Aberrant promoter methylation of *PPP1R3C* and *EFHD1* in plasma of colorectal cancer patients
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日本大学大学院医学研究科博士課程
外科系消化器外科学専攻

高根　希世子

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Contents

1) Abstract

Aberrant DNA methylation is a common epigenetic alteration involved in colorectal cancer (CRC). In our previous study, we identified genes hypermethylated in CRC through methylated DNA immunoprecipitation-on-chip analysis of CRC cell lines combined with array analysis of gene re-expression by 5-aza-2′-deoxycytidine treatment. In this study, we aim to find out any of these methylation genes to be utilized for CRC detection. Among 12 candidate genes which showed aberrant hypermethylation frequently in >75% of 149 CRC samples but did not in normal samples in the previous analysis, we designed primers for methylation-specific PCR and pyrosequencing for seven genes. Among them, *PPP1R3C* and *EFHD1* were rarely hypermethylated in peripheral blood cells but frequently hypermethylated in another set of 24 CRC tissue samples and their corresponding plasma samples. In plasma samples, *PPP1R3C* was methylated in 81% (97/120) of CRC patients, but only in 19% (18/96) of non-cancer patients ($P=6x10^{-20}$, Fisher's exact test). In combined analysis with *EFHD1*, both genes were methylated in 53% (64/120) of CRC patients, but only in 4% (4/96) of non-cancer patients ($P=2x10^{-16}$), giving high specificity of 96%. At least one of the two genes was methylated in 90% (108/120) of CRC patients, and 36% (35/96) of control patients, giving high sensitivity of 90%. Compared with low positive ratios of CEA (2/12 at stage

I, 12/30 at stage II) and CA19-9 (0/12 at stage I, 4/30 at stage II) for early stage CRCs, methylation-positive ratio was significantly higher: *PPP1R3C* methylation at 92% (11/12) for stage I and 77% (23/30) for stage II, and methylation of at least one gene at 100% (12/12) for stage I and 87% (26/30) for stage II. *PPP1R3C* methylation or its combined use of *EFHD1* methylation was highly positive in CRC plasma samples, and they might be useful in detection of CRC, especially for early stage CRCs.

2) Introduction

For cancer treatment, early detection of disease leads to favorable outcomes for patients, and it is important to develop screening tests with high sensitivity and specificity, especially for early stage cancer(1). In colorectal cancer (CRC) screening, stool blood tests and measurement of tumor markers in serum, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA19-9), are conventional methods that have been used. The fecal occult blood test, however, has a low specificity, ranging from 0.3–0.5%(2). CEA and CA19-9 are not frequently positive in CRC at early stages (I and II), and their sensitivities are <50%(3). These methods are not satisfactory for early cancer detection, and a new, non-invasive technique to

detect early stage malignancies would be useful as a first screening test, before the need of invasive examinations e.g. barium enemas and colonoscopies(4-5).

Cell-free DNA derived from solid tumor cells circulates in the blood stream; therefore, detection of tumor DNA in plasma/serum could be an attractive method for cancer screening(6). For example, detection of mutated RAS gene fragments(7) and microsatellite aberrations(8) in plasma/serum of cancer patients have been demonstrated. But these methods can detect only a fraction of cancer cases with specific genomic aberrations such as RAS mutations, and the development of screening methods to detect the majority of cancer cases are urgently needed. Aberrant DNA methylation of promoter CpG islands is a common epigenetic alteration to inactivate tumor suppressor genes in CRC and in other cancers(9-10). Detection of genetic mutations is rather difficult to apply to cancer screening because it is necessary to examine many possible mutation sites per gene. When DNA methylation is analyzed, only one promoter region per gene needs to be examined.

In detection of aberrantly methylated DNA in plasma samples, Lofton-Day et

al. identified three blood-based molecular biomarkers including *TMEFF2*, *NGFR* and *SEPT9* that were useful for CRC screening(11). Thereafter, the concentration of *SEPT9* methylated DNA could be measured with higher sensitivity and specificity and detected in a majority of CRCs at all stages and colorectal locations(12).

A subgroup of CRC has been shown to have aberrant CpG island methylation at a significantly higher frequency, so-called CpG island methylator phenotype (CIMP)(13-14). We(15) and other groups(16-18) performed comprehensive methylation analysis of CRC samples and reported three DNA methylation epigenotypes of CRC: high-methylation, intermediate-methylation and low-methylation. In the analysis, we performed methylated DNA immunoprecipitation-on-chip analysis of CRC cell lines combined with array analysis of gene re-expressions by 5-aza-2-deoxycytidine treatment, and established methylation genes to epigenotype CRC(15). These epigenotyping genes included two major groups of genes: Group-1 genes specifically methylated in high-methylation/CIMP+ CRCs, and Group-2 genes methylated in both high- and intermediate-methylation CRCs. These genes threrefore classify CRC into

three epigenotypes: high-methylation/CIMP+ CRCs with methylation of Group-1 and Group-2 genes, intermediate-methylation CRCs with methylation of Group-2 genes, and low-methylation CRCs without methylation of either group of genes. Besides these genes, another type of genes was found to be aberrantly methylated in all or most CRC cases regardless of epigenotype(15).

In our previous methylation analysis of CRC, 60 methylation in CRC were established and their methylation levels were analyzed quantitatively in 149 CRC and nine normal colon samples. Among them, 12 genes were not hypermethylated in any of the normal colon samples, but were frequently methylated (>75%) in CRC cases: *COL4A2* (147/149), *TSPYL5* (141/149), *TMEFF2* (141/149), *RASSF2* (134/149), *SPG20* (130/149), *EDIL3* (130/149), *CIDEB* (128/149), *ADAMTS1* (128/149), *EFHD1* (127/149), *STOX2* (126/149), *PPP1R3C* (118/149) and *UCHL1* (115/149) (15).

In this study, we aim to find out whether any of commonly hypermethylated genes could be utilized for CRC detection using plasma DNA samples. For candidate genes showing aberrant methylation in >75% of CRC samples but in none of normal samples in the previous analysis, we first checked

methylation status of peripheral blood cells, and genes rarely methylated in peripheral blood cells underwent subsequent methylation analysis using plasma DNA samples of CRC and non-cancer patients. Methylation was analyzed using methylation-specific PCR(19) in conjunction with pyrosequencing(20), which was used for the validation of the methylation-specific amplification. It was found that *PPP1R3C* (acts as a glycogen-targeting subunit for PP1 and regulates its activity) (21) methylation alone or in combination with *EFHD1* (an EF-hand domain-containing protein that displays increased expression during neuronal differentiation) (22) methylation showed high sensitivity and specificity, and these genes could be used to detect CRC, especially at early stage.

3) Materials and Methods

i) Clinical samples

Peripheral blood was collected from 96 patients undergoing surgical operations for benign diseases including inguinal hernia, appendicitis and gallbladder stones (non-cancer group), and from 120 patients undergoing

surgical operations for CRC (CRC group). Corresponding primary CRC tissue samples were also collected from 24 CRC patients. All samples were collected with written informed consent and the surgery was done in the Department of Digestive Surgery, Graduate School of Medicine, Nihon University. Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Frozen materials were microscopically examined for the determination of cancer cell content by pathologists, and it was confirmed that all 24 samples contained at least 40% cancer cells. DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacture's protocol. Peripheral blood was put in an ethylenediaminetetraacetic acid vacutainer coated tube and centrifuged at 1,200g at room temperature for 15 minutes. From 3 mL of the supernatant plasma, cell-free genomic DNA was extracted using QIAamp Circulating Nucleic Acid Kit (Qiagen). The Ethics Committees of Nihon University, Chiba University and The University of Tokyo certified this study.

ii) Characteristics of the study population

The 120 CRC patients were 67.7±11.4 years old (mean ± standard error),

ranging 30-88, and included 71 males and 49 females, whereas the 96 non-cancer patients were 63.0±13.6, ranging 24-87 (P=1, t-test versus CRC patients), and included 67 males and 29 females (P=0.1, Fisher's exact test versus CRC patients). 20 (17%) CRC patients were neoadjuvantly treated. Tumor locations were 41 (34%) at proximal colon (10 in cecum, 15 in ascending colon, 16 in transverse colon), 37 (31%) at distal colon (4 in descending colon, 33 in sigmoid colon), and 42 (35%) at rectum. For AJCC (American Joint Committee on Cancer) stages, 12 (10%) were at Stage I, 30 (25%) at Stage II, 12 (10%) at Stage III, and 66 (55%) at Stage IV.

iii) Bisulfite treatment of genomic DNA

By bisulfite treatment, unmethylated cytosine is converted to uracil, i.e. recognized as thymine (T) after PCR reaction, but methylated cytosine is not converted, i.e. cytosine (C) after PCR reaction. Unmethylated DNA and methylated DNA are therefore distinguishable by detecting the difference of T and C in the sequence after bisulfite treatment. Bisulfite conversion of 500 ng of genomic DNA from each tissue sample was performed using Zymo EZ DNA Methylation Kit (Zymo Research, Irvine, CA), and the DNA was eluted

in 30 μL of 10 mEq Tris buffer. For plasma samples, genomic DNA isolated from 3 mL of plasma was treated with bisulfite in the same manner. To check the quality of bisulfite-converted DNA sample as PCR template, upstream region of MYOD(23) was amplified by PCR and the PCR product was visualized using ethydium bromide after agarose gel electrophoresis. Primers for MYOD were 5′-TGATTAATTT AGATTGGGTT TAGAGAAGGA-3′ (forward) and 5′-CTCCCTCTAT CCCCTAACAA ACTT-3′ (reverse). PCR product length was 97 bp and annealing temperature was 62 °C. This region contains no CpG site, and should therefore be amplified regardless of methylation status.

Methylation control samples (0% and 100%) were prepared as previously described(15). Briefly, human peripheral lymphocyte DNA was amplified using GenomiPhi v2 DNA amplification kit (GE Healthcare Life-Science, Uppsala, Sweden). The amplified DNA was not methylated at all in any CpG sites, and was used as unmethylated (0%) control. The amplified DNA was methylated by SssI methylase and used as fully methylated (100%) control. These control samples were also treated with bisulfite using Zymo EZ DNA Methylation Kit.

iv) Methylation-specific PCR.

Methylation status was determined by methylation-specific PCR(19). To design primers, Pyro Q-CpG software (Qiagen) was used to obtain the genomic DNA sequence after bisulfite conversion, by converting C at non-CpG sites to T and keeping C at CpG sites as C. Forward and reverse primers were designed to contain multiple C's, especially at the 3' end of primer. When annealing temperature is high enough, the primers would anneal to methylated allele only, and unmethylated allele should not be recognized and amplified.

Methylation genes in CRC were selected from genes identified in our previous study(15), in which bisulfite sequencing primers were designed in the 5′ region of each gene. The PCR products were 200-400 bp, and were analyzed in the methylation assay using MALDI-TOF-MS (Matrix Assisted Laser Desorption Ionization-Time Of Flight-Mass Spectrometry)(24). In this study, primers for methylation-specific PCR were designed within these regions, with PCR products being ≤100 bp, because these analyzed regions were located in 5′ CpG islands of genes and confirmed to be aberrantly

methylated in CRC. PCR was performed using 5μL of bisulfite-modified DNA as a template, and FastTaq polymerase (Roche, Basel, Switzerland). The annealing temperature for the PCR was determined to amplify 100% methylation control sample only, and not to amplify 0% methylation control sample. For 12 candidate genes, *COL4A2*, *TSPYL5*, *TMEFF2*, *RASSF2*, *SPG20*, *EDIL3*, *CIDEB*, *ADAMTS1*, *EFHD1*, *STOX2*, *PPP1R3C* and *UCHL1,* such primers could be designed for seven genes, *COL4A2*, *TSPYL5*, *EDIL3*, *ADAMTS1*, *EFHD1*, *STOX2* and *PPP1R3C*. Primer sequences for these genes and the number of analyzed CpG sites are shown in Table 1.

v) Pyrosequencing analysis

To confirm that methylation-specific PCR specifically amplified the methylated allele, the methylation status of the PCR product was quantitatively sequenced using pyrosequencing as previously described(25). Briefly, the biotinylated PCR product was bound to streptavidin Sepharose beads HP (GE Healthcare Life Sciences), washed and denatured using a 0.2 mol/L NaOH solution. After addition of 0.3 μmol/L sequencing primer to the purified, single-stranded PCR product, pyrosequencing was carried on

PyroMark Q24 MD System (Qiagen) with Pyro Q-CpG software (Qiagen) according to the manufacturer's instructions. Primer sequences and conditions, and the number of analysed CpG sites are shown in Table 1. Methylation control samples (0% and 100%) were analyzed in every assay to check that no PCR product was obtained in the 0% control sample and that the fully methylated allele was amplified in the 100% control sample.

vi) Evaluation of protein markers CEA and CA19-9

At clinical diagnosis of CRC, serum CEA and CA19-9 levels were evaluated by ELISA. CEA and CA19-9 were considered to be positive when CEA was ≥5 ng/mL and CA19-9 was ≥40 U/mL.

vii) Statistical analysis

P-values were calculated to compare CRC patients and non-cancer patients. Student t-test was used for age, and Fisher's exact test was used for analysis of sex. P-values were also calculated to compare methylation(+) group and methylation(-) group. Student t-test was used for age, and Fisher's exact test was used for analysis of sex, AJCC stage, neoadjuvant chemotherapy, and

tumor locations (Tables 2 and 3). In each AJCC stage, methylation frequency in plasma DNA samples was also compared with frequencies of CEA-positive and CA19-9-positive using Fisher's exact test (Fig. 5). When $P<0.05$, the correlation was considered as statistically significant. Student t-test and Fisher's exact test were performed using R software (www.r-project.org/).

4) Results

i) Selection of candidate marker genes

To detect aberrantly methylated alleles, bisulfite-treated genomic DNA was amplified using methylation-specific PCR primers designed to generate PCR products ≤100 bp. To validate that methylation-specific PCR products resulted from amplification of methylated alleles, and not from unexpected amplification of unmethylated DNA or DNA with partial methylation in primer regions, sequence primers were designed within the product regions and the methylation level of the PCR products were analyzed using pyrosequencing. Such primers for methylation-specific PCR and pyrosequencing were successfully designed for seven of the 12 genes; *COL4A2*, *TSPYL5*, *EDIL3*, *ADAMTS1*, *EFHD1*, *STOX2* and *PPP1R3C*

(Table 1).

In pyrosequencing, the signal intensity should be high enough (≥5) and methylation rate should be high enough (60-100%) if methylated allele was successfully amplified. If methylation rate was low (<60%), that would be due to unexpected amplification of unmethylated allele in methylation-specific PCR, and the sample would therefore be regarded as methylation(-). But all the analysed samples showed methylation rate as high as 60-100% when the signal intensity was higher than 5, and they were regarded as methylation(+). When no signal was detected in pyrosequence, that should be due to no amplification in methylation-specific PCR, and the sample was regarded as methylation(-). When the signal intensity was too low to accurately calculate methylation rate, that would be regarded as insufficient amplification by methylation specific PCR, we set the threshold at 5. To check the quality of sample DNA, bisulfite-converted DNA was amplified using primers for MYOD upstream region. MYOD primers were designed in the regions without CpG sites, and therefore amplify the region regardless methylation status. All the analyzed samples showed amplification of the MYOD region, indicating that lack of amplification is

due to absence of methylation, not due to poor DNA quality.

ii) Selection of genes using normal peripheral blood cell samples

Considering that plasma DNA samples can be easily contaminated with DNA originating from normal peripheral blood cells, we first analyzed the methylation status of the seven genes in peripheral blood cell samples from four non-cancer patients. Methylation of *PPP1R3C* and *EFHD1* was rarely detected in peripheral blood cells, but the other five genes, *STOX2*, *EDIL3*, *COL4A2*, *TSPYL5* and *ADAMTS*1, were frequently methylated in these cells (Fig. 1). Given that false positive results could potentially be obtained if these latter five genes were analyzed in plasma DNA samples, *PPP1R3C* and *EFHD1* were selected for subsequent analyses.

iii) Methylation of *PPP1R3C* and *EFHD1* in plasma and tumor samples from CRC patients

PPP1R3C and *EFHD1* were analyzed using plasma samples from 24 CRC patients and their corresponding CRC tissue samples (Fig. 2A). *PPP1R3C* and *EFHD1* were methylated in 22 (92%) and 19 (79%) of the 24 CRC tissue

samples, respectively. While these two genes were frequently methylated in 149 CRC tissue samples in the previous study(15), it was confirmed that they were also frequently methylated in this additional set of CRC tissue samples. When plasma DNA samples from these CRC patients were analyzed, *PPP1R3C* and *EFHD1* were frequently methylation-positive (+), at 79% (19/24) for each gene (Fig. 2A). In this time, 24 mucosal tissues weren't analyzed because cell-free DNA can't be extracted in normal tissues.

When the two genes were combined, all 24 CRC tissue samples (100%) and 22 plasma DNA samples (94%) were methylation(+) for at least one of the two genes (Fig. 2B). This suggested that high sensitivity could be obtained if these two genes were analyzed for CRC detection.

A small number of cases were methylation(+) in plasma DNA samples despite methylation(-) in CRC tissue samples. These might be due to unexpected methylation in peripheral blood cells contaminated in plasma samples or it might be due to heterogeneity of tumor tissues, that is, plasma DNA derived from a part of CRC might be methylated while the analyzed piece of CRC tissue might not be methylated.

iv) Comparison between CRC patients and non-cancer patients

Next, *PPP1R3C* and *EFHD1* were analyzed using plasma samples from 120 CRC patients and 96 non-cancer patients. *PPP1R3C* was methylated in 81% (97/120) of CRC patients (Fig.3), which was at a similar frequency determined for the initial 24 samples (Fig. 2A). The methylation(+) ratio for non-cancer patients was 19% (18/96) (P=6×10−20, Fisher's exact test). *EFHD1* was methylated in 62% (75/120) of CRC patients and in 22% (21/96) of non-cancer patients (P=3×10−9) (Fig. 3).

If analyses of these two genes were combined, then at least one gene was methylated in 90% (108/120) of CRC patients and in 36% (35/96) of non-cancer patients (P=4×10−17). Both *PPP1R3C* and *EFHD1* genes were methylated in 53% (64/120) of CRC patients, but in only 4% (4/96) of non-cancer patients (P=2×10−16) (Fig. 4A).

When a single gene was used for CRC detection using plasma samples, *PPP1R3C* gave better results than *EFHD1*. For *PPP1R3C*, 97 of 120 CRC patients (81% sensitivity) and 78 of 96 non-cancer patients (81% specificity) were diagnosed correctly. The sensitivity and specificity could be improved when *EFHD1* was combined with *PPP1R3C*. If methylation of at least one

gene was regarded as methylation(+), as many as 108 of 120 CRC patients would have been diagnosed correctly, with 90% sensitivity. If methylation of both genes was regarded as methylation(+), as many as 92 of 96 non-cancer patients would have been diagnosed correctly, with 96% specificity, while the sensitivity would be 53% (Fig. 4B).

v) Comparison with protein markers, CEA and CA19-9

To evaluate the usefulness of the two CRC detection markers, the positive ratio was compared with two protein markers, CEA and CA19-9 (Fig. 5). CEA and CA19-9 were positive in 64% (77/120) and 34% (41/120) of CRC cases, respectively. *PPP1R3C* methylation showed a higher positive ratio, 81% (97/120), than the two protein markers. At early clinical stages, positive ratio of *PPP1R3C* methylation was significantly higher than the protein markers (Fig. 5A). For stage I CRC, 92% (11/12) samples were *PPP1R3C* methylation(+), whereas only 17% (2/12) were CEA(+) ($P=3\times10^{-4}$, Fisher's exact test) and 0% (0/12) was CA19-9(+) ($P=5\times10^{-6}$). For stage II CRC, 77% (23/30) were *PPP1R3C* methylation(+), whereas only 40% (12/30) were CEA(+) (P=0.004) and 13% (4/30) were CA19-9(+) ($P=7\times10^{-7}$).

Sensitivity of *EFHD1* methylation was also significantly higher than the protein markers for stage I CRC. Seven of 12 (58%) were *EFHD1* methylation(+), whereas 17% were CEA(+) ($P=3\times10^{-4}$) and 0% was CA19-9(+) ($P=5\times10^{-6}$ (Fig. 5B).

When *EFHD1* methylation was combined with *PPP1R3C* analysis and methylation of at least one gene was regarded as methylation(+), the positive ratio at early clinical stages was further increased. All 12 (100%) were methylation(+) for stage I CRC ($P=3\times10^{-5}$ against CEA and $P=4\times10^{-7}$ against CA19-9). For stage II CRC, 87% (26/30) were methylation(+) ($P=2\times10^{-4}$against CEA and $P=6\times10^{-9}$against CA19-9) (Fig. 5C). Even when methylation of both *PPP1R3C* and *EFHD1* was regarded as methylation(+), resulting in very high specificity, the positive ratio for stage I CRC was still significantly higher than that of the protein markers. Six of 12 CRCs (50%) were methylation(+), whereas 17% were CEA(+) ($P=3\times10^{-5}$) and 0% was CA19-9(+) ($P=4\times10^{-7}$).

vi) Statistical analysis for methylation markers of early stages

Likelihood ratio for a positive finding (LR+) and positive predictive value

(PPV) for Stage1 and 2 was analyzed by these methylation markers (Table 4). LR+ of *PPP1R3C* and *EFHD1* were 4.31 and 1.14, respectively. LR+ of at least one gene which was methylated was 2.48. LR+ of both these genes which were methylated was 12.0. On the other hand, PPV of *PPP1R3C* and *EFHD1* were 0.84 and 0.54, respectively. PPV of at least one gene was methylated at 0.76 PPV of both these genes which were methylated was 0.94.

vii) Comparison with other clinicopathological factors

Methylation status of *PPP1R3C* and *EFHD1* was compared with other clinicopathological factors including sex, age, tumor stage and tumor locations (Tables 2 and 3). For both genes, sex, age, tumor stage, presence or absence of neoadjuvant chemotherapy and tumor locations did not show significant difference between methylation(+) and methylation(-) cases.

4) Discussion

PPP1R3C encodes a protein phosphatase 1 (PP1) regulatory subunit which forms complexes with glycogen phosphorylase, glycogen synthase, and phosphorylase kinase necessary for its regulation of PP1 activity. Little is

known about its function and its potential deregulation in human cancer(21). In 2009, Bonazzi et al. assessed the *PPP1R3C* CpG island presented a proportion of methylation in 57% of the melanoma cell lines (26), but a relevance of colon cancers didn't suggest so far. Furthermore *EFHD1* is an EF-hand domain-containing protein that displays increased expression during neuronal differentiation (22) but relation of *EFHD1* and colon cancers has never been suggested yet.

Aberrant DNA methylation of promoter CpG islands is one of major epigenetic alterations in CRC(9-10). Some genes are methylated in CRC commonly regardless of epigenotypes and could possibly be suitable as CRC detection markers. Among these commonly methylated genes, those methylated in normal colon samples or in peripheral blood cells were excluded. *PPP1R3C* and *EFHD1* were subsequently analysed using plasma DNA samples of 120 CRC and 96 non-cancer patients in this study, using methylation-specific PCR in combination with pyrosequencing for the validation of specific amplification of methylated DNA. Detection of *PPP1R3C* methylation alone or its combination with *EFHD1* methylation in plasma DNA samples was found to show high sensitivity and specificity, and

their positive ratios in early stage CRCs were substantially higher than that of CEA and CA19-9.

In 2004, Müller et al. assessed *SFRP2* methylation in faecal DNA to diagnose CRC using MethyLight analysis; its sensitivity and specificity were as high as 77% and 77%, respectively, although they analyzed only 13 CRC and 13 control samples(5). In 2005, Chen et al. analyzed VIM methylation in faecal DNA from 94 CRC and 198 control samples using methylation-specific PCR; its specificity was as high as 90%, while sensitivity was 46%(27). As for methylation in plasma DNA, Lofton-Day et al. searched for CRC-specific methylated DNA in plasma and reported that the sensitivity and specificity of *TMEFF2*, *NGFR* and *SEPT9* were 65% and 69%, 51% and 84%, and 69% and 86%, respectively(11). When *PPP1R3C* methylation was used alone in this study, its sensitivity (81%) and specificity (81%) were considerably high, compared with these reports.

Several groups analyzed *SEPT9* methylation in plasma samples for CRC detection. Some reports showed considerably high sensitivity (90%–96%) and specificity (85%–88%)(12, 28), while other groups reported relatively lower sensitivity (48%–72%) but higher specificity (86%–95%)(29-31). In 2009,

deVos et al. measured *SEPT9* methylation using real-time PCR-based analysis, in which three independent experiments per sample were performed. High-sensitivity method, where at least 1 of 3 PCR reactions was positive, resulted in 72% sensitivity and 86% specificity. But high-specificity method, where at least 2 of 3 PCR reactions were positive, resulted in 56% sensitivity and 95% specificity(30). This indicated that the results were dependent on the decision criteria, and that specificity would be increased by lowering sensitivity. Our results had similar tendencies. In high-sensitivity analysis where methylation of at least one gene was regarded as methylation(+), sensitivity improved to 90% while specificity was 64%. In high specificity analysis where methylation of both the *PPP1R3C* and *EFHD1* genes was regarded as methylation(+), specificity improved to as high as 96% while sensitivity was 53%. These suggested that in addition to *SEPT9* methylation, *PPP1R3C* methylation alone or in combination with *EFHD1* methylation could be detection markers for CRC detection with high sensitivity and high specificity.

CRC is one of the leading causes of cancer deaths in the world, and diagnosis at an early onset followed by surgical intervention is currently the best way

to cure the disease and decrease mortality. It is therefore important to develop detection markers to detect asymptomatic CRCs at earlier stages, while the positive ratios of CEA and CA19-9 were reported to be relatively low in early stage CRCs(3). Our previous studies of DNA methylation in CRC and precancerous lesions revealed that accumulation of aberrant DNA methylation was mostly completed by the adenoma stage(15, 32), suggesting the possible usefulness of assessing aberrant DNA methylation in plasma DNA derived from solid tumors in detecting early stage CRCs. Warren et al. reported that the sensitivity of *SEPT9* methylation was 71% for stage I CRCs(12). In another report, *SEPT9* methylation was the sensitivity in 60% of stage I CRCs, which could be increased to 84% using a high sensitivity method(28). *PPP1R3C* methylation in this study gave similar, or even better results in detecting early stage CRCs. Methylation of *PPP1R3C* alone was the sensitivity in 92% of stage I CRCs. Using a more sensitive method to detect methylation of at least one of the *PPP1R3C* and *EFHD1* genes, the sensitivity increased to 100% for stage I CRCs. Even in a method with high specificity of 96%, methylation of both genes was sensitivity in 50% of stage I CRCs, which was significantly higher than positive ratios of

CEA (17%) and CA19-9 (0%). Moreover, in Stage 1 and 2, LR+ and PPV of both these genes, were methylated at 12.0 and 0.84 (Table 4). This indicated that detection of aberrant methylation in plasma DNA was a powerful method to diagnose CRC, especially for early stage CRCs, and that *PPP1R3C* and *EFHD1* were useful biomarkers for the method. Accordingly, even if a fecal occult blood test of a patient who has hemorrhoids is positive, using these biomarkers may be possible to find an adenoma, or an early stage tumor, and doctors can recommend doing colonoscopy to patients.

A subset of high-methylation CRC cases showed CpG island methylator phenotype (CIMP). A subgroup of methylation genes including CIMP markers were specifically hypermethylated in CIMP-positive high-methylation CRC, and methylation of these genes was associated with female, older age, and proximal tumor location significantly(15). But the genes analyzed in this study were extracted from genes hypermethylated commonly in CRC regardless of epigenotypes, and these genes did not show any significant difference about sex, age, or tumor location(15). In good agreement to these previous observation, methylation of *PPP1R3C* and *EFHD1* in plasma DNA samples were detected commonly in CRC patients,

regardless of sex, age, or tumor location (Table 2 and 3).

5) Conclusion

In summary, detection of methylation of *PPP1R3C* alone or in combination with *EFHD1* in plasma DNA showed high sensitivity and specificity in CRC detection, and may be useful detection markers for CRC, especially for detection of early stage CRCs.

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Table

Table 1. Primer sequences for methylation-specific PCR and for pyrosequencing

Primer sequence	anneal (°C)	product (bp)	analyzed CpG sites	primer position
ADAMTS1 (bottom strand)				
Fwd: GTTTCGAGATTTCGGAGTTCGTTTCGC	64	97	5	+538~+512
Rev*: AAACTCCAATACAACGAACTATACCCG			2	+470~+442
Seq: TTTTTTATGTAGTTGTTTAGTT			2	+510~+499
STOX2 (top strand)				
Fwd*: TGGGGTAGTTGTTAAGGTTTTCGCGTC	61	97	3	+301 ~ +327
Rev: CACCAAACTACCTTAAATTAAAACGCG			2	+371 ~ +397
Seq: CATCAAACTTCTCATTTTCATATA			4	+375 ~ +352
EDIL3 (bottom strand)				
Fwd: GATTAAGAGTTAGACGGTTATCGAGC	64	79	3	+452~+427
Rev*: CGCGACGACCCCTAACCAACCGAAATCACG			5	+403~+374
Seq: GGTTATAGAGAGTTTTATGATTT			2	+437~+415
COL4A2 (bottom strand)				
Fwd: TTTATCCTCGGTTTCGGTTC	64	72	3	+529 ~ +510
Rev*: CTCCCATCACCCCTACATACG			1	+478 ~ +458
Seq: GAGAAGAGGGGATAG			4	+507 ~ +493
PPP1R3C (top strand)				
Fwd: TCGTTTCGGGGCGATTACGTTGTC	65	100	5	-123 ~ -120
Rev*: CCTAAAACCAATCGCCGAACCTCG			3	-47 ~ -24
Seq: GAGGGTTGGAGTTTTAGTTGG			3	-114 ~-94
EFHD1 (top strand)				
Fwd: TTTCGAGTTTGCGAGGAGCGCGTC	68	90	5	+4 ~ +27
Rev*: CATAACGACGAATCGCAAAACGCG			5	+70 ~+93
Seq: CGTCGTTAGTTAGTTTTTTG			6	+24 ~ -43

TSPY5 (top strand)				
Fwd: TATAGTTGTACGTTCGTGACGTC	61	75	4	-17 ~ +6
Rev*: CCTAACGCCAACTCTCGATCG			3	+38 ~ +58
Seq: GGTTGTAGTGGAGAGATT			4	+10 ~ +27

The position of the transcription start site (TSS) was regarded as +1. The DNA strand used for the template was shown by *top/bottom*. *Fwd/Rev*, forward and reverse primers for methylation-specific PCR. *C* and *G*, C in forward primer and G in reverse primer to distinguish methylated DNA from unmethylated DNA. *Primers biotinylated for pyrosequencing. *Seq*, sequence primer for pyrosequencing.

Table 2. *PPP1R3C* methylation and clininopathological factors

	Methylated	Unmethylated	*p*-value
Number	97	23	
Age (yrs)	67.9±11.4	67.0±11.8	0.9
Sex (male/female)	59/38	12/11	0.7
AJCC stage			0.1
I/II/III/IV	11/23/9/54	1/7/3/12	
NAC (+ / -)	17/80	3/20	0.5
Tumor location			XXXX
Proximal	36	5	
Ce/A/T	9/11/16	1/4/0	
Distal	29	8	
D/S	3/26	1/7	
Rectum	32	10	

Age, CEA and CA19-9 were shown by mean ± standard deviation. AJCC, American Joint Committee on Cancer. NAC, Neoadjuvant chemotherapy. Tumor locations were classified into proximal colon including cecum (*Ce*), ascending (*A*) and transverse colon (*T*), distal colon including descending (*D*) and sigmoid colon (*S*), and rectum. *P*-values were analyzed using the Fisher's exact test, the Student's *t*-test for age, CEA and CA19-9, or the Kruskal-Wallis test for tumor location. **P*-value < 0.05.

Table 3. *EFHD1* methylation and clininopathological factors

	Methylated	Unmethylated	*p*-value
Number	75	45	
Age (yrs)	67.0±10.7	71.2±12.3	0.5
Sex (male/female)	44/31	27/18	0.4
AJCC stage			0.5
I/II/III/IV	7/18/8/42	5/12/4/24	
NAC (+ / -)	13/62	7/38	0.7
Tumor location			XXXX
Proximal	26	15	
Ce/A/T	7/9/10	3/6/6	
Distal	20	17	
D/S	2/18	2/15	
Rectum	29	13	

Age, CEA and CA19-9 were shown by mean ± standard deviation. AJCC, American Joint Committee on Cancer. NAC, Neoadjuvant chemotherapy. Tumor locations were classified into proximal colon including cecum (*Ce*), ascending (*A*) and transverse colon (*T*), distal colon including descending (*D*) and sigmoid colon (*S*), and rectum. *P*-values were analyzed using the Fisher's exact test, the Student's *t*-test for age, CEA and CA19-9, or the Kruskal-Wallis test for tumor location. **P*-value < 0.05.

Table 4. Statistical analysis for methylation markers of 42 cases of Stage 1 and 2

	PPP1R3C	EFHD1	PPP1R3C or EFHD1	PPP1R3C and EFHD1
LR+	4.31	2.72	2.48	12.0
LR-	0.23	0.51	0.15	0.52
PPV	0.65	0.54	0.52	0.84
NPV	0.30	0.30	0.30	0.30

LR+: likelihood ratio for a positive finding LR-: likelihood ratio for a negative finding
PPV: positive predictive value NPV: negative predictive value

Figure

Figure1

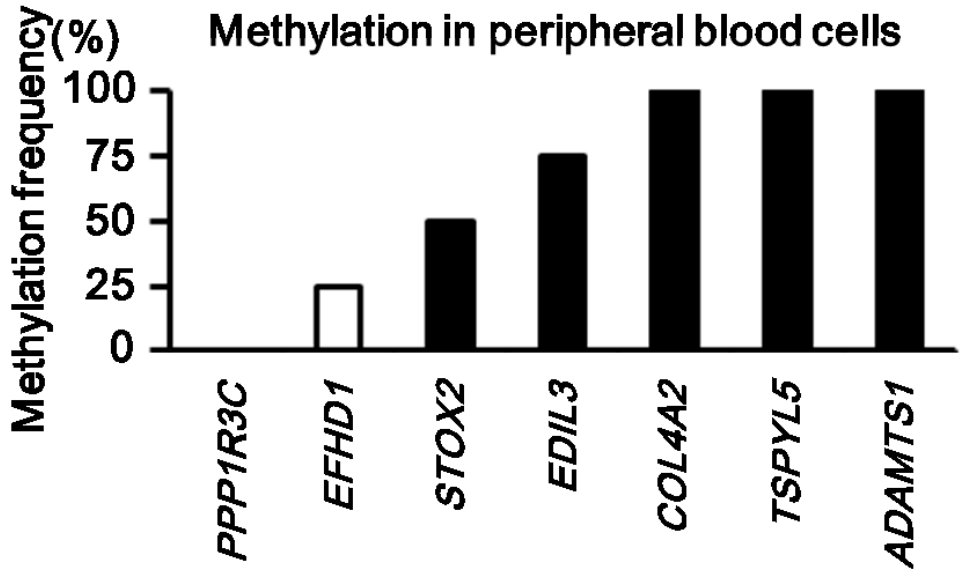
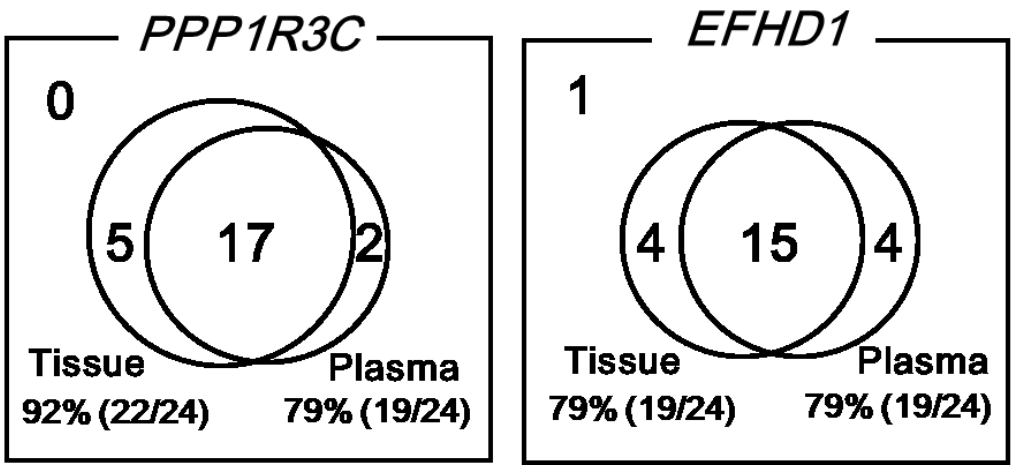


Figure2

A


PPP1R3C
0
5
17
2
Tissue
92% (22/24)
Plasma
79% (19/24)
EFHD1
1
4
15
4
Tissue
79% (19/24)
Plasma
79% (19/24)

B

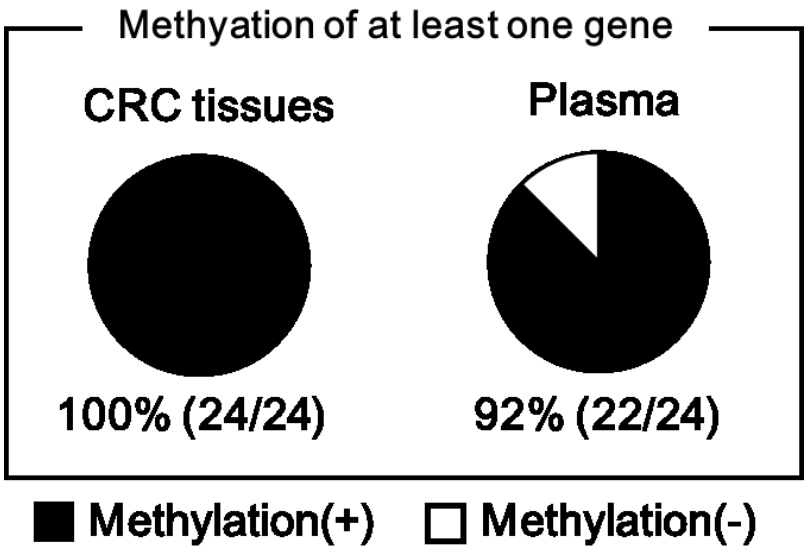
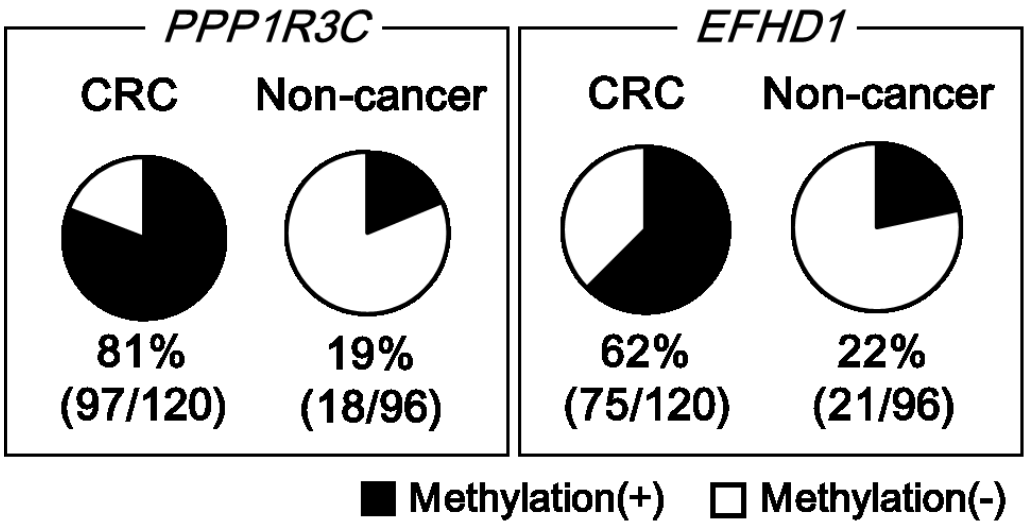
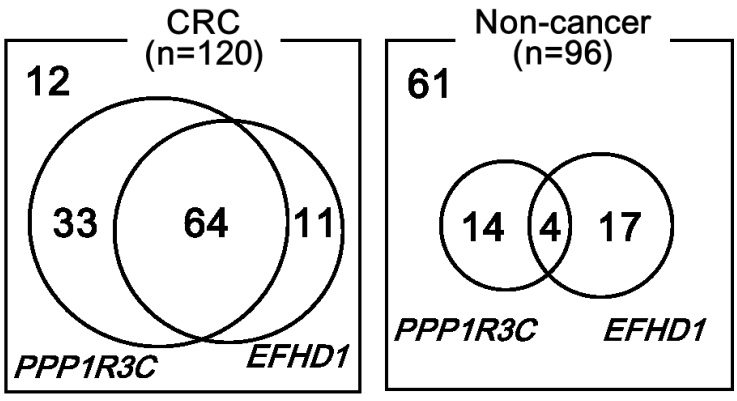

Methyation of at least one gene
CRC tissues
100% (24/24)
Plasma
92% (22/24)
Methylation(+)
Methylation(-)

Figure3



Figuere4

A



B

	PPP1R3C	*EFHD1*	*PPP1R3C* or *EFHD1*	*PPP1R3C* and *EFHD1*
Sensitivity	81%	63%	90%	53%
Specificity	81%	78%	64%	96%

Firure5

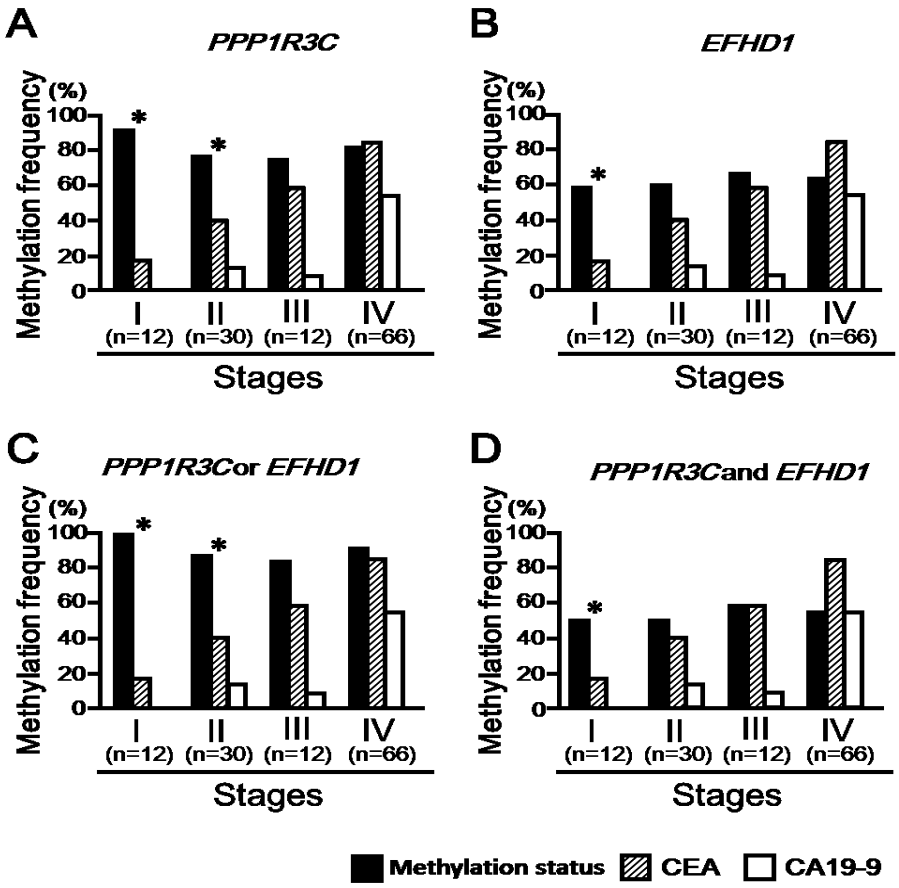


Figure Legends

Fig. 1. Screening of seven genes using peripheral blood cells. Methylation status was analyzed using peripheral blood cell samples from four non-cancer patients. *PPP1R3C* and *EFHD1* showed no or infrequent methylation (open box), but the other five genes showed methylation frequency in peripheral blood cells (closed box).

Fig. 2. Screening of two genes using CRC tissue and corresponding plasma samples. (A) Methylation status in plasma and CRC tissue samples from 24 CRC patients. In another set of 24 CRC tissue samples than those in Figure 1, *PPP1R3C* and *EFHD1* were confirmed to be frequently methylated at 92% (22/24) and 79% (19/24), respectively. The corresponding plasma samples were also frequently methylated at 79% (19/24) for each gene. (B) Positive ratio for the methylation of at least one gene. Among 24 patients, at least one of the two marker genes was methylated in 24 (100%) CRC tissue and 22 (92%) plasma samples.

Fig. 3. Methylation in plasma samples from 122 CRC patients and 96 non-cancer patients. *PPP1R3C* was methylated in 81% (97/120) of CRC patients and in 19% (18/96) of non-cancer patients ($P=6\times10^{-20}$, Fisher's exact test). *EFHD1* was methylated in 62% (75/120) of CRC patients and in 22% (21/96) of non-cancer patients ($P=3\times10^{-9}$).

Fig. 4. Sensitivity and specificity of methylation markers. A) Combination of the two genes. Positive ratio of methylation of both markers was 53% (64/120) for CRC patients, but only 4% (4/96) for non-cancer patients ($P=2\times10^{-16}$, Fisher's exact test), giving high specificity. Positive ratio of methylation of at least one of the two genes was 90% (108/120) for CRC patients, but only 36% (35/96) for non-cancer patients ($P=4\times10^{-17}$), giving high sensitivity. B) Sensitivity and specificity. Methylation of PPP1R3C gave better sensitivity and specificity, 81% and 81% respectively, than did *EFHD1*. When the positive ratio of methylation of at least one gene was analyzed, sensitivity was increased to 90%. When the positive ratio of methylation of both genes was analyzed, specificity was as high as 96%.

Fig. 5. Comparison of the methylation markers with tumor markers, CEA and CA19-9. Closed box, methylation. Hatched box, CEA. Open box, CA19-9. While positive ratios for CEA and CA19-9 were 64% (77/120) and 34% (41/120), respectively, methylation showed a higher methylation frequency, especially at early clinical stages. *P<0.05, between methylation and CEA and between methylation and CA19-9. A) Methylation of *PPP1R3C*. For stage I, 11 of 12 (92%) CRCs were *PPP1R3C* methylation(+), whereas 2 of 12 CRCs (17%) were CEA(+) ($P=3\times10^{-4}$, Fisher's exact test), and 0% (0/12) were CA19-9(+) ($P=5\times10^{-6}$). For stage II, 23 of 30 CRCs (77%) were *PPP1R3C* methylation(+), whereas 40% (12/30) were CEA(+) (P=0.004) and 13% (4/30) were CA19-9(+) ($P=7\times10^{-7}$). (B) Methylation of *EFHD1*. For stage I, 7 of 12 (58%) CRCs were *EFHD1* methylation(+) ($P=3\times10^{-4}$ against CEA, $P=5\times10^{-6}$against CA19-9). (C) Methylation of at least one gene. For stage I, 12 CRCs (100%) were methylation(+) ($P=3\times10^{-5}$ against CEA, $P=4\times10^{-7}$ against CA19-9). For stage II, as many as 26 out of 30 (87%) CRCs were methylation(+) ($P=2\times10^{-4}$ against CEA, $P=6\times10^{-9}$ against CA19-9). (D) Methylation of both *PPP1R3C* and *EFHD1*. For stage I, 6 CRCs (50%) were methylation(+) ($P=3\times10^{-5}$ against CEA, $P=4\times10^{-7}$ against CA19-9).

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要約

背景：ステージ 1 及び 2 の大腸癌患者における既存の腫瘍マーカー (CEA, CA19-9) の陽性率は 30%以下であり、スクリーニング及び診断検査としての有用性は低い。一方で、癌より血中に遊離する cell free DNA (cfDNA) のバイオマーカーとしての有用性が再認識され、癌診断への臨床応用が期待されている。

方法：以前に報告した大腸癌組織 149 検体においてプロモーター領域の CpG アイランドが高率にメチル化されている 44 遺伝子より、エピゲノムマーカーとして測定可能な新規メチル化マーカー候補遺伝子を選択した。これらの新規メチル化マーカー候補遺伝子について、大腸癌患者 120 人と健常者 96 人の血漿より cfDNA を抽出し、バイサルファイト処理後に methylation-specific PCR を行い、パイロシークエンス法で配列を決定して CpG アイランドのメチル化の頻度を比較検討した。

結果：新規メチル化マーカー候補遺伝子として *PPP1R3C* 及び *EFHD1* の 2 種類を選択した。それぞれのマーカーの組織及び血漿のメチル化陽性率は高い相関を示した($P < 0.001$)。*PPP1R3C* の血漿中における感度は 81%、特異度 81%、*EFHD1* では感度 62%、特異度 78%であった。また、上記 2 遺伝子のうち少なくとも 1 遺伝子のメチル化で陽性とした場合の感度は 96%、特異度は 53%であった。一方、2 遺伝子がともにメチル化している場合は感度 64%、特異度 90%であった。さらにステージ 1 及び 2 においても、同一患者での CEA 及び CA19-9 の

感度・特異度と比較して有意に高い正診率であった。

結論：大腸癌患者において、血中 cfDNA のメチル化はバイオマーカーとして有用であり、既存の腫瘍マーカーと比較して大腸癌スクリーニング及び診断に有用である。

研究業績

高根　希世子

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Ⅱ　論文	①原著論文	1 (単　1)
	②症例報告	1 (単　1)
	③総説	なし
Ⅲ　著書		なし

以上

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Ⅱ　論文

①原著論文

1、Kiyoko Takane, Yutaka Midorikawa, Koichi Yagi, Ayako Sakai, Hiroyuki Aburatani, Tadatoshi Takayama, and Atsushi Kaneda: Aberrant promoter methylation of *PPP1R3C* and *EFHD1* in plasma of colorectal cancer patients, Cancer Medicine, DOI: 10.1002/cam4.273, 2014

②症例報告

1、Kiyoko Takane, Yutaka Midorikawa, Shintaro Yamazaki, Takahiro Kajiwara, Naoki Yoshida, Yoshiaki Kusumi, Tadatoshi Takayama: Gastrointestinal stromal tumor with nephrotic syndrome as a paraneoplastic syndrome: a case report, J Med Case Rep. 2014;

8: 108. doi: 10.1186/1752-1947-8-108

③総説

なし

Ⅲ　著書

なし