples, 51 adenoma samples, and 504 cancer samples. All datasets using raw *idat* files were preprocessed using R package minfi (vision 1.28.4) [48]. The sites which failed detection at P = 0.01 were rewrote by nearest neighbor average to ensure an adequate number of sites for analysis. 656 white blood data was collect from GEO (accession number GSE40279).

**Comparison of the ability of discrimination between normal, LGA, HGA, and CRC tissue**

For random forest prediction, we used R package randomForest (vision 4.6.14) with the number of trees set at 5,000 [49]. For neural network prediction, we used R package nnet (vision 7.3.12) with number of units in the hidden layer as 2, weight decay as 10-4, and with a maximum number of iterations at 400 [50]. The R package pROC (vision 1.14.0) was used for ROC analysis to compare the abilities of various models to distinguish between hyper- and hypo-methylated sites by the area under the curve (AUC) analysis [51].

**t-SNE analysis, PCA analysis and Gene Enrichment analysis**

tSNE analysis was performed by R package tsne (vision 0.1-3) [52]. PCA was performed by R function princomp and visualized by first two principal components. KEGG and GO enrichment were analyzed online by DAVID 6.8 (<https://david.ncifcrf.gov>) [53, 54]. Ingenuity Pathway Analysis (IPA) was also used for enrichment analysis for more elaborate results with the P value cutoff set at 0.05 [55].

## **List of abbreviations**

LGA: Low-grade adenoma

HGA: High-grade adenoma

LGA vs Normal: Comparison of low-grade adenoma with normal tissue

HGA vs Normal: Comparison of high-grade adenoma with normal tissue

HGA VS LGA: Comparison of high-grade adenoma with low-grade adenoma

DMR: Different methylation region

DMS: Different methylation site

ROC: Receiver operating characteristic

AUC: Area under the curve

IPA: Ingenuity Pathway Analysis

KEGG: Kyoto Encyclopedia of Genes and Genomes

GO: Gene Ontology

t-SNE: t-distributed stochastic neighbor embedding

PCA: Principal components analysis

mBV: Mean beta values

FDR: False discovery rate

SNP: Single nucleotide polymorphism

UTR5: 5′ untranslated region

CHR: Chromosome

## **Declarations**

**Ethics approval and consent to participate**

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Peking University Third hospital (IRB number: 206H005). Informed written consent was obtained from all patients and volunteers prior to the procedure.

**Consent for publication**

Not applicable

**Availability of data and materials**

All methylation array data are available at GEO under accession number GSE139404. Other public data involved in this study included GSE68060, GSE68838, GSE77954, GSE77965, GSE81211, GSE101764, GSE107352, GSE75546, GSE40279, and E-MTAB-6450.

**Competing Interests**

The authors disclose no potential competing interests.

**Funding**

This study is funded by Youth Innovation Promotion Association CAS (2016098), Major State Basic Research Development Program (2014CB542006), Key Research Program of the Chinese Academy of Sciences (KJZD-EW-L14), and National Key Research and Development Plan of China (2016YFA0201404).

**Authors’ Contributions**

JF developed the analysis method and drafted the manuscript. JL recruited patients, provided clinical expertise on colorectal adenomas, and interpreted the results. JF, HZ, and SG performed data analysis. SG, DZ reviewed and edited the manuscript and provided respective scientific expertise in result interpretation. CT and YZ conducted array experiments. WW collected and prepared tissue samples and collected the results of clinical assays. SD funded and supervised all clinical procedures for enrolled patients. DZ and CZ designed the study, supervised all experiments and analysis, provided molecular and cellular biology advice, and reviewed and edited the manuscript.

**Acknowledgements**

The authors gratefully acknowledge Dr. Steven J Schrodi, Dr. Emily A. Andreae and Dr. Ingrid Glurich from Center for Precision Medicine Research (CPMR), Marshfield Clinic Research Institute (MCRI) for reviewing, commenting and editing their manuscript.

## **Reference**

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2018. CA Cancer J Clin 2018;68:7-30.

2. Chen W, Zheng R, Baade PD, et al. Cancer statistics in China, 2015. CA Cancer J Clin 2016;66:115-32.

3. Kuipers EJ, Grady WM, Lieberman D, et al. Colorectal cancer. Nat Rev Dis Primers 2015;1:15065.

4. Guo S, Diep D, Plongthongkum N, et al. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. Nat Genet 2017;49:635-42.

5. Wang X, Wang L, Guo S, et al. Hypermethylation reduces expression of tumor-suppressor PLZF and regulates proliferation and apoptosis in non-small-cell lung cancers. FASEB J 2013;27:4194-203.

6. Guo S, Yan F, Xu J, et al. Identification and validation of the methylation biomarkers of non-small cell lung cancer (NSCLC). Clin Epigenetics 2015;7:3.

7. Zhao Y, Xue F, Sun J, et al. Genome-wide methylation profiling of the different stages of hepatitis B virus-related hepatocellular carcinoma development in plasma cell-free DNA reveals potential biomarkers for early detection and high-risk monitoring of hepatocellular carcinoma. Clin Epigenetics 2014;6:30.

8. Haikun Zhang PD, Shicheng Guo, Chengcheng Tao, Wenmin Zhao, Jiakang Wang, Ramsey Cheung, Augusto Vilanueva, Huiguo Ding, Steven J. Schrodi, Dake Zhang, Changqing Zeng. Circulating cell-free DNA based low-pass genome-wide bisulfite sequencing aids non-invasive surveillance to Hepatocellular carcinoma. Science Advance (Submitted) 2019.

9. Patai AV, Molnár B, Kalmár A, et al. Role of DNA methylation in colorectal carcinogenesis. Dig Dis 2012;30:310-5.

10. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology 2008;135:1079-99.

11. Hidaka H, Higashimoto K, Aoki S, et al. Comprehensive methylation analysis of imprinting-associated differentially methylated regions in colorectal cancer. Clinical epigenetics 2018;10:150-.

12. Shi YX, Wang Y, Li X, et al. Genome-wide DNA methylation profiling reveals novel epigenetic signatures in squamous cell lung cancer. BMC Genomics 2017;18:901.

13. Lindqvist BM, Wingren S, Motlagh PB, et al. Whole genome DNA methylation signature of HER2-positive breast cancer. Epigenetics 2014;9:1149-62.

14. Raggi C, Invernizzi P. Methylation and liver cancer. Clin Res Hepatol Gastroenterol 2013;37:564-71.

15. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. Nat Rev Genet 2012;13:484-92.

16. Morris MR, Latif F. The epigenetic landscape of renal cancer. Nat Rev Nephrol 2017;13:47-60.

17. Herman JG, Merlo A, Mao L, et al. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. Cancer Research 1995;55:4525.

18. Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Research 1997;57:808.

19. Yoshiura K, Kanai Y, Ochiai A, et al. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. Proceedings of the National Academy of Sciences 1995;92:7416.

20. Witold K, Anna K, Maciej T, et al. Adenomas - Genetic factors in colorectal cancer prevention. Rep Pract Oncol Radiother 2018;23:75-83.

21. Zauber AG, Winawer SJ, O'Brien MJ, et al. Colonoscopic polypectomy and long-term prevention of colorectal-cancer deaths. N Engl J Med 2012;366:687-96.

22. Schlemper RJ, Riddell RH, Kato Y, et al. The Vienna classification of gastrointestinal epithelial neoplasia. Gut 2000;47:251-5.

23. Rex DK, Johnson DA, Anderson JC, et al. American College of Gastroenterology guidelines for colorectal cancer screening 2009 [corrected]. Am J Gastroenterol 2009;104:739-50.

24. Perez-Silva JG, Araujo-Voces M, Quesada V. nVenn: generalized, quasi-proportional Venn and Euler diagrams. Bioinformatics 2018;34:2322-4.

25. Clemmensen C, Muller TD, Woods SC, et al. Gut-Brain Cross-Talk in Metabolic Control. Cell 2017;168:758-74.

26. Church TR, Wandell M, Lofton-Day C, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut 2014;63:317-25.

27. Kulis M, Esteller M. DNA methylation and cancer. Adv Genet 2010;70:27-56.

28. Straussman R, Nejman D, Roberts D, et al. Developmental programming of CpG island methylation profiles in the human genome. Nat Struct Mol Biol 2009;16:564-71.

29. Swami T, Weber HC. Updates on the biology of serotonin and tryptophan hydroxylase. Curr Opin Endocrinol Diabetes Obes 2018;25:12-21.

30. Xiaolong G, Junhai P, Yichang L, et al. Intestinal Crosstalk between Microbiota and Serotonin and its Impact on Gut Motility. Current Pharmaceutical Biotechnology 2018;19:190-5.

31. Berke JD. What does dopamine mean? Nat Neurosci 2018;21:787-93.

32. Jeong S, Zheng B, Wang H, et al. Nervous system and primary liver cancer. Biochim Biophys Acta Rev Cancer 2018;1869:286-92.

33. Wang K, Zhao XH, Liu J, et al. Nervous system and gastric cancer. Biochim Biophys Acta Rev Cancer 2020;1873:188313.

34. Pan Y, Liu G, Zhou F, et al. DNA methylation profiles in cancer diagnosis and therapeutics. Clin Exp Med 2018;18:1-14.

35. Deng Y, Wang Z, Gu S, et al. Cloning and characterization of a novel human alcohol dehydrogenase gene (ADHFe1). DNA Seq 2002;13:301-6.

36. Moon JW, Lee SK, Lee YW, et al. Alcohol induces cell proliferation via hypermethylation of ADHFE1 in colorectal cancer cells. BMC Cancer 2014;14:377.

37. Tae CH, Ryu KJ, Kim SH, et al. Alcohol dehydrogenase, iron containing, 1 promoter hypermethylation associated with colorectal cancer differentiation. BMC Cancer 2013;13:142.

38. Tóth K, Sipos F, Kalmár A, et al. Detection of methylated SEPT9 in plasma is a reliable screening method for both left- and right-sided colon cancers. PLoS One 2012;7:e46000.

39. Tóth K, Wasserkort R, Sipos F, et al. Detection of methylated septin 9 in tissue and plasma of colorectal patients with neoplasia and the relationship to the amount of circulating cell-free DNA. PLoS One 2014;9:e115415.

40. Luo H, Zhao Q, Wei W, et al. Circulating tumor DNA methylation profiles enable early diagnosis, prognosis prediction, and screening for colorectal cancer. Sci Transl Med 2020;12.

41. Wang D, Yan L, Hu Q, et al. IMA: an R package for high-throughput analysis of Illumina's 450K Infinium methylation data. Bioinformatics 2012;28:729-30.

42. Qu X, Sandmann T, Frierson H, Jr., et al. Integrated genomic analysis of colorectal cancer progression reveals activation of EGFR through demethylation of the EREG promoter. Oncogene 2016;35:6403-15.

43. consortium B. Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. Nat Biotechnol 2016;34:726-37.

44. Kang K, Bae JH, Han K, et al. A Genome-Wide Methylation Approach Identifies a New Hypermethylated Gene Panel in Ulcerative Colitis. Int J Mol Sci 2016;17.

45. Barrow TM, Klett H, Toth R, et al. Smoking is associated with hypermethylation of the APC 1A promoter in colorectal cancer: the ColoCare Study. Journal of Pathology 2017;243:366-75.

46. Damaso E, Castillejo A, Arias MDM, et al. Primary constitutional MLH1 epimutations: a focal epigenetic event. Br J Cancer 2018;119:978-87.

47. Bormann F, Rodriguez-Paredes M, Lasitschka F, et al. Cell-of-Origin DNA Methylation Signatures Are Maintained during Colorectal Carcinogenesis. Cell Rep 2018;23:3407-18.

48. Aryee MJ, Jaffe AE, Corrada-Bravo H, et al. Minfi: a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays. Bioinformatics 2014;30:1363-9.

49. Wiener ALaM. Classification and Regression by randomForest. R News 2002;2:18-22.

50. Ripley WNVaBD. Modern Applied Statistics with S. Fourth ed. New York: Springer; 2002.

51. Robin X, Turck N, Hainard A, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.

52. Hinton GE. Visualizing High-Dimensional Data Using t-SNE. Journal of Machine Learning Research 2008;9:2579-605.

53. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009;4:44-57.

54. Huang DW, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic acids research 2009;37:1-13.

55. Kramer A, Green J, Pollard J, Jr., et al. Causal analysis approaches in Ingenuity Pathway Analysis. Bioinformatics 2014;30:523-30.

## **Figures and table**

Figure 1. Genome-wide DNA methylation of low-grade adenoma (LGA), high-grade colorectal adenoma (HGA) and normal colorectal tissue. (A): tSNE analysis highlights the data structure and sample relationship among the sample groups. (B): PCA analysis confirms the data structure and sample relationship of the tSNE analysis. (C): Average methylation levels of Normal (N), LGA, and HGA samples. (D): Density plot reveals the distribution of the whole array probes for N, LGA, and HGA samples. (E): Number of sites in β ranging from 0.7 to 0.9. (F): Heatmap of the 209 hyper-methylated DMSs of in-house datasets and samples from 504 public cancer datasets. (G): DMR between LGA and normal tissues, HGA and normal tissue, and HGA and LGA. (H): Venn graph highlights the relationships among all DMRs.

Figure 2. Enrichment analysis shows the top 5-10 terms associated with methylation differences between LGA and HGA. (A) GO and KEGG analysis of the genes with DMRs associated with LGA and HGA. (B) GO analysis of the genes with alterations in DMRs including differences in DMRs only in HGA vs LGA, only in LGA vs Normal, and areas where HGA vs LGA and LGA vs Normal overlapped.

Figure 3. Hyper-methylated CpG sites showed better diagnostic performance than the hypo-methylated pattern. (A): Cluster analysis based on hyper-DMSs among normal, adenoma and cancer samples. (B): Cluster analysis based on hypo-DMSs among normal, adenoma and cancer samples. (C): Random forest prediction performance based on hyper- and hypo-DMSs. (D): Neural network prediction performance based on hyper- and hypo-DMSs. (E): tSNE analysis highlights the data structure and sample relationship based on hyper-DMSs. (F): tSNE analysis highlights the data structure and sample relationship based on hypo-DMSs. (G): Average methylation level of hyper- and hypo-DMSs (H): ROC curve of hyper-mBV and hypo-mBV.

Figure 4. DNA methylation *ADHFE1* and *ACSS3* in Normal, Adenoma, and Cancer. (A): Pathway of ethanol degradation II [55]. (B): Relationship between DNA methylation and gene expression of *ADHFE1*. (C): Relationship between DNA methylation and gene expression of *ACSS3*. (D): Left panel is identification of cutoff where the X axis is sample number of classification error; Right panel is DNA methylation of *ADHFE1* in normal, adenoma, and cancer samples. (E): Heatmap of sites within *ADHFE1* promoter in normal, adenoma and cancer samples. (F): ROC of the prediction of *ADHFE1* for colorectal adenoma and cancer. (G): DNA methylation of *ACSS3* in normal, adenoma, and cancer samples.

Figure 5. Comparison of *ADHFE1* with *SEPT9.* (A): ROC comparison of *ADHFE1* and *SEPT9*. (B): DNA methylation of *SEPT9* in normal, adenoma, and cancer samples.

Table 1. Prediction performance based on hyper-DMS and hypo-DMS to distinguish between disease and normal colorectal tissues.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Model | Methylation | Observation | Prediction | | Sensitivity | Specificity |
| Disease | Normal |
| Random Forest | hyper | Disease | 532 | 23 | 0.959 | 0.860 |
| Normal | 39 | 239 |
| hypo | Disease | 507 | 48 | 0.914 | 0.601 |
| Normal | 111 | 167 |
| Neural Network | hyper | Disease | 537 | 18 | 0.968 | 0.727 |
| Normal | 76 | 202 |
| hypo | Disease | 406 | 149 | 0.732 | 0.701 |
| Normal | 83 | 195 |