Tumor-induced DNA methylation in the white blood cells of patients with colorectal cancer

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Abstract. The secretions of cancer cells alter epigenetic regulation in cancer stromal cells. The present study investigated the methylation changes in white blood cells (WBCs) caused by the secretions of colorectal cancer (CRC) cells. Changes in the DNA methylation of peripheral blood mononuclear cells (PBMCs) from normal individuals co-cultured with CRC cells were estimated using a methylation microarray. These changes were then compared against the DNA methylation changes and mRNA levels observed in the WBCs of patients with CRC. Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1 (*PLOD1)* and matrix metalloproteinase 9 (*MMP9*) were selected to assess the DNA methylation of the WBCs from CRC patients using real-time methylation-specific PCR. The majority of the genes analyzed presented high levels of mRNA in the WBCs of the patients with CRC and DNA methylation in the co-cultured PBMCs. Intragenic methylation revealed the strongest association ($P=8.52x10^{-21}$). For validation, *MMP9* and *PLOD1* were selected and used to test WBCs from 32 patients with CRC and 57 normal controls. The intragenic *MMP9* methylation was commonly found ($P<0.0001$) with high sensitivity (90.63%) and high specificity (96.49%), and a positive predictive value of 93.33% and a negative predictive value of 93.22%. *PLOD1* methylation was revealed to have lower sensitivity (30.00%) but higher specificity (97.92%). In addition to circulating WBCs, MMP9 protein expression was observed in infiltrating WBCs and the metastatic lymph nodes of patients with CRC. In conclusion, CRC cells secrete factors that induce genome wide DNA methylation changes in the WBCs of patients with CRC. These changes, including intragenic *MMP9* methylation in WBCs, are promising CRC biomarkers to be tested in future CRC screening studies.

Introduction

Several epigenetic biomarkers in peripheral blood and circulating cell-free DNA in serum and plasma have been reported for cancer diagnosis. The DNA methylation biomarkers of various cancers include: *Adenomatous polyposis coli protein (APC), glutathione S-transferase Pi 1 (GSTP1), Ras association domain family member 1A (RASSF1A)*, and *retinoic acid receptor (RAR)-β2* for breast cancer; *BRCA1, HIC ZBTB transcriptional repressor 1, paired box 5, progesterone receptor*, and *thrombospondin 1* for ovarian cancer; *Cyclin D2, plasminogen activator urokinase, suppressor of cytokine signaling 1, thrombospondin*, and *von Hippel-Lindau tumor suppressor* for pancreatic cancer; *GSTP1, RASSF1*, and *RARB2* for prostate cancer; *P15, P16*, and *RASSF1A* for hepatocellular carcinoma; and *APC, O-6-methylguanine-DNA methyltransferase, RASSF2A, WNT inhibitory factor*-1, and Septin 9 (*SEPT9)* for colorectal cancer (CRC). All of the reported biomarkers exhibit different sensitivities and specificities for cancer detection (1). Furthermore, in colon, liver, lung, and nasopharynx cancers, significant DNA hypomethylation of long interspersed nucleotide element-1 (LINE-1) has been reported (2). Each candidate gene has been investigated using appropriate methods; PCR and pyrosequencing are suitable for quantitatively measuring DNA methylation levels. Typically, hypermethylation and hypomethylation are

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Abbreviations: CRC, colorectal cancer; WBCs, white blood cells; PBMCs, peripheral blood mononuclear cells; MSP, methylation-specific PCR; LINE-1, long interspersed nucleotide element-1; CU-DREAM, connection up- and downregulation expression analysis of microarrays; FFPE, formalin-fixed paraffin-embedded; H&E, hematoxylin and eosin; PPV, positive predictive value; NPV, negative predictive value

Key words: CRC, WBCs, matrix metalloproteinase 9 methylation

measured at specific loci from the promoter regions. However, the molecular mechanism underlying DNA methylation changes in circulating cells, cancer cells or white blood cells (WBCs), has not been determined.

In a previous study, we reported LINE-1 hypermethylation in micrometastatic lymph nodes and the surrounding cells in patients with breast cancer. Secretions from breast cancer cells were shown to increase LINE-1 methylation in cancer stromal cells (3). Therefore, we hypothesize that cancer secretions can alter the DNA methylation of circulating WBCs.

In CRC, cancer cells grow uncontrollably in the colon or rectal area (4). The majority of CRC develops from the inner lining of organs called polyps, with ~5% of polyps becoming cancerous (5,6). CRC is the third most common cancer and the fourth leading cause of death worldwide (7). The majority of cases of CRC occur in patients >50 years of age (8). In addition, several factors can increase the risk of CRC, including diet, obesity, lack of physical activity, alcohol consumption, tobacco use, family history of CRC, and hereditary conditions (9). Currently, the gold standard tool for CRC screening is colonoscopy, which has high sensitivity for the detection of CRC (10).

In the present study, whether CRC secretions cause methylation changes in the circulating WBCs of patients with CRC was examined. Connection up- and downregulation expression analysis of microarrays (CU-DREAM) (11) was used to compare the methylation profile of normal control peripheral blood mononuclear cells (PBMCs) after co-culturing with CRC cell lines and the RNA expression profiles of WBCs from patients with CRC. Methylation changes in the candidate genes in the WBCs of patients with CRC were evaluated using real-time methylation-specific PCR (RT-MSP).

Materials and methods

Ethical statement. The present study was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University (IRB no. 326/60). All samples were obtained from patients diagnosed with CRC between January 2016 and January 2018 in Chulalongkorn Memorial Hospital. All study subjects provided written informed consent.

Blood samples and formalin-fixed paraffin-embedded (FFPE) tissue. The blood samples for the co-culture model were obtained from 3 healthy males and 2 healthy females with no immune disorders or chronic diseases. The blood samples for the methylation tests were obtained from 32 CRC patients and 57 normal controls. Various FFPE tissues were examined in the present study by immunohistochemical staining using anti-matrix metalloproteinase (MMP)-9 antibodies; the tissue samples were comprised of normal colon biopsies (5 cases), tumor colon biopsies (5 cases) and a complete metastatic lymph node (1 case). Normal controls and CRC patients were investigated directly by colonoscopy. The biopsies were stained with hematoxylin and eosin (H&E) for histopathological confirmation of the components by a pathologist.

PBMC isolation. Firstly, whole blood was diluted with an equal amount of 1X phosphate-buffered saline (PBS). The diluted blood solution was placed on top of Ficoll-Paque gradient media (GE Healthcare Bio-Sciences) in tubes for centrifugation at room temperature for 20 min at 1,020 x g. Next, the PBMCs were carefully collected from the interface layer between the blood plasma and Ficoll solution. The collected PBMCs were washed with 1X PBS and then centrifuged at 640 x g twice, and were then resuspended in freezing media [10% DMSO in fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.)] at a concentration of $1x10^7$ PBMCs/ml (12).

Cell lines and co-culture conditions. The following human CRC cell lines were obtained from the American Type Culture Collection (ATCC): SW480 (ATCC® CCL-228™), representing early-stage CRC in a 50-year-old male and HT29 (ATCC® HTB-38™), representing early-stage CRC in a 44-year-old female. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 0.1 mg/ml streptomycin, and 100 U/ml penicillin at 37˚C in a humidified atmosphere (95% air: 5% CO_2). The cell lines were grown in 25 CC culture flasks (Corning Inc.) and were harvested at 80% confluence with 0.05% trypsin. The cell lines were washed with 1X PBS.

The co-culture technique was used to study the effects of substances released from CRC cells and PBMCs. Cancer cells and PBMCs were co-cultured in Transwell® culture plates (Costar; Corning Inc.). CRC cells were seeded in 24-well culture plates ($5x10^4$ cells/well), which were then filled with medium (DMEM with 10% FBS) and incubated in a CO_2 incubator at 37˚C in a humidified atmosphere for 24 h. PBMCs were co-cultured with CRC cells in permanent membrane cultures ($1x10^5$ cells/well), which were incubated for 4 h in a CO_2 incubator at 37˚C in a humidified atmosphere before harvesting for DNA extraction.

DNA extraction. In the present study, genomic DNA was extracted from two sample types: Co-cultured PBMCs and blood samples. The co-cultured PBMCs were centrifuged at 700 x g for 15 min and the supernatant was discarded. The PBMC pellets were then washed with 1X PBS and centrifuged 700 x g for 15 min. The blood samples were added to an equal amount of red blood cell lysis buffer to lyse the red blood cells, then centrifuged at 700 x g for 15 min; the resulting supernatant was discarded. The WBC pellets were washed with 1X PBS and centrifuged at 700 x g for 15 min twice. All cell pellets were supplemented with 500 μl of extraction buffer containing 10% SDS and proteinase K 0.5 mg/ml. Then, the cell solutions were mixed together and incubated for 72 h in a water bath at 50˚C. The DNA was extracted using a phenol: Chloroform: Isoamyl alcohol (25:24:1) solution and the mixtures were centrifuged at 4˚C and 14,000 x g for 15 min. The upper aqueous phase was carefully collected and transferred to a fresh tube, and then absolute ethanol was added for DNA precipitation before centrifugation at 4˚C and 14,000 x g for 15 min. The supernatant was discarded and the DNA was washed with 70% ethanol at 4˚C then centrifuged at 14,000 x g for 15 min twice. The obtained DNA was air-dried and then dissolved in distilled water. DNA samples were converted by sodium bisulfite treatment using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp.) according to the manufacturer's instructions.

Methylation microarray. The genome-wide DNA methylation profiling of the co-cultured PBMCs was carried out using

the recently developed Illumina Infinium MethylationEPIC BeadChip (Illumina, Inc.), which accesses the DNA methylation profile across ~850,000 CpGs. Samples in the present study included duplicate female control PBMCs and duplicate male control PBMCs. The duplicate female PBMCs were co-cultured with HT29 cells and the duplicate male PBMCs were co-cultured with SW480 cells. The methylation profiling obtained in the present study is available in the Gene Expression Omnibus (GEO) database under the reference, GSE110274.

Retrieval of data from GenBank. Gene expression profiles obtained by microarray were obtained from GEO datasets (www.ncbi.nlm.nih.gov/gds) using keywords including 'colorectal cancer' and 'blood'. GSE11545 (Applied Biosystems; Thermo Fisher Scientific, Inc.) (13) and GSE10715 (Affymetrix; Thermo Fisher Scientific, Inc.) (14) were used to represent the expression profiling of peripheral blood samples. GSE110274 (Illumina, Inc.) is the methylation profile obtained from the present experiment with co-cultured PBMCs.

CU-DREAM. In the present study, gene expression probes were always in genes and were tagged with their host genes. Unlike the expression probes, some methylation probes were in genes, while some methylation probes were not. The methylation probes were classified by their location. Intragenic probes were in genes and upstream probes were located 5,000 bp upstream of genes. All intragenic and upstream probes were tagged with their host genes. A comparison between experimental and control groups resulted in upregulated (up) or downregulated (dn) or 'neural' at every probes. A gene was said to be 'up' if there was at least a single probe in which the level of the experimental group was significantly higher (Student's t-test). The association between gene expression and methylation was identified by means of 2x2 contingency tables. A table consisted of 4 numbers denoted by a, b, c and d. The first and the second rows were up- or down-methylated genes and the rest, respectively. The first and the second columns were up- or down-expressed genes and the rest, respectively. Each 2x2 contingency table produced odds ratios (ORs) and Chi-square P-values (11).

RT-MSP of MMP9 and procollagen-lysine,2-oxoglutarate 5-dioxygenase 1 (PLOD1). The present study examined the methylation status of genes including *MMP9* and *PLOD1* in the WBCs from patients with CRC and normal controls using RT-MSP. The PCR Master Mix was prepared using PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, Inc.) with primer sets specific for methylated bisulfite-modified DNA for the quantification of methylation levels and unmethylated bisulfite-modified DNA as an internal control. The PCR reactions of *MMP9* and *PLOD1* were quantified using Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Inc.). For each gene, the primers were designed from the CpG position in the intragenic region. The primer sequences for *MMP9* amplification were: Forward methylated primer, 5'-TTGTTATTTTTTTTTTATTTTCGAGGGTC-3' and reverse methylated primer, 5'-GTAATACTACACCAAAACAAACCG-3'; forward unmethylated primer, 5'-GTTTGTTATTTTTTTTTTATTTTTGAGGGTT-3' and reverse unmethylated primer, 5'-TTTTTCATAATACTACACCAAAACAAACCA-3'. The thermocycling conditions for *MMP9* were as follows: 45 cycles of 95˚C for 45 sec and 54˚C for 45 sec. The primer sequences for *PLOD1* amplification were: Forward methylated primer, 5'-TAAAAGGTTATTTGATTTTTGATGTC-3' and reverse methylated primer, 5'-TCAAAAAAACAAAAAAACCTACG-3'; forward unmethylated primer, 5'-TTGTAAAAGGTTATTTGATTTTTGATGTT-3' and reverse unmethylated primer, 5'-TTTTTCTCAAAAAAACAAAAAAACCTACA-3'. The thermocycling conditions for *PLOD1* were as follows: 45 cycles of 95˚C for 45 sec and 54˚C for 45 sec.

The DNA methylation levels for each sample were measured and compared to standard curves for methylated *MMP9* and *PLOD1*. Firstly, the standard curves were created using completely methylated bisulfite converted DNAs (Qiagen, Inc.) diluted to give DNA concentrations of 10, 1, 0.1 and 0.01 ng/μl, respectively. The diluted DNA samples were then subjected to RT-MSP, and the *MMP9* and *PLOD1* methylation levels were calculated from the obtained Cq values. The formulae for the standard curves for methylated *MMP9* and *PLOD1* were $y=3x10^{28}xX^{-20.22}$ (Fig. S1) and $y=4x10^{24}xX^{-17.09}$ (Fig. S2), respectively. Thus, the Cq values for each sample can be measured for the absolute quantification of DNA methylation levels (15).

Immunohistochemistry. FFPE tissue samples were embedded in paraffin according to the protocol used in the Surgical Pathology Department. Sections (3 μm) were fixed in formalin solution and dehydrated in alcohol solution. The sections were then incubated with rabbit polyclonal anti-MMP9 (dilution 1:500; cat. no. HPA001238; Sigma-Aldrich; Merck KGaA) for 1 h for detection. Immunohistochemical staining was performed using an ultraView Universal 3,3'-diaminobenzidine tetrahydrochloride (DAB) Detection kit (Roche Diagnostics) containing horseradish peroxide and DAB, followed by counterstaining with hematoxylin on a BenchMark XT (Roche Diagnostics) automated system. The positive WBCs can be visualized by a brown colored precipitate.

Statistical analysis. All statistical analyses were performed using SPSS for Windows, version 17.0 (SPSS Inc.). The mean and the standard error of the mean (SEM) were calculated and Student's t-tests were performed to determine significant differences in methylation changes in the WBCs from patients with CRC and normal controls. The P-values obtained were two-sided and $P<0.05$ was considered to indicate a statistically significant difference. Receiver operating characteristic (ROC) curve analysis was carried out to differentiate the capacities of *MMP9* and *PLOD1* for methylation changes between patients with CRC and normal controls.

Results

Bioinformatics analysis of DNA methylation and mRNA array profiling. The flowchart in Fig. 1 illustrates the process followed in the present study. The methylation profile of Group 1 (GSE110274) from co-cultured PBMCs and the RNA expression profiles of Groups 2 and 3 (GSE11545 and GSE10715) from peripheral blood samples were interpreted to identify the

genes in common using CU-DREAM analysis. The candidate genes were hypermethylated and upregulated genes from group (a) with a P<0.01 and an OR of >1. The results of the analysis are presented in Tables I and SI, along with the P-values, ORs, and 95% confidence intervals (95% CIs). Genes from the PBMCs that were methylated at intragenic locations as a result of co-culturing with CRC cell lines were found to be upregulated in the WBCs of CRC patients ($P<3x10^{-14}$). An association between the DNA methylation of upstream locations and upregulation was also observed ($P<4x10^{-3}$).

The candidate genes were selected from overlapping genes from the 3 data groups and were classified by biological process based on gene ontology, as shown in Table II. The validation genes were selected based on gene function, biological process, and the reported literature. *PLOD1* was selected as a validation gene from genes overlapping the 3 data groups: GSE110274, GSE11545, and GSE10715. *MMP9* was selected as a validation gene from the two data groups: GSE110274 and GSE10715.

MMP9 and PLOD1 methylation test by RT-MSP. MMP9 and *PLOD1* were selected to validate the DNA methylation of the WBCs from 32 CRC patients (Table SII) and 57 normal controls by RT-MSP. The present study measured the Cq values of each sample and then calculated the absolute methylation levels from the standard curves derived from the Cq values of the diluted methylated DNA. The concentration of methylated *MMP9* of the samples ranged from 0.0004 to 3.5088 ng/μl. The mean and SEM of the methylated *MMP9* levels from the CRC patients and normal controls were 0.7226±0.1369 and 0.0487±0.0061 ng/μl, respectively (P<0.0001), as shown in Fig. 2A. The comparison of methylated *MMP9* levels in CRC patients and normal controls revealed high sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 90.63, 96.49, 93.33 and 93.22%, respectively, with a cut-off at 0.1445 and area under the ROC curve of 0.9846, as shown in Fig. 2C. Furthermore, the concentration of methylated *PLOD1* of the samples ranged from 0.0044 to 3.4091 ng/μl. The mean and SEM levels of methylated *PLOD1* from the CRC patients and normal controls were 0.4204±0.1259 and 0.0967±0.0131 ng/μl, respectively (P=0.0019), as shown in Fig. 2B. The examination of methylated *PLOD1* revealed low sensitivity and high specificity values of 30.00 and 97.92%, respectively, with a cut-off at 0.3301 and area under the ROC curve of 0.7167, as shown in Fig. 2D.

Immunohistochemical staining differentiates colorectal tissue. The present study validated the expression of MMP9 in WBCs via the immunohistochemical staining of CRC samples. The samples were stained with H&E and immunostained using anti-MMP-9 antibody. The normal epithelial area of CRC patients showed a low number of MMP9-positive WBCs (Fig. 3A). A large number of MMP9-positive WBCs were observed to infiltrate the WBCs of CRC patients (Fig. 3B). The metastatic lymph node from a late-stage CRC patient also showed a large number of MMP9-positive WBCs in the lymph node containing CRC metastases (Fig. 3C).

Discussion

DNA methylation in circulating cells can be derived from two cell types: Circulating tumor cells and WBCs (16). The

Table I. Connection up- and down- regulation expression analysis of microarrays of DNA methylation changes (GSE110274) and mRNA levels in the white blood cells of patients with colorectal cancer (GSE11545 and GSE10715).

Experiments	MET+, RNA+	MET+, RNA-	MET-, RNA+	MET-, RNA-	Lower 95% CI	Upper 95% CI	Odds ratio	P-value
Intragenic_ Group1_up & Group2_up	760	7,252	292	5,369	1.68	2.22	1.93	$8.52x10^{-21}$
Intragenic_ Group1_up & Group3_up	1,273	8,994	636	6,609	1.33	1.63	1.47	$3.70x10^{-14}$
Upstream_ Group1_up & Group3_up	824	5,556	1,064	9,971	1.26	1.53	1.39	$2.17x10^{-11}$
Upstream_ Group1_up & Group2_up	489	5,297	561	7,295	1.06	1.36	1.20	$4.54x10^{-3}$

The results of the analysis are presented as P-values, odds ratios, and 95% CIs. MET+, RNA+ represents the number of genes was included hypermethylated in Group 1 and up-regulated in Group 2 or Group 3. MET+, RNA- represents the number of genes was included hypermethylated in Group 1 and not up-regulated in Group 2 or Group 3. MET-, RNA+ represents the number of genes was included unmethylated in Group 1 and up-regulated in Group 2 or Group 3. MET-, RNA- represents the number of genes was included unmethylated in Group 1 and not up-regulated in Group 2 or Group 3. Group 1, GSE110274 of methylation profiling from co-cultured PBMC; Group 2, GSE11545 of expression profiling from peripheral blood; Group 3, GSE10715 of expression profiling from peripheral blood.

Table II. Candidate genes determined by biological process from Gene Ontology.

Biological Process	Gene name
Biological adhesion (GO:0022610)	VCAN, ICAM3, ITGA2B, ITGA5, PLOD1, PLXND1, CENTB1
Biological regulation (GO:0065007)	CAPZB, ECE1, NUCB1, SSH2, STK24, TNFRSF1A, TYK2, ZFPM1, LPPR2, PLXND1
Cellular component organization or biogenesis (GO:0071840)	ATXN2, BAP1, CAPZB, CFL1, CLN6, COTL1, PLXND1, TBL1X, TRIP6, VPS4A, SSH2, STK24
Cellular process (GO:0009987)	ACRBP, ACTR1A, ARF3, ARHGAP27, ARHGDIB, ATXN2, B3GNT8, BAP1, CAPZB, CBL, CFL1, CKAP4, CLN6, COTL1, CTSD, DDEF1, DGAT1, ECE1, ELL, EMILIN2, FMNL1, GDI1, GNB2, HIRA, ICAM3, LILRB2, LPAR2, LPPR2, LRRC4, MTRF1L, OTUD5, PARVG, PLP2, PLXND1, PXN, RNF24, RRAGC, SLC16A3, SPEN, SSH1, STAT5B, STXBP2, TBL1X, TCF7L2, TIMP2, TYK2, UBA7, VCAN, WDR42A, ZFPM1, ZNF672, EXOC3, LPP, SSH2, STK24
Developmental process (GO:0032502)	ACRBP, ADAM8, B3GNT8, EFHD2, FGD2, FGD3, PLXND1, PXN, SSH1, STAT5B, TNFRSF1A, TRIP6, TYK2, VCAN, WDR42A, ZFPM1, ANKRD11, LPP, PXN, SSH2, STK24, TRIOBP
Immune system process (GO:0002376)	AZU1, IRF9, STAT5B, TNFRSF1A, TYK2, ZFPM1
Localization (GO:0051179)	ACTR1A, ARF3, EHD1, EXOC7, PLXND1, PRAF2, SLC16A3, SLC35A4, SORL1, STXBP2, TYK2, EXOC3, RIN3
Locomotion (GO:0040011)	PLXND1, SEMA4A, TYK2
Metabolic process (GO:0008152)	AZU1, B3GNT8, BAP1, CAPN1, CDA, CKAP4, CLPTM1, DGAT1, DRAP1, ECE1, ELL, GTPBP1, IRF9, LCAT, LPPR2, LRRC4, MED16, MTRF1L, NADK, PCGF1, PLXND1, PRAF2, RNF24, RRAGC, RYBP, SLC2A4RG, SLC35A4, SORL1, SPEN, STAT5B, TBL1X, TCF7L2, TIMP2, TYK2, UBA7, VPS4A, WDR42A, ZNF672, GALNT2, MXD4, SND1, STK24, ZFPL1, ZFPM1
Multicellular organismal process (GO:0032501)	ACRBP, ADAM8, ATXN7L1, BAP1, ECE1, EHD1, GDI1, GNB2, GPSM3, PLXND1, SSH1, STXBP2, TNFRSF1A, VCAN, ZFPM1, ANKRD11, SSH2, STK24
Reproduction (GO:0000003)	ACRBP, ADAM8, B3GNT8
Response to stimulus (GO:0050896)	LPPR2, LRRC4, RRAGC, SEMA4A, STAT5B, TCF7L2, TIMP2, TNFRSF1A, TYK2, STK24

All candidate genes were classified by biological process into 12 categories. The validation genes were selected based on gene function, biological process and the reported literature. *PLOD1*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; *MMP9*, matrix metalloproteinase 9.

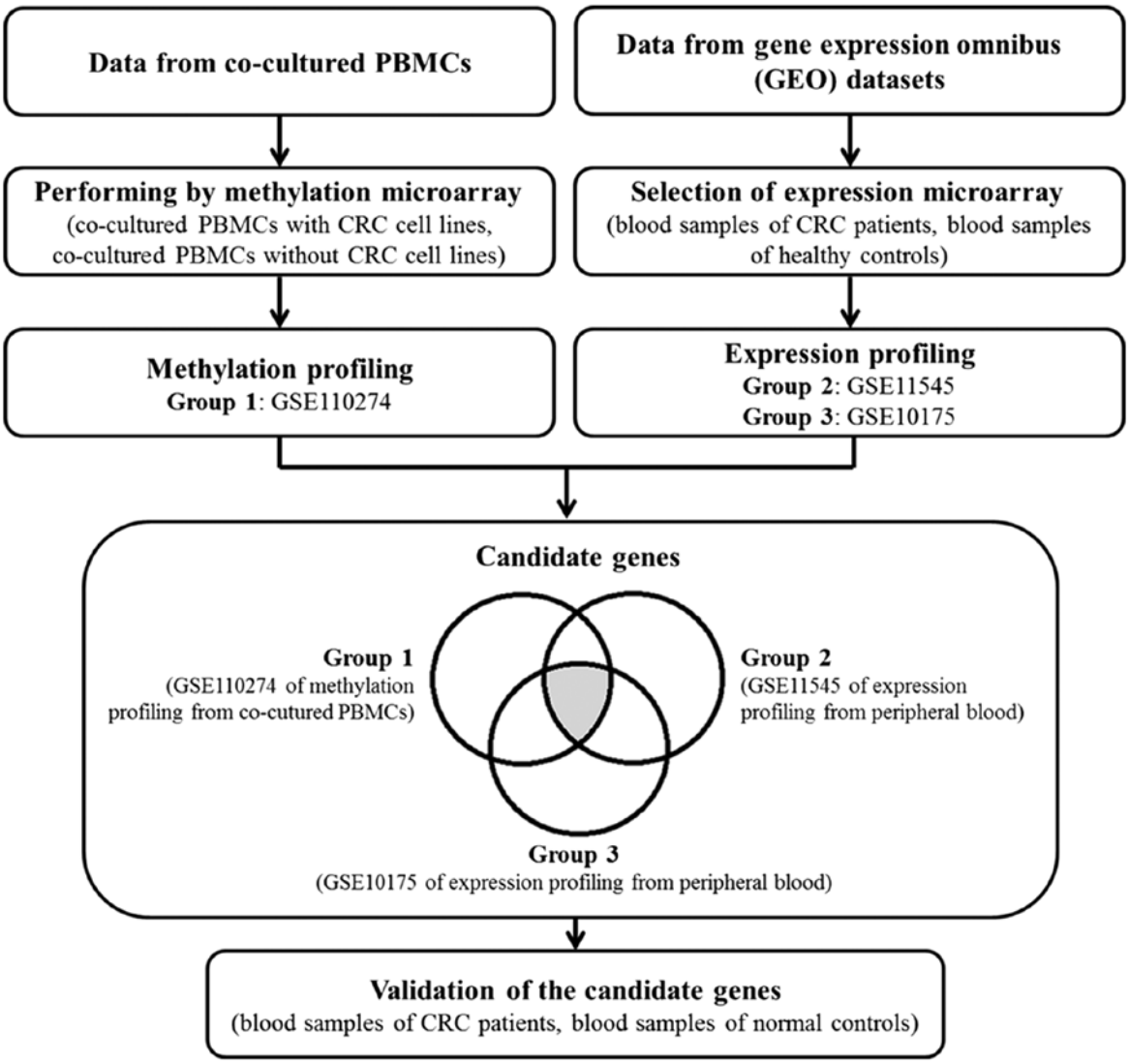

Figure 1. A flow chart of the methods employed in the present study. Data were processed and the candidate genes were collected. Group 1 represents DNA methylation changes (GSE110274), and Groups 2 and 3 represent the mRNA levels of the WBCs of patients with CRC (GSE11545 and GSE10715, respectively). All groups were analyzed by connection up- and downregulation expression analysis of microarrays. The association between gene expression and methylation was identified by means of 2x2 contingency tables. Each 2x2 contingency table produced ORs and χ^2 P-values. The candidate genes were collected from the overlapping genes of the 3 data groups, which were hypermethylated and upregulated genes with a P<0.01 and an OR of >1. Then, the present study selected genes of interest from gene function, biological process and the reported research literatures. The validated genes were quantified methylation using reverse transcription-quantitative methylation-specific PCR in WBCs of patients with CRC compared with normal controls. OR, odds ratio; WBCs, white blood cells; CRC, colorectal cancer; PBMCs, peripheral blood mononuclear cells.

number of circulating tumor cells is generally low and is dependent on the tumor size and metastasis status. Therefore, circulating cancer cell-derived tumor markers, in general, have low sensitivity. Previously, we demonstrated that breast cancer cells secrete factors that promote the expression of Mucin 1 (MUC1) by plasma cells (B lymphocytes in peripheral tissues). We also detected a large number of MUC1-positive plasma cells in micrometastatic lymph nodes (3). Therefore, tumor-induced molecular changes in WBCs can be used as a highly sensitive tumor marker. As cell secretion is a biologically active process, a large number of WBCs receive secretory molecules from cancer cells, regardless of the tumor size. The present study proved that in addition to infiltrating cells, CRC secretions can alter circulating WBCs and these changes can be used as sensitive circulating tumor markers. However, further exploration is required to determine whether the induced WBCs were infiltrating WBCs that migrated into the circulation or if CRC secretions play an endocrine role, targeting circulating WBCs.

The nature of CRC secretions inducing DNA methylation in WBC genomes remains to be explored. Several cytokines, including *fibroblast growth factor 2, granulocyte-macrophage colony stimulating factor, interleukin (IL)-1β, IL-6, macrophage inflammatory protein-1α, platelet-derived growth factor-BB, tumor necrosis factor-α*, and *vascular endothelial growth factor* have been reported at higher levels in the blood of patients with CRC (17). These cytokines potentially play a role in the DNA methylation of WBCs. Nevertheless, there are other types of cancer cell secretory molecules, including microRNA, exosomes, cells, mRNA, DNA, and proteins (18-20). All of these molecules should be evaluated to determine if they can induce DNA methylation.

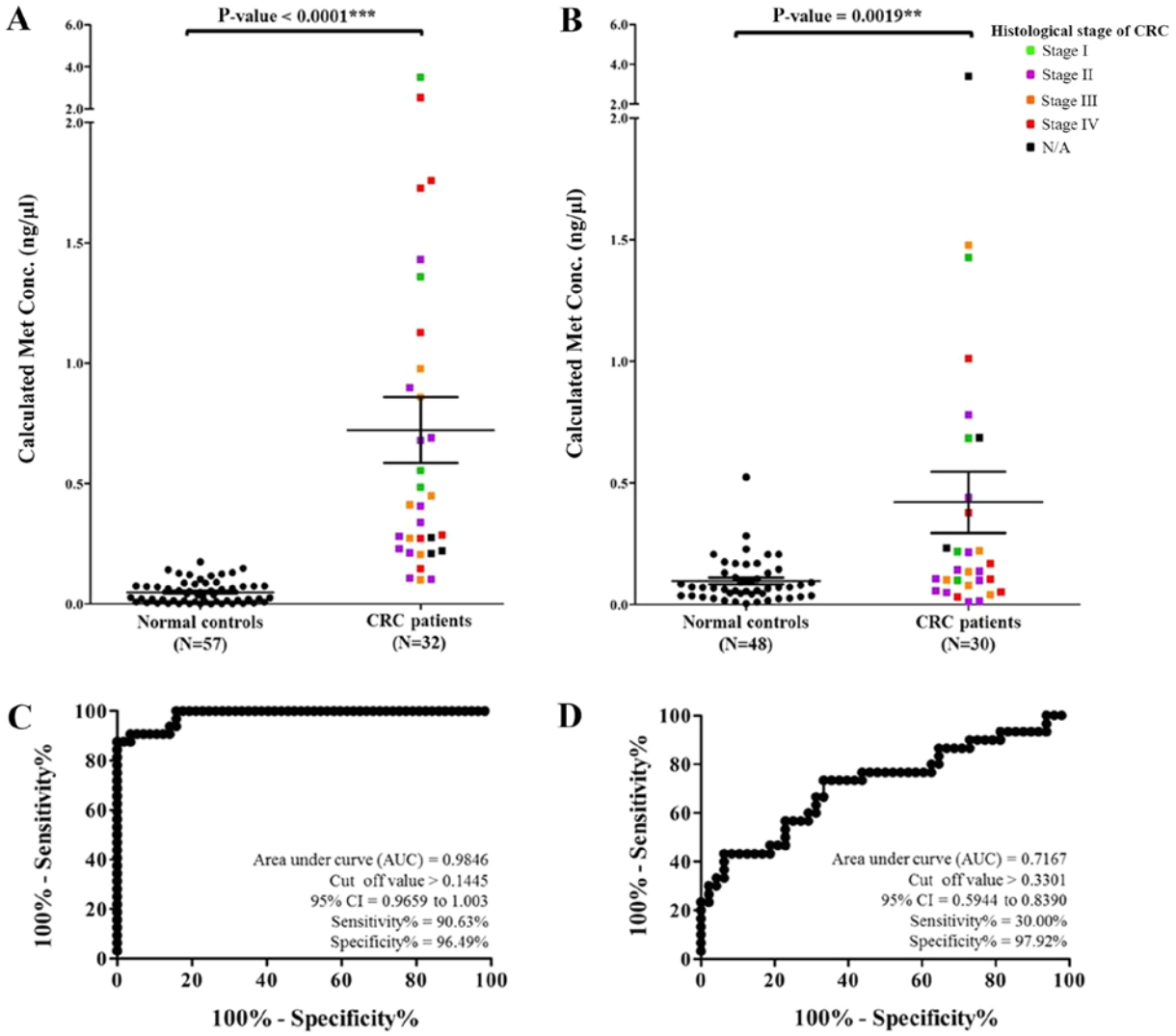

Figure 2. Methylation in white blood cells as determined by reverse transcription-quantitative methylation-specific PCR. (A) Scatter plot of *MMP9* methylation between 32 patients with CRC and 57 normal controls. The levels of *MMP9* methylation are expressed as the mean ± standard error of the mean. ***P<0.0001. (B) Scatter plot of *PLOD1* methylation between 30 CRC patients and 48 normal controls. The levels of *PLOD1* methylation are expressed as the mean ± standard error of the mean. **P=0.0019. (C) ROC curve of *MMP9* methylation exhibiting a satisfactory validated gene with high sensitivity (90.63%) and high specificity (96.49%). (D) ROC curve of *PLOD1* methylation showing an unsatisfactory validated gene with low sensitivity (30.00%) but high specificity (97.92%). *MMP9*, matrix metalloproteinase 9; CRC, colorectal cancer; *PLOD1*, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; ROC, receiver operator characteristic.

The most commonly known role of DNA methylation is the downregulation of gene expression via the methylation of promoters. CRC secretions were reported to methylate intragenic or upstream locations and increase mRNA levels. Gene body methylation sequences are frequently affected in highly expressed genes (21). The hypomethylated intragenic LINE-1 was reported to decrease the expression of cancer-associated genes in the formation of L1-RNA-AGO2 complexes (22,23). Similarly, the hypermethylated intragenic LINE-1 upregulates genes from these complexes as well (3). Hypermethylation in the upstream region was reported to play a role in regulating alternative promoters (24,25). We hypothesized that DNA methylation of upstream sequences might lead to the use of alternate but stronger promoters, resulting in upstream methylation increasing mRNA levels.

PLOD1 and *MMP9* were selected to represent hypermethylated and upregulated genes for validation. The present study selected both genes from the results that had the highest OR and P-value. Both genes were hypermethylated at intragenic locations in PBMCs and upregulated in WBCs. Primers were designed for the methylation tests according to *MMP9* and *PLOD1* oligonucleotide gene probes. The results of the methylation test presented hypermethylation. The present study then examined the protein expression of *MMP9* in the WBCs of colon cancer tissues and lymph nodes of colon cancer patients. The immunohistochemistry results demonstrated positive staining of MMP9 in WBCs. Thus, these examinations produced similar results. The methylation of the *MMP9* gene is in the gene body and upregulation of gene body methylation has been reported previously (21,22).

PLOD1 is related to collagen synthesis and assembly. This gene has been reported in the literature as being involved in CRC, especially in tumor progression (26,27). *MMP9* is involved in the degradation of collagen from extracellular

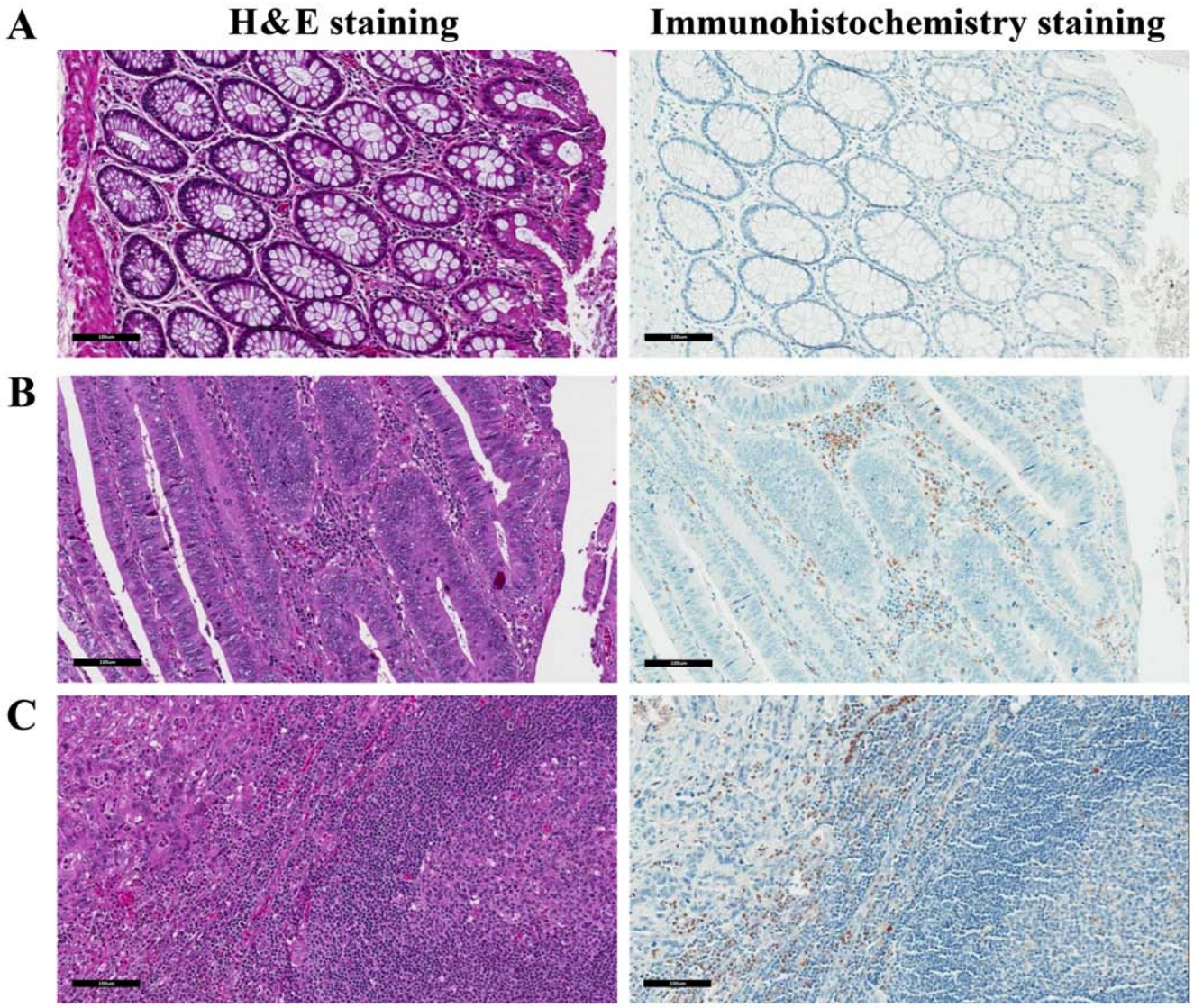

Figure 3. Formalin-fixed paraffin-embedded samples of patients with CRC were stained with H & E, and immunohistochemistry staining with the anti-MMP9 antibody (magnification, x200). (A) Normal colon tissue of a patient with CRC exhibited few MMP9 positive WBCs (5-10%). (B) A large number of CRC infiltrating MMP9 positive WBCs (60-70%). (C) Metastatic lymph node of late-stage disease showed a large number of CRC infiltrating MMP9 positive WBCs (50-60%). CRC, colorectal cancer; MMP9, matrix metalloproteinase 9; WBCs, white blood cells; H & E, hematoxylin and eosin.

matrix components in normal physiological processes. Several studies have revealed a correlation between *MMP9* and CRC, including in tumor angiogenesis, tumor invasion and inflammatory response (28-32). Epigenetic modifications are significantly involved with gene expression in cancer development (33). Thus, CRC progression may alter the epigenetic regulation of these genes.

To the best of our knowledge, this is the first report of *MMP9* and *PLOD1* methylation in the PBMCs of CRC. To apply these biomarkers for screening tests, the present study selected WBC isolation to examine methylation status, which is easier and faster than PBMC isolation. We tested the colon tissues of patients with colon cancer. The results of protein expression between WBCs and PBMCs revealed similar levels therefore we chose WBCs instead of PBMCs.

Intragenic methylation of *MMP9* is a good candidate for further clinical studies, including CRC screening, due to its high sensitivity (90.63%), specificity (96.49%), PPV (93.33%), and NPV (93.22%). Conversely, the validation results from the *PLOD1* methylation test were unsatisfactory, with low sensitivity (30.00%) but high specificity (97.92%). These methylation changes were not found to correlate with tumor grade or stage. Many biomarkers have been reported for CRC screening. For example, methylation tests have been reported for the hypermethylated DNA promoter regions of *ALX homeobox 4*, *bone morphogenetic protein 3*, *neuronal pentraxin 2*, *RARb*, *Syndecan 2, SEPT9*, and *Vimentin*, with an overall sensitivity of 90.70% and specificity of 72.50% in plasma samples (34). In particular, *SEPT9* was reported to have a sensitivity of 48.20-90.00% and specificity of 88.00-91.50% (35,36). In global DNA, hypomethylation of LINE-1 was reported to have a sensitivity of 52.78% and specificity of 86.81% in blood samples (2,37). The majority of cases of hereditary CRC syndrome involve point mutations of tumor-suppressor genes, such as *APC* and *p53*, with detection rates of 30.40 and 34.20%, respectively, and oncogenes, such as *K-ras*, with a detection rate of 34.00% and a mutation frequency of 75.00% in tumor tissues found in serum (38,39). In gene expression tests, *MMP*2 and *MMP*9 were significantly

expressed in plasma samples (40). In the protein expression tests using ELISA in blood samples, TIMP metallopeptidase inhibitor-1 was reported to have a sensitivity of 63.00% and specificity of 98.00% (41).

In conclusion, CRC cells release secretions that induce DNA methylation changes in circulating WBCs. Both upstream and intragenic methylation of the genes were revealed to be associated with higher levels of the mRNAs. Finally, CRC-induced DNA methylation in circulating WBCs is a promising tumor marker for cancer screening studies.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author on reasonable request.

Authors' contributions

PB performed the experiments, analyzed and interpreted the data, and wrote the manuscript. PA, NKo and VA collected the specimens. CA and CP conducted the microarray and bioinformatics analyses. Nki and AM contributed to the conception and design of the study, and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University (IRB no. 326/60). All study subjects provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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