Accumulation of aberrant DNA methylation during colorectal cancer development

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Abstract

Despite the recent advances in the therapeutic modalities, colorectal cancer (CRC) remains to be one of the most common causes of cancer-related death. CRC arises through accumulation of multiple genetic and epigenetic alterations that transform normal colonic epithelium into adenocarcinomas. Among crucial roles of epigenetic alterations, gene silencing by aberrant DNA methylation of promoter regions is one of the most important epigenetic mechanisms. Recent comprehensive methylation analyses on genome-wide scale revealed that sporadic CRC can be classified into distinct epigenotypes. Each epigenotype cooperates with specific genetic alterations, suggesting that they represent different molecular carcinogenic pathways. Precursor lesions of CRC, such as conventional and serrated adenomas, already show similar methylation accumulation to CRC, and can therefore be classified into those epigenotypes of CRC. In addition, specific DNA methylation already occurs in the normal colonic mucosa, which might be utilized for prediction of the personal CRC risk. DNA methylation is suggested to occur at an earlier stage than carcinoma formation, and may predict the molecular basis for future development of CRC. Here, we review DNA methylation and CRC classification, and discuss the possible clinical usefulness of DNA methylation as biomarkers for the diagnosis, prediction of the prognosis and the response to therapy of CRC.

Key words: Colorectal cancer; Colorectal adenoma; Aberrant crypt foci; Genetic mutation; Epigenotype; DNA methylation; Colorectal carcinogenesis

Core tip: Colorectal cancer (CRC) is a heterogeneous disease which involves several distinct molecular carcinogenic pathways. Recent comprehensive genome-wide analyses clarify detailed DNA methylation statuses of cancer-related genes in CRC. We and others have investigated the association between DNA methylation and genetic alterations, and performed classification of CRC/their precursors, including conventional adenomas, serrated adenomas, non-polypoid colorectal neoplasms and aberrant crypt foci. In addition, we also evaluated the usefulness of DNA methylation markers as surrogate biomarkers for diagnosis, prognosis and therapeutic application of CRC. Here, we review the DNA methylation status and classification of CRC to understand the roles of DNA methylation in colorectal carcinogenesis.

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INTRODUCTION
Colorectal cancer (CRC) arises through accumulation of multiple acquired genetic and epigenetic alterations that cause malignant transformation of normal colonic epithelium to adenocarcinoma[12,13]. These carcinogenic processes were first described in the model of the adenoma-carcinoma sequence[14], and somatic mutations of tumor-suppressor genes (e.g., APC, p53 and DCC) and activating mutations in the KR-AS oncogene are well-known genetic alterations involved in this model[13-15] (Table 1).

CRC can be biologically divided into those with microsatellite instability (MSI), characterized by DNA replication and repair defects, and those with chromosomal instability (CIN), characterized by aneuploidy, multiple chromosomal rearrangements and accumulation of somatic mutations in oncogenes[16]. These genomic instabilities have been reported to be closely associated with the molecular heterogeneity of CRC[17], which is a factor responsible for the significant variability in the prognosis and treatment response among patients with the same stage of CRC[18]. Since the distinct molecular subtypes of CRC are difficult to be accurately distinguished histologically or clinically, technologies that can detect significant molecular alterations in CRC on genome-wide scale had been expected to be developed. Recently, exome sequencing analyses revealed the involvement of many somatic mutations of genes, including SMAD4, FBXW7, TCF7L2, and FAM123B[11,19]. Although hundreds of mutations, on average, are found in genomes of CRC, only a small set of functionally important genes are proposed to be involved with cancer formation as driver genes in individual cancer[20]. Whereas key mutational changes are necessary for the initiation and progression of CRC, the number of genes silenced by epigenetic mechanisms is greater than the number of genetic mutations in CRC[21], suggesting a crucial role of epigenetic alterations.

Recently, we and other groups performed epigenotyping of CRC, by unsupervised hierarchical clustering method using comprehensive and quantitative methylation data (Table 2). These results demonstrated that CRC can be clearly clustered into three DNA methylation epigenotypes[12-14]. Interestingly, each of the epigenotypes showed a unique association with a variety of genetic mutations (i.e., BRAF, KR-A3 and TP53) and the genomic instability status of sporadic CRC, indicating that they develop through distinct carcinogenic pathways. Moreover, the intermediate-methylation subtype with KR-A3 mutation showed a poorer prognosis than other subtypes, suggesting that the DNA methylation status could be used as prognostic markers. Subsequently, we conducted epigenotyping of conventional adenomas and serrated adenomas, and showed that these precursor lesions of CRC can also be classified into the three epigenotypes[22]. These findings suggested that epigenotype development occur at an earlier stage than carcinoma formation, and is already completed at the adenoma stage. In this review, we focus on the importance of DNA methylation and provide an overview of the classification of CRC and their precursors, and discuss the clinical applications of aberrant DNA methylation as biomarkers for the diagnosis, prediction of the prognosis and the response to therapy of CRC.

ABERRANT DNA METHYLATION ON GENE PROMOTERS
Two major types of epigenetic alterations closely linked to CRC are aberrant DNA methylation and covalent histone modifications[16]. Gene silencing by aberrant DNA methylation of its promoter region is one of the most important epigenetic mechanisms to inactivate the expression of tumor-suppressor genes. While the majority of CpG sites in the genome are known to be methylated in normal mammalian cells, unmethylated CpG sites are typically present in the genomic regions known as CpG islands. CpG islands are reported to overlap the promoter regions in 60%-70% of genes and tend to be protected from methylation; however they can be aberrantly methylated during the carcinogenic process[17]. Investigation of genes using aberrant methylation as markers is useful to identify novel tumor-suppressor genes and methylation markers for cancer classification[18-23]. Therefore, several methods for genome-wide analysis have been developed since the 1990s[24-28]. These epigenetic alterations have been noted to play crucial roles not only in cancer progression, but also in cancer initiation, since the alterations have been identified in the pre-cancerous “normal” tissues that could modify cancer risk[22,29]. Recently, genome-wide DNA methylation analysis tools have been developed to reveal the detailed epigenetic backgrounds of CRC[12-14]. Importantly, gene silencing resulting from aberrant DNA methylation cooperates with other genetic mechanisms to alter the key molecular pathways critical in colorectal carcinogenesis[25] (Figure 1).

CLASSIFICATION OF CRC USING DNA METHYLATION INFORMATION
CpG island methylator phenotype
In 1999, Toyota et al. reported that some CRCs show a significantly high frequency of aberrant DNA methylation in specific CpG islands, named CpG island methylator phenotype (CIMP). CIMP-positive CRC shows DNA hypermethylation at a specific subset of genomic loci[21-23] and is highly enriched for an activating mutation of BRAF[34,35]. Hypermethylation of CpG islands in gene promoter regions results in transcriptional repression. For example, CIMP-mediated gene silencing of the mismatch repair gene MLH1 by promoter hypermethylation is the molecular basis for MSI in sporadic microsatellite-unstable CRC, and most sporadic microsatellite-unstable
CRC are therefore CIMP-positive\(^\text{[34]}\). CIMP-positive CRC inversely correlates with CRC with CIN\(^\text{[37,38]}\), tends to occur in the proximal colon, and is commonly observed in women\(^\text{[32]}\), suggesting that they appear to develop distinct carcinogenic pathway from CIMP-negative CRC.

**DNA methylation markers and CRC epigenotypes**

Since the first CIMP markers were identified by Toyota et al\(^\text{[20,21]}\), many other CIMP markers have been described, e.g., MLH1, NEUROG1, SOCS1, RUNX3, IGF2 and CACNA1C\(^\text{[18-22,35]}\). Using quantitative real-time PCR, Ogino et al\(^\text{[39]}\) selected five CIMP markers to distinguish high from low levels of CIMP-mediated gene promoter methylation, and found that CIMP-low CRC tends to be associated with male sex and KRAS mutations. CIMP-low appears to be independent of the MSI status, suggesting that CIMP-low might be a different subtype of CRC from CIMP-high and CIMP-0. However, no clear difference was observed between CIMP-low and CIMP-0, because these methylation markers were specific for CIMP-high and not ideal for identification of the CIMP-low subtype. Sensitive and specific markers for CIMP-low were needed to be determined.

According to the results of unsupervised two-way hierarchical clustering based on the quantitative DNA methylation data of 27 previously reported gene promoter and genetic alterations, including mutations of BRAF, KRAS, and p53, Shen et al\(^\text{[13]}\) proposed that CRC can be classified into three subsets, CIMP1, CIMP2, and CIMP-negative. This report successfully showed the existence of three clusters of CRC with different molecular characteristics: (1) CIMP1 with MSI-high (80%), BRAF mutation (53%) and high-methylation; (2) CIMP2 with KRAS mutation (92%) and different methylation; and (3) CIMP-negative with p53 mutation (71%) and absence of these methylations. Integrated genetic and epigenetic analysis was found to be important, and genetic markers performed better than epigenetic markers in their classification of CRC\(^\text{[13]}\).

To clarify whether CRC can be classified into more than two subsets using information on methylation accumulation alone, we performed comprehensive two-way unsupervised hierarchical clustering, using quantitative methylation data of genome-wide selected novel markers that were established through MeDIP-chip analysis. We demonstrated that CRC can be clearly classified into three distinct epigenotypes: high-, intermediate-, and low-methylation epigenotypes (HME, IME, and LME). HME was strongly correlated to the presence of the BRAF mutation (71%) and MSI-high (76%), and IME was

### Table 1 Colorectal carcinogenic pathways and genetic alterations

| Pathway                     | MSI | Methylation | KRAS | BRAF | TP53 | Reports                  |
|-----------------------------|-----|-------------|------|------|------|--------------------------|
| Adenoma-carcinoma sequence | -   | +/-         | ++   | -    | +    | Grady et al\(^\text{[41]}\)  |
| Serrated pathway           | +   | +++         | +    | ++   | +/-. | Vogelstein et al\(^\text{[40]}\) |
| De novo pathway            | -   | -           | -    | -    | -    | Howkins et al\(^\text{[32]}\) |

MSI: Microsatellite instability.

### Table 2 Reports on colorectal cancer classification by methylation information

| Ref.           | Marker selection      | Methylation analysis methods | Classification method | Methylation phenotypes |
|----------------|-----------------------|------------------------------|-----------------------|------------------------|
| Toyota et al\(^\text{[20]}\) | Genome-wide (MCA-RDA) | COBRA                        | Methylation frequency | CIMP+                  |
| Weisenberger et al\(^\text{[35]}\) | MethyLight markers   | Methylight                   | Hierarchical clustering | CIMP+                  |
| Ogino et al\(^\text{[39]}\)    | Reported markers      | Methylight                   | Methylation frequency | CIMP-high              |
| Shen et al\(^\text{[13]}\)     | Reported markers      | Pyrosequence                 | Hierarchical clustering | CIMP-L                 |
| Yagi et al\(^\text{[14]}\)     | Genome-wide (MeDIP-chip) | MassARRAY                  | Hierarchical clustering | HME                   |
| Hinoue et al\(^\text{[12]}\)  | Genome-wide (Infinium 27k) | Methylight                  | Hierarchical clustering | CIMP-H                |

CIMP: CpG island methylator phenotype; MCA: Methylated CpG island amplification; RDA: Representation difference analysis; COBRA: Combined bisulfite restriction analysis; MSP: Methylated DNA immunoprecipitation; LME: Low-methylation epigenotype; IME: Intermediate-epigenotype; HME: High-methylation epigenotype.
Colorectal cancer (CRC) arises through accumulation of multiple acquired genetic and epigenetic alterations. Adenomas and CRCs can be classified into several subgroups based on the status of DNA methylation and associated genetic mutations, suggesting that the different types of CRC developed through different molecular carcinogenetic pathways. DNA methylation accumulation occurs during aberrant cell expansion and is usually completed at adenoma stage. Non-polypoid colorectal neoplasms are hypothesized to develop through de novo pathway, whereas the epigenetic features of laterally spreading tumors have not yet been fully investigated.

Figure 1  Carcinogenetic pathway and classification of colorectal cancer. Colorectal cancer (CRC) arises through accumulation of multiple acquired genetic and epigenetic alterations. Adenomas and CRCs can be classified into several subgroups based on the status of DNA methylation and associated genetic mutations, suggesting that the different types of CRC developed through different molecular carcinogenetic pathways. DNA methylation accumulation occurs during aberrant cell expansion and is usually completed at adenoma stage. Non-polypoid colorectal neoplasms are hypothesized to develop through de novo pathway, whereas the epigenetic features of laterally spreading tumors have not yet been fully investigated.

遗传和表观遗传学特征

遗传和表观遗传学特征的Serrated腺瘤

Serrated腺瘤是通过KRAS突变（63%）的发生。在我们的分析中，p53突变在HME中没有检测到，但在IME和LME中检测到。它值得注意的是，甲基化标记物被分为两种类型：(1) Group-1标记物包括大多数已知的CIMP标记物，显示在HME中具体发生的遗传和表观遗传学特征；(2) Group-2标记物包括新型甲基化标记物，显示在HME和IME中。它也值得注意的是，带有IME KRAS突变(+)-CRC的患者表现出显著的更差的预后。

在我们的报告中，Hinoue et al.[12]进行DNA甲基化 profiling的CRC使用Illumina Infinium DNA甲基化微阵列，报告说CRC可以被分为三种不同的表观遗传学特征(CIMP-H, CIMP-L, 和Non-CIMP)，与以前的报告一致[13,14]。遗传和表观遗传学特征的CIMP-H/CIMP-L CRC和HME/IME CRC是一致的，并与观察到的CIMP1/CIMP2 CRC[13]和HME/IME CRC[14]一致。根据p53突变的频率，他们认为非-CIMP CRC可以被分类到两个不同的子组中，其中一个显著的更高的频率p53突变(65%)和频繁发生在直肠的外侧，和那个与存在缺乏的各自特定的DNA甲基化和基因突变，和更频繁地出现频率的整个区域。

### CLASSIFICATION OF PRECURSOR LESIONS OF CRC

#### Genetic and epigenetic alterations of serrated adenomas

The majority of sporadic CRC is thought to develop from conventional adenomas through the adenoma-carcinoma sequence[3], whereas the serrated pathway has been considered to be an alternative pathway distinct from the adenoma-carcinoma sequence. The serrated pathway is known to involve mutation of BR-AF, MLH1 methylation and CIMP[14,18]. While serrated adenomas are commonly CIMP-high and carry the BR-AF mutation[34,40-45], conventional adenomas rarely exhibit these genetic and epigenetic alterations[40]. In addition, the risk factors for CIMP-high serrated adenomas are reported to be similar to those of CRC with CIMP[45]. Serrated adenoma is therefore considered to be a precursor of CIMP-positive CRC. Although CIMP-high and BR-AF mutation were frequently observed at the adenoma stage, the prevalence of MLH1 methylation was lower than that of CRC with CIMP[40-42,45]. Interestingly, MLH1 methylation was more frequently observed in proximal, large serrated adenomas[46], suggesting that MLH1 methylation is a late event in the serrated pathway, and heralds the transition from serrated adenoma to CRC, involving the mutator phenotype.

#### Epigenotype of conventional adenomas

While the genetic and epigenetic features among conventional and serrated adenomas have been demonstrated to be widely different, existence of DNA methylation phenotypes within conventional adenomas and their correlation to genetic mutations were not fully investigated. We investigated whether conventional adenomas could be classified into epigenotypes, and our CRC classification markers successfully classified conventional adenomas into two distinct epigenotypes, IME and LME[18]. There were no remarkable differences in the morphological and
pathological features among the two epigenotypes. While IME adenomas showed a significantly high frequency of the KRAS mutation (62%), LME adenomas did not show any genetic alterations, similar to the case of LME CRC. Interestingly, there was no difference in the methylation level between IME adenoma and IME cancer, suggesting that accumulation of aberrant DNA methylation is mostly completed at the adenoma stage. This indicated that additional aberration(s) other than DNA methylation are needed for adenomas to transform into CRC. The progression of adenomas to CRC is postulated to be associated with p53 abnormalities[9], and DNA methylation of some genes, e.g., MGMT, CXLC12, TIMP3, ID4, and IRF8, might also be involved in the development to CRC[66,67].

**Genetic and epigenetic alterations of non-polypoid colorectal neoplasms**

Non-polypoid colorectal neoplasms that do not exhibit a macroscopic protruding appearance have been documented not only in Japan[46-53], but also in western countries[54,55]. They are characterized by lateral extensions along the luminal wall with a low vertical axis, and such tumors with a diameter of > 10 mm are called laterally spreading tumors (LSTs)[48]. The incidence of genetic alterations such as KRAS, BRAF and p53[49,56-58] and MSI[59] were less common in these non-polypoid colorectal neoplasms than those in conventional adenomas. In addition, a high percentage of these lesions are reported to exhibit high-grade dysplasia and rapidly invade the submucosal layer despite their small sizes[50-52,60]. Therefore, non-polypoid colorectal neoplasms are hypothesized to develop through an alternative carcinogenic pathway (i.e., de novo pathway) different from the adenoma-carcinoma sequence and the serrated pathway. LSTs are usually categorized into two subtypes based on their macroscopic morphology: the granular type and non-granular type[48]. Whereas the epigenetic features of LST have not yet been fully investigated, Hiraoaka et al[61] reported frequent methylation of CIMP-markers and frequent KRAS mutation in the granular type, but not in the non-granular type. LST might be composed of several subtypes which exhibit distinct molecular pathway, and further investigations are needed to reveal the genetic and epigenetic features of non-polypoid colorectal neoplasms and their association with colorectal carcinogenesis.

**CLASSIFICATION OF ABERRANT CRYPT FOCD**

**Aberrant crypt foci and colorectal carcinogenesis**

Aberrant crypt foci (ACF) are microscopic mucosal abnormalities, a subset of which postulated to be the earliest precursors of CRC[62]. ACF show increased expression of proliferative markers[63], and a significant correlation has been reported to exist between the presence of ACF and synchronous advanced neoplasia[62,64,65], suggesting a positive role of these lesions in colorectal carcinogenesis. Therefore, ACF have been recognized as a useful surrogate biomarker for CRC surveillance[62] and been used in recent chemoprevention trials[73-77]. Histopathologically, human ACF can be sub-classified into two categories: dysplastic and heteroplastic[62]. Dysplastic ACF resemble adenomas and sometimes lack mucin production[76,77], and are more common in familial adenomatous polyposis (FAP) patients than in sporadic CRC patients[77]. In contrast, heteroplastic ACF resemble hyperplastic polyps and lack dysplasia, and are highly identified in sporadic CRC patients.

**Genetic and epigenetic alterations in aberrant crypt foci**

Although all ACF from FAP patients carry the APC mutation[69], both the dysplastic and heteroplastic ACF from sporadic CRC patients frequently carry KRAS mutation, but not the APC mutation[67,80]. While BRAF mutation has rarely been identified in ACF[79,82], Rosenberg et al[85] reported that heteroplastic ACF with serrated pathology exclusively exhibit BRAF mutation. Although there was a report that CIMP-high was less frequently observed in ACF in sporadic CRC patients[80], the methylation status of ACF has not been well investigated. In our DNA methylation analysis in heteroplastic ACF, ACF showed frequent KRAS mutation, consist with previous reports. The levels of aberrant DNA methylation were significantly lower compared to adenomas[83], suggesting that DNA methylation accumulation might be requested during aberrant cell expansion in adenoma formation, but not in ACF formation.

**DNA METHYLATION IN APPARENTLY NORMAL MUCOSA**

Some of genes showing aberrant methylation in CRC, such as ESR1, IGF2 and TUSC3 are also methylated in histologically normal colonic epithelium. Aberrant DNA methylation of these genes is considered to increase in an age-dependent manner, and approximately half of them have also been shown to be involved in the pathogenesis of CRC[84-86].

The concept of “field cancerization” was proposed to explain the multiple primary lesions, local recurrence and increased susceptibility of normal tissue to malignant transformation[87]. The field changes occur at the molecular level, and these abnormalities of the normal colonic epithelium could be potential biomarkers for assessing the personal risk for future CRC development. Suzuki et al[88] reported that a higher incidence of hypermethylation and down-regulation of the JFRP genes, negative regulators of the WNT signaling pathway, were observed in the normal colonic mucosa from patients with CRC, than in that from patients without CRC. In addition, Kawakami et al[89] reported that higher methylation levels of age-related markers, such as ESR1 and MYOD, were observed in the normal colonic mucosa from patients with CIMP-positive CRC than in that from patients without CRC. It was reported, in contrast, that lower methylation levels
of these markers were observed in the normal colonic mucosa from patients with CRC than from patients without CRC. Recently, genome-wide DNA methylation analysis revealed that the gene methylation levels involved in the metabolic pathways of carbohydrates, lipids and amino acids were significantly different among normal colonic mucosa specimens obtained from patients with and without CRC. While DNA methylation accumulation is expected to contribute to field cancerization in the colon, further studies are necessary to establish useful surrogate biomarkers for CRC surveillance.

CLINICAL APPLICATION OF DNA METHYLATION MARKERS

Early CRC detection could contribute to a reduction of CRC-related mortality. However, strategies such as colonoscopy are invasive, whereas the less-invasive fecal blood test shows low sensitivity and specificity. Identification of noninvasively testable, high-quality biomarkers for CRC is therefore necessary. Recent genome-wide analyses were conducted to identify candidate DNA methylation markers for early CRC detection, by comparing the DNA methylation levels between CRC and/or adenomas, and matched normal colonic mucosa. For example, Mori et al. reported that the methylation status of VSX2 showed a high discriminative accuracy (83% sensitivity and 92% specificity). These potential biomarkers may allow reliable discrimination of CRC patients from tumor-free patients. Several clinical studies have been carried out to confirm the usefulness of stool and blood DNA-based methylation markers for early CRC detection. In any application, classification marker genes are specifically methylated in some epigenotypes, therefore, genes that are commonly methylated in all CRCs, regardless of the epigenotype, would be useful markers for early CRC detection.

The MSI status has been proposed as a biomarker for determination of the prognosis and/or the effectiveness of FU chemotherapy in advanced CRC patients. KR-RAF mutation in advanced CRC has been reported to be associated with a poor prognosis, and the usefulness of determining its presence for predicting a lack of response to EGFR-targeted therapy is well proven. Our previous study revealed that IME CRC with KR-RAF mutation is associated with a poor prognosis. DNA methylation biomarkers for prediction of the therapeutic responses of CRC, however, have not been identified yet. Additional studies are needed to establish methylation biomarkers for application in clinical practice, e.g., for prediction of the prognosis and of the responses to therapy of CRC.

CONCLUSION

Recent comprehensive genome-wide methylation analyses revealed that sporadic CRC can be classified into three distinct epigenotypes. Each of these CRC epigenotypes cooperates with specific genetic alterations, suggesting that they develop through different molecular carcinogenic pathways. Serrated adenomas are commonly CIMP-high and carry BRAF mutation, thus postulated to be precursor lesions of CIMP-positive, MSI-high proximal CRCs. MLH1 methylation has been suggested to be a late event in the serrated pathway, and heralds the transition from serrated adenoma to CIMP-positive CRC. Conventional adenomas can also be classified into two distinct epigenotypes. DNA methylation accumulation is mostly completed by the adenoma stage, and conventional adenomas are hypothesized to be precursors of CIMP1/IME/CIMP-low and CIMP0/LME/Non-CIMP CRCs. ACF showed significantly lower methylation levels than adenomas, suggesting that DNA methylation accumulation is a prerequisite for aberrant cell expansion in adenoma formation, but not in the formation of ACF.

DNA methylation may predict the molecular basis of CRC, and these markers might be present as useful surrogate markers for the diagnosis, prediction of the prognosis and the response to therapy of CRC. Some genes already showed aberrant methylation in apparently normal colonic mucosa, and their methylation may be related to field cancerization of CRC and predict cancer risk. Continued efforts to investigate the associations between molecular mechanisms of CRC and genetic/epigenetic alterations may allow us to understand colorectal carcinogenesis, and lead to the translation of these insights into clinical practice.

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Définitions

**Sakai E et al.** DNA methylation and CRC

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