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Review

Epigenetic alterations in colorectal cancer: the CpG island methylator phenotype

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Summary. DNA methylation is one of the key mechanisms of epigenetic modification, and genome-wide hypomethylation and CpG island hypermethylation are characteristics of cancer cells. The CpG island methylator phenotype (CIMP) is a distinctive subtype of colorectal cancers (CRCs) that show concordant hypermethylation of numerous promoter CpG island loci. CIMP-positive CRCs are associated with a proximal location in the colon, microsatellite instability, *BRAF* mutation and a relatively poor clinical outcome. CIMP-positive CRCs have their own precursor lesions, serrated adenomas, distinct from conventional adenomas which progress and transform into CIMP-negative CRCs. Although the existence of CIMP-positive CRCs is generally accepted, there has been controversy over technical issues with gene markers, the methodology used to define CIMP, and the prognostic or predictive role of CIMP. This review addresses recent advances in the field of CIMP-related research.

Key words: Colorectal cancers, CpG island methylator phenotype, DNA methylation, Epigenetics

Introduction

Epigenetic modifications are heritable changes in gene expression that are not accompanied by changes in the DNA sequence. Epigenetic change occurs through various mechanisms, such as DNA methylation (promoter CpG island hypermethylation), histone modification, chromatin looping and condensation, and small non-coding RNAs (Laird, 2005). Promoter CpG island hypermethylation is one of the best-known epigenetic modifications and is associated with many physiologic and pathologic conditions. In physiologic conditions, promoter CpG island hypermethylation regulates genomic imprinting, X-chromosome inactivation, embryonic development, and silencing of repetitive DNA elements and germ cell-specific genes. In pathologic conditions, DNA promoter CpG island hypermethylation is involved in neurodevelopmental and degenerative disorders, autoimmune diseases, and in almost all types of cancers (Bird, 2002; Jones and Baylin, 2007; De Carvalho et al., 2010; Meissner, 2010; Portela and Esteller, 2010)

Colorectal cancer (CRC) is well-studied in terms of DNA methylation changes. Initially, attention was focused on DNA hypomethylation of specific genes or genome-wide hypomethylation, which was found in colorectal adenomas and CRCs (Feinberg and Vogelstein, 1983; Goelz et al., 1985). Later, the Baylin lab was the first to pay attention to promoter CpG island hypermethylation of single genes in human neoplasia, and these researchers reported Calcitonin gene hypermethylation in colon adenomas and CRCs (Silverman et al., 1989). After that publication, many reports exploring promoter CpG island hypermethylation of single genes were published, culminating in the Issa lab first demonstrating concordant hypermethylation of multiple genes or CpG island loci in a subset of CRCs and introducing the concept of the CpG island methylator phenotype (CIMP) in CRCs (Toyota et al., 1999). CIMP-positive CRCs, which constitute 8-20% of all CRCs, are known to be associated with older age at diagnosis, proximal location in the colon, microsatellite instability (MSI) and BRAF mutation (Toyota et al., 1999; Hawkins et al., 2002; van Rijnsoever et al., 2002; Weisenberger et al., 2006; Lee et al., 2008a). However, the exact causes of CIMP are not yet known, and there is still controversy regarding the clinical usefulness of this category. Because of the lack of a universal standard or

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consensus on gene marker panels, marker threshold values, and laboratory techniques to define CIMP, it is a challenge to pool data from case-series or population-based studies that explore CIMP and its clinicopathologic, epidemiological, or molecular features in CRCs. In this paper, we review the literature documenting the clinicopathological and molecular features of CIMP-positive CRCs to provide a summary of current knowledge on CIMP in CRCs.

Promoter CpG islands and CIMP

DNA methylation occurs on the 5' cytosine moiety in the context of CpG nucleotides in mammals (Bestor, 2000). In the human genome, CpG nucleotides are not evenly distributed but are aggregated in certain regions, called CpG islands. CpG islands are defined as sequences > 200-500 bps in length with greater than 50% CG content and a ratio of observed CpG to expected CpG of >0.6 (Gardiner-Garden and Frommer, 1987). Approximately 37% of CpG islands are localized to gene promoters, and up to 70% of known genes have CpG islands in the region from -2 kilobases (kb) to +1 kb of their transcription start site (Takai and Jones, 2002; Saxonov et al., 2006). In normal cells, the promoter CpG island loci are scarcely methylated, but these protected promoter CpG island loci undergo aberrant hypermethylation in association with cancer development. The majority of the genes that undergo aberrant promoter CpG island hypermethylation in cancer cells are inactive in normal cells, but a minority of the genes that are active in normal cells can undergo aberrant hypermethylation, resulting in transcriptional inactivation in their malignant counterparts (Berman et al., 2012) (Fig. 1). Methylation of promoter CpG island loci is associated with transcriptional repression, but the relationship between DNA methylation and transcriptional activity in non-CpG island promoters is less clear.

Although it is possible that promoter CpG island hypermethylation is a stochastic process that is selected for in neoplastic cells, hypermethylation might result from specific defects in the methylation process. Through determination of the methylation status of multiple genes in a large number of CRC samples, it was recognized that in a subset of CRCs, methylation was simultaneously detected at multiple genes and that methylation of two separate genes was statistically correlated in a given tumor sample (Toyota et al., 1999). Akin to the mutator phenotype in which the rate of acquisition of genetic defects is accelerated, the hypermethylator phenotype is associated with an elevated frequency of promoter CpG island hypermethylation. Issa and colleagues were the first to recognize this subtype of CRCs and coined the name CIMP to identify a subset of CRCs with widespread concordant hypermethylation of multiple promoter CpG island loci (Toyota et al., 1999). However, the concept of CIMP was met with substantial skepticism from several researchers who argued that this phenomenon could be a misinterpretation of age-dependent methylation, a by-product of the neoplastic process, or a statistical artifact (Bestor, 2003; Yamashita et al., 2003; Anacleto et al., 2005). However, many studies confirmed the clinical relevance of CIMP in hospital- or population-based samples (Samowitz et al., 2005; Ogino et al., 2007a; Lee et al., 2008a; Dahlin et al., 2010). Recently, genome-wide methylation studies confirmed genome-scale differences of CpG island methylation in CIMP-positive and CIMP-negative CRCs (Hinoue et al., 2012; Xu et al., 2012). Furthermore, CIMP-positive tumors and CIMP-negative tumors are now recognized as having

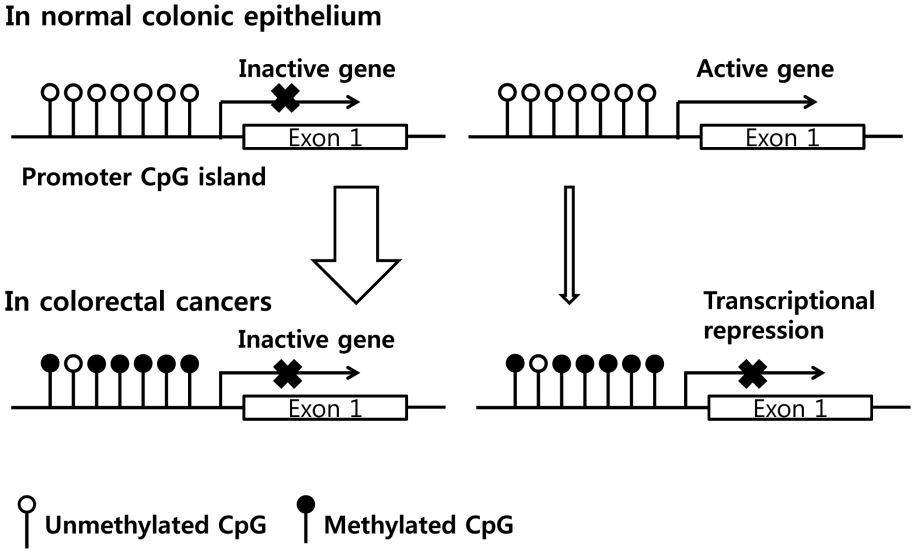

Fig. 1. Compared to active genes in normal cells, inactive genes in normal cells are much more likely to undergo aberrant hypermethylation of their promoter CpG island loci during the process of multistep colorectal carcinogenesis.

evolved along different morphological progression routes: the serrated neoplasia pathway and the adenoma-carcinoma sequence pathway, respectively (Hawkins and Ward, 2001; Jass, 2002; Kwon et al., 2010; Kang, 2011)

A variety of methylation marker panels and methodologies are used to define and classify different CIMPs

Because of the lack of reference methodology and reference panels of DNA methylation markers to determine CIMP-positive CRCs, researchers use their own methodologies and marker panels to define CIMP-positive CRCs, which leads to inconsistencies in whether a tumor is appropriately defined as a CIMP-positive CRC. The varied methods and gene marker panels used for the determination of CIMP are summarized in Table 1. Originally, Issa's group used the combined bisulfite restriction analysis and a panel of seven cancer-specific DNA methylation markers to diagnose CIMP (Toyota et al., 1999). However, Issa's group subsequently reduced the number of markers from seven to five, which include *MINT1, MINT2, MINT31, CDKN2A (p16)* and *MLH1* (Issa, 2004). In 2006, the Laird team developed a MethyLight-based method for the determination of CIMP using a panel of five DNA methylation markers, *CACNA1G, IGF2, NEUROG1, RUNX3* and *SOCS1*, which were selected from 92 cancer-specific DNA methylation markers (Weisenberger et al., 2006). On the MethyLight platform, the new five-marker panel has a superior performance to that of the classic five-marker panel (*MINT1, MINT2, MINT31, CDKN2A* and *MLH1*) in that the new CIMP panel easily detected a heavily methylated subset of CRCs that encompasses almost all *BRAF* mutants and sporadic MSI-positive CRCs. In the same year, another MethyLight-based method using a five-marker panel (*CACNA1G, CDKN2A, CRABP1, MLH1* and *NEUROG1*) was proposed by Ogino et al. (2006a), who later combined their own five-marker panel with the Laird team's five-marker panel into an eight-marker panel (*CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3,* and *SOCS1*) (Ogino et al., 2007a,b). The eight-marker panel was shown to outperform the five-marker panels in comparisons of the association of CIMP-positive CRCs with characteristics of CRCs that have previously been reported to be associated with CIMP-positive status (Ogino et al., 2006a-c, 2007a,b; Kim et al., 2009). Meanwhile, Ogino et al. noted the existence of an intermediate-methylation subgroup, referred to as CIMP-low, and found that CIMP-low tumors, defined as tumors with 1/5 to 3/5 (five-marker panel) or 1/8 to 5/8 methylated markers (eight-marker panel), had close associations with the *KRAS* mutation and male gender but no association with MSI (Ogino et al., 2006a-c; Ogino and Goel, 2008). These results contrasted with the close association of

Table 1. Summary of gene panels and methodologies used to define CpG island methylator phenotype in colorectal cancer.

Study	Gene panel	Methodology	Features of CIMP-positive tumors
Toyota et al., 1999	MINT1, MINT2, MINT12, MINT17, MINT25, MINT27, and MINT31	COBRA	Proximal colon location and MSI
Issa, 2004	p16, MINT1, MINT12, MINT31, and MLH1	COBRA	Poorly differentiated mucinous carcinoma, KRAS or BRAF mutations, and MLH1-associated MSI
Ogino et al., 2006a	CACNA1G, CDKN2A, CRABP1, MLH1, and NEUROG1	MethyLight	Female gender, MSI, BRAF mutations and wild-type KRAS
Weisenberger et al., 2006	CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1	MethyLight	Female gender, proximal colon location, MSI, BRAF mutation and wild-type KRAS
Ogino et al., 2007a	CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1	MethyLight	Female gender, older age at onset, proximal colon location, MSI, BRAF mutation, and wild-type KRAS
Shen et al., 2007a	MINT1, MINT2, MINT12, MINT17, MINT27, MINT31, MLH1, p14, THBS1, THBS2, MGMT, MEGALIN, COX2, RIZ1, p16, RUNX3, RASSF1A, DAPK, TIMP3, TERT, NEUROG1, SOCS1, ER, MYOD, N33, SFRP1, and HPP1	COBRA or pyrosequencing	MSI, BRAF mutation, wild-type KRAS, and wild-type TP53
Yagi et al., 2010	CACNA1G, LOX, and SLC30A10	MassArray (Sequenom)	Female gender, older age at onset, proximal colon location, mucinous histology, poor differentiation, MSI, BRAF mutation, wild-type KRAS, and negative staining in p53 immunohistochemistry
Ahn et al., 2011	MINT1, MINT2, MINT31, p16, MLH1, p14, SFRP1, SFRP2, and WNT5A	Pyrosequencing	Proximal colon location and MSI
Hinoue et al., 2012	FAM78A, MYOCD, KCNC1, FSTL1, SLC6A4	Infinium Human Methylation 27 Beadchip	Proximal colon location, female gender, wild-type TP53, BRAF mutation, MLH1 methylation-associated MSI

COBRA, combined bisulfite restriction analysis; CIMP, CpG island methylator phenotype; MSI, microsatellite instability

CIMP-positive tumors with *BRAF* mutations, MSI and female gender. In 2007, Shen et al. conducted clustering analysis, integrating both epigenetic changes of 27 DNA methylation markers and genetic changes, including the *BRAF, KRAS*, and *TP53* mutations and MSI, which generated three clusters, CIMP1, CIMP2 and CIMP-negative. CIMP2 tumors, an intermediate-methylation subgroup, showed a close association with the *KRAS* mutation and no association with MSI (Shen et al., 2007a-c). In 2010, Yagi and Kaneda developed a MassARRAY (MALDI-TOF mass spectrometry)-based method to define CIMP status, using a two-step CIMP marker panel composed of 7 markers (panel-1, *CACNA1G, LOX, SLC30A10*; and panel-2, *ELMO1, FBN2, HAND1, THBD,* and *SLC30A10* again) (Yagi et al., 2010). Panel-1 and panel-2 were designed to be specific for the detection of high- and intermediate-methylation epigenotypes, respectively. In 2012, the Laird team performed comprehensive genome-wide methylation profiling of primary CRC samples and adjacent non-neoplastic colonic tissue samples, using the Illumina Infinium HM27 DNA methylation assay, and conducted model-based unsupervised clustering on DNA probes which showed the most variable DNA methylation values across the tumor tissue samples (Hinoue et al., 2012). Four DNA methylation-based epigenotypes of CRC were identified, including CIMP-positive (cluster 1), CIMP-low (cluster 2), and non-CIMPs (clusters 3 and 4). The Laird team developed Infinium-based CIMP-defining marker panels *(FAM78A, FSTL1, KCNC1, MYOCD,* and *SLC6A4; B3GAT2, FOXL2, KCNK13, RAB31,* and *SLIT1*) for CIMP-positive tumors and CIMP-low tumors, respectively.

Through comprehensive, genome-wide profiling studies, it became clear that CIMP-positive tumors exhibit increased DNA hypermethylation in both the number of methylated promoter CpG island loci and the size of methylated regions compared to CIMP-low and non-CIMP tumors. In other words, CIMP-positive tumors are not hypermethylated at specific genomic loci but at overall genomic regions (Hinoue et al., 2012; Xu et al., 2012). Thus, comprehensive genome-wide profiling studies disprove the existence of two CIMP subgroups, the so-called CIMP1 and CIMP2, but indicate that CIMP2, designated by the Issa group, does not represent high-methylation epigenotype but intermediate-methylation epigenotype or CIMP-low. The literature that documents the prevalence of CIMP in CRC, gene panels, marker thresholds, and techniques for the detection of the altered DNA methylation used to define CIMP, was recently summarized and reviewed by Hughes et al. (2012).

Clinicopathologic and molecular characteristics of CIMP-positive tumors

When the concept of CIMP was introduced by the Issa group, they reported that CIMP is significantly associated with a proximal location in the colon, the *KRAS* mutation, wild type *TP53*, and MSI (Toyota et al., 1999). Subsequent studies found a close association of CIMP-positive CRCs with older age, female gender, mucinous histology, smoking, and *BRAF* mutation (Samowitz et al., 2005; Weisenberger et al., 2006; Goel et al., 2007; Ogino et al., 2007a; Nagasaka et al., 2008). However, the frequent *KRAS* mutation has been clarified to be a feature of CIMP-low (or CIMP2 or the intermediate methylation epigenotype) but not CIMP-positive tumors (or CIMP1 or the high-methylation epigenotype). Regardless of the type of marker panels and methodologies used to determine CIMP, CIMP-positive CRCs demonstrate consistent associations with a proximal location in the colon, MSI, and a high *BRAF* mutation rate (Toyota et al., 1999; Samowitz et al., 2005; Weisenberger et al., 2006; Barault et al., 2008), whereas associations of CIMP-positive CRCs with older age, female gender, and mucinous histology vary among studies (Lee et al., 2008a,b; Dahlin et al., 2010; Zlobec et al., 2011). The reasons for inconsistent associations include not only different marker panels and methodologies used to define CIMP, but also differences in the relative proportion of MSI-positive tumors within CIMP-positive tumors. CIMP-positive tumors are heterogeneous in terms of MSI and are composed of both the CIMP-positive and MSI-positive ($CIMP^+MSI^+$) subtype and the CIMP-positive and MSI-negative ($CIMP^+MSI^-$) subtype. These two subtypes show differences in some clinicopathological features, including male to female ratio and age at diagnosis or prognosis (Samowitz et al., 2005; Kim et al., 2009; Sanchez et al., 2009; Dahlin et al., 2010). In previous studies that compared clinicopathological and molecular features between subtypes of CRCs generated by combinatory statuses of CIMP and MSI, patients with the $CIMP^+MSI^+$ subtype tended to have an older age at diagnosis, higher female to male ratio, and better prognosis compared to those with the $CIMP^+MSI^-$ subtype (Samowitz et al., 2005; Kim et al., 2009; Sanchez et al., 2009; Dahlin et al., 2010). When we analyzed 734 cases of CRC for their CIMP and MSI status and correlated molecular subtypes with clinicopathological features, we found that the average age of patients with the $CIMP^+MSI^+$ subtype was significantly higher than that of patients with the $CIMP^+MSI^-$ subtype (68.8 years (n=19) vs. 61.8 years (n=29), Student's t-test, P=0.029) and that patients with the $CIMP^+MSI^-$ subtype showed worse clinical outcomes than those with the $CIMP^+MSI^+$ subtype (hazard ratio, 2.363 (0.864-6.463)). Thus, the more $CIMP^+MSI^+$ tumors included in the CIMP-positive tumors, the more likely patients with CIMP-positive tumors will be older at diagnosis and the higher the female to male ratio will be compared to patients with CIMP-negative tumors.

CIMP-positive tumors tend to more frequently show a serrated appearance and eosinophilic cytoplasm and less frequent tumor necrosis than do CIMP-negative tumors. Furthermore CIMP-positive tumors produce

larger amounts of extraglandular mucin, with cell balls and papillary rods more frequently floating in the mucinous lakes than CIMP-negative tumors. Of the histopathological variants of CRCs defined by the 2010 WHO classification of tumors of the digestive system (Bosman et al., 2010), serrated adenocarcinoma and cribriform comedo-type adenocarcinoma are morphological correlates of CIMP-positive CRCs (Jass, 2007). Serrated adenocarcinoma was initially described as CRC, showing morphologic and histochemical resemblances with a hyperplastic polyp (Jass and Smith, 1992). Serrated adenocarcinoma is frequent in females and the proximal colon and has morphologic characteristics differing from those of conventional CRCs, such as epithelial serration, eosinophilic cytoplasm, vesicular nuclei, absence of luminal necrosis and mucin production (Fig. 2) (Tuppurainen et al., 2005). Cribriform comedo-type adenocarcinoma, which was introduced as a new distinct histological subtype of CRC in the WHO classification of tumors of the digestive system (Bosman et al., 2010), is characterized by large cribriform glands with central necrosis, and usually showing CIMP and no MSI (Chirieac et al., 2005).

Different clinicopathological features of MSI-positive CRCs depend on CIMP status

MSI is caused by a defect in mismatch repair proteins, including MLH1, MSH2, MSH6 and PMS2 (Boland and Goel, 2010). A hereditary form of MSI-positive CRC, Lynch syndrome, is caused by a germline mutation of the mismatch repair genes and is represented in the $CIMP^{-}MSI^{+}$ subtype. By contrast, sporadic MSI-positive CRC is caused by promoter hypermethylation of the *hMLH1* gene in the context of CIMP. Regardless of

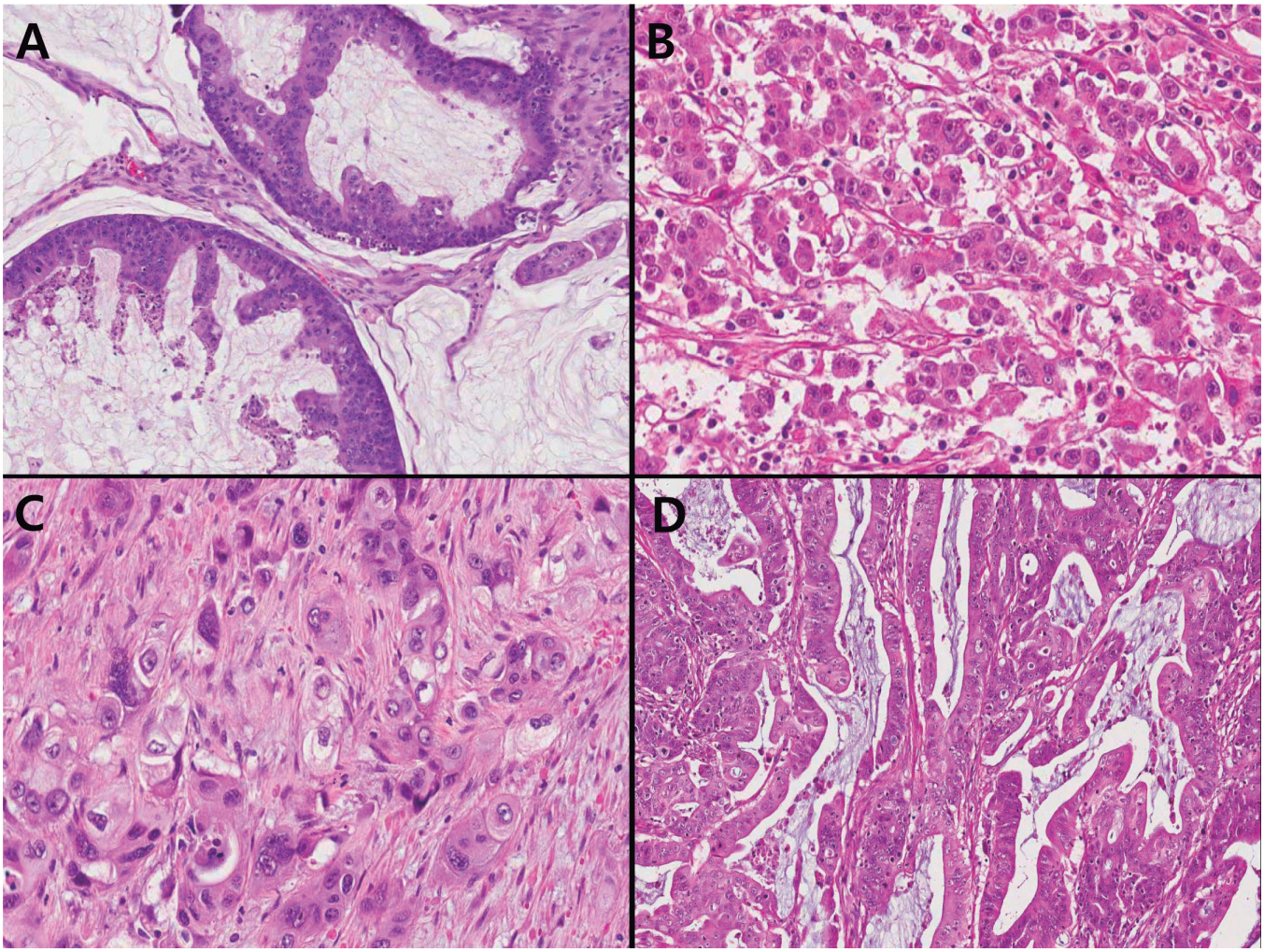

Fig. 2. Histologic findings of CIMP-positive colorectal cancers. **A.** Extracellular mucin production. **B.** Eosinophilic cytoplasm. **C.** Vesicular nuclei. **D.** Serrated crypt morphology. A, D, x 100; B, C, x 200

whether they are sporadic or Lynch-associated, MSI-positive tumors have clinicopathological features distinct from MSI-negative tumors; MSI-positive tumors show a proclivity toward a proximal location in the colon, mucinous histology, Crohn-like peritumoral lymphocyte infiltrate, intratumoral lymphocyte infiltrate, expansile growth, and better clinical outcome. However, MSI-positive tumors are heterogeneous and composed of $CIMP^{+}MSI^{+}$ and $CIMP^{-}MSI^{+}$ tumors, and these two subtypes show clear differences in certain clinicopathological features. Compared to $CIMP^{-}MSI^{+}$ tumors, $CIMP^{+}MSI^{+}$ tumors appear in patients who are older at onset, are more often poorly differentiated, have a higher BRAF mutation rate and a worse clinical outcome, and more frequently show histological characteristics such as extracellular mucin production, Crohn-like lymphoid reaction, increased intraepithelial lymphocytes, serrated crypts, signet ring cell appearance, rhabdoid appearance and medullary appearance (Ogino et al., 2006a-c; Jass, 2007; Bae et al., 2011; Pancione et al., 2011). The $CIMP^{-}MSI^{+}$ subtype exhibits a younger age of onset, better prognosis, and conventional CRC histology. These differences reflect an epigenotypic difference between $CIMP^{+}MSI^{+}$ and $CIMP^{-}MSI^{+}$ tumors because $CIMP^{+}MSI^{+}$ tumors harbor another group of genes inactivated by CIMP plus a group of genes inactivated by MSI that are shared with $CIMP^{-}MSI^{+}$ tumors.

Cytokeratin 20 (CK20) and CDX2 are normally expressed in colon epithelial cells and are considered to be of great importance in the differentiation of colonic metastases from other metastatic tumors such as lung, breast, pancreas, and bile duct carcinomas (Park et al., 2007). However, CK20 and CDX2 are not expressed in all CRCs, and decreased or absent expression of CDX2 or CK20 is frequent in CRCs associated with MSI or CIMP (McGregor et al., 2004; Rozek et al., 2005; Lugli et al., 2008; Baba et al., 2009; Zlobec et al., 2011). Recent studies indicate that CIMP, rather than MSI status, is related to decreased levels of CK20 and CDX2 (Baba et al., 2009; Zlobec et al., 2011). Thus, it is expected that decreased levels of CK20 and CDX2 are more frequent in $CIMP^{+}MSI^{+}$ tumors than in $CIMP^{-}MSI^{+}$ tumors; however, this is not yet documented in the literature.

Multistep carcinogenesis in CIMP – the serrated carcinoma pathway

For decades, based on the premise that CRC is a homogeneous entity, the evolution of CRC was understood to proceed along a relatively uniform and linear sequence of steps, with *APC* inactivation initiating adenoma and additional genetic changes (including *KRAS* activation and *TP53* inactivation) promoting the emergence of increasingly aggressive subclones, finally resulting in malignant transformation (Fearon and Vogelstein, 1990). Almost all CRCs were traditionally thought to develop from pre-existing conventional adenomas, including tubular, tubulo-villous, or villous adenomas—a sequence that is called the "adenoma-carcinoma sequence" (Morson, 1962; Day and Morson, 1978). However, the extensive promoter CpG island hypermethylation and *BRAF* mutation that characterize

Table 2. Summary of studies exploring the prognostic value of CIMP in colorectal cancers.

Study	Patients	Gene panel (methodology)	Note
Zlobec et al., 2011	302 stage I-IV CRCs	CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (pyrosequencing)	Worse prognosis in CIMP-positive CRCs (CSS, univariate analysis)
Ogino et al., 2009	649 stage I- IV colon cancers	CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1 (MethyLight)	Better prognosis in CIMP-positive CRCs (CSS, Multivariate analysis)
Ahn et al., 2011	161 stage III CRCs	MINT1, MINT2, MINT31, MLH1, p16, p14, SFRP1, SFRP2, and WNT5A (pyrosequencing)	Worse prognosis of CIMP-positive CRCs in proximal colon group (DFS, Multivariate analysis)
Dahlin et al., 2010	574 stage I- IV CRCs	CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1 (MethyLight)	Worse prognosis of CIMP-positive CRCs in MSI-negative group (CSS, Univariate analysis). No significance on multivariate analysis
Sanchez et al., 2009	391 stage I-IV CRCs	CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (MethyLight)	Worst prognosis of CIMP+MSI? CRCs (DFS, Univariate analysis). No significant association between CIMP and DFS on Multivariate analysis
Ward et al., 2003	532 stage I-IV CRCs	p16 (MSP) and MINT1, MINT2, MINT12 and MINT31 (COBRA)	Significant association between CIMP and poor prognosis in MSI-negative CRC patients (CSS, Multivariate analysis)
Shen et al., 2007c	185 stage III-IV CRCs	MINT1, MINT31, p14, and p16 (COBRA)	CIMP cases had a significantly shorter survival (OS, multivariate analysis)
Kim et al., 2009	318 stage I-IV CRCs	CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, and SOCS1 (MethyLight)	No significant association between CIMP and poor prognosis (OS, multivariate analysis)

CRC, colorectal cancer; COBRA, combined bisulfite restriction analysis; MSP, methylation-specific PCR; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; CSS, cancer-specific survival; DFS, disease-free survival; OS, overall survival

CIMP-positive CRCs are rarely found in conventional tubular or villous adenomas but are frequently found in sessile serrated adenoma/polyps in the proximal colon (Wynter et al., 2004; Yang et al., 2004). CIMP-positive CRCs do not follow the conventional "adenoma-carcinoma sequence" but instead have an alternative multistep carcinogenesis pathway, termed the "serrated neoplasia pathway". The latter pathway refers to a progression pattern of neoplasms of the colon and rectum that involves hyperplastic polyps and serrated adenomas, resulting in the development of serrated adenocarcinoma. Although this pathway was initially suggested on morphological grounds, over the past decade, the increasing recognition of the biological and genetic similarities in lesions of this pathway has reinforced this concept. Sessile serrated adenoma/polyps and serrated adenocarcinomas have the shared-in features of female preponderance, proximal location, serrated crypts and the common molecular features of frequent *BRAF* mutation and CIMP (Yang et al., 2004; O'Brien et al., 2006).

Clinical application of CIMP

Whether the identification of CIMP-positive tumors has prognostic or predictive value is controversial; thus, clinical application of CIMP is primitive. Several studies have explored the relationship between CIMP status and survival in CRCs, but their results were contradictory (Table 2). Most studies have shown that CIMP-positive CRCs are associated with a poor clinical outcome (Shen et al., 2007c; Ahn et al., 2011), and some studies have failed to show an independent role of CIMP in CRC prognosis (Kim et al., 2009; Sanchez et al., 2009; Dahlin et al., 2010). Because CIMP-positive CRCs exhibit different clinical behaviors depending on their MSI status, some studies have attempted to analyze the prognostic value of CIMP within MSI-negative CRCs and have claimed that there is an independent association with poor prognosis (Ward et al., 2003; Dahlin et al., 2010). However, in other studies, significant associations between CIMP and poor prognosis within the MSI-negative context were generally recognized in univariate analyses but not in multivariate analysis (Kim et al., 2009; Dahlin et al., 2010). When we analyzed 678 cases of MSI-negative CRCs for their CIMP status and correlated CIMP status with prognosis, CIMP-positive status was significantly associated with worse overall survival by univariate analysis, but the relationship of CIMP with poor prognosis was insignificant by multivariate analysis (article in preparation). CIMP status has also been assessed as a predictive marker for 5-fluorouracil (5-FU) responsiveness; however, there are conflicting data surrounding the correlation of CIMP status with responsiveness to adjuvant 5-FU therapy (Table 3). Two studies have suggested a beneficial response to 5-FU chemotherapy; in the van Rijnsoever et al. study (Van Rijnsoever et al., 2003), CIMP-positive status predicted survival benefit from 5-FU chemotherapy in stage III CRC patients, and in the Min et al. study, CIMP-positive status was closely associated with longer recurrence-free survival in stage II/III CRC patients receiving 5-FU chemotherapy (Min et al., 2011). However, Jover et al. reported loss of the survival benefit in CIMP-positive stage II-III CRCs by 5-FU treatment compared to CIMP-negative CRCs (Jover et al., 2011). Furthermore, Ogino et al. and Shen et al. reported that CIMP-positive CRCs

Table 3. Summary of studies exploring the predictive value of CIMP in colorectal cancers.

Study	Patients	Gene panel (methodology)	Note
Van Rijnsoever et al., 2003	Stage III CRCs (103 surgery alone + 103 5FU-based adjuvant chemotherapy)	p16, MINT2, and MDR1 (MSP)	CIMP-positive status predicted survival benefit from 5-FU chemotherapy in CRC (OS, Multivariate analysis)
Ward et al., 2003	164 stage II-III CRCs	p16 (MSP) and MINT1, MINT2, MINT12 and MINT31 (COBRA)	No predictive value of CIMP in patients receiving 5-FU adjuvant chemotherapy (CSS, Multivariate analysis)
Shen et al., 2007c	188 stage III-IV CRCs	MINT1, MINT31, p14, and p16 (COBRA)	CIMP cases had a poor clinical outcome following 5-FU-based adjuvant therapy (OS, Multivariate analysis)
Ogino et al., 2007b	30 stage IV CRCs with no microsatellite instability	CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3, SOCS1, MINT1, MINT31, IGFBP3,MGMT, and WRN (MethyLight)	CIMP cases had a poor clinical outcome following 5-FU and Irrinotecan-based chemotherapy (CSS, Univariate analysis)
Jover et al., 2011	196 stage II-III CRCs	CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (pyrosequencing)	No predictive value of CIMP in 5-FU adjuvant therapy (DFS, Multivariate analysis)
Min et al., 2011	124 stage II-III CRCs	CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (MethyLight)	CIMP-positive cases showed better RFS than that of CIMP-negative cases (Univariate analysis)

MSP, methylation-specific PCR; COBRA, combined bisulfite restriction analysis; OS, overall survival; CSS, cancer-specific survival; DFS, disease-free survival

showed poor clinical outcome after 5-FU based chemotherapy (Ogino et al., 2007b; Shen et al., 2007b). However, all of the results are derived from retrospective observations, so a prospective randomized controlled study is necessary to validate the predictive value of CIMP.

Causes of CIMP

Although the causes of CIMP in CRC remain unknown, genetic factors are known to be involved in causing CIMP in glioma or glioblastoma. The *IDH1* or *IDH2* mutations, which are found in >75% of low-grade gliomas and secondary glioblastoma, have been shown to enhance hypermethylation of genome-wide promoter CpG island loci, leading to the genesis of CIMP in glial tumors (Yan et al., 2009; Turcan et al., 2012). Genetic mutations of *IDH1*/*IDH2* disable the production of alpha-ketoglutarate, instead causing the production of 2-hydroxyglutarate, which inhibits the activities of alpha-ketoglutarate-dependent dioxygenases, including alpha-ketoglutarate-dependent histone demethylases and the TET family of 5-methylcytosine hydroylases (Dang et al., 2009; Zhao et al., 2009). However, genetic mutations of these genes are not found in genome-wide comprehensive studies of CRCs.

The close association between the *BRAF* mutation and CIMP leads us to speculate that the *BRAF* mutation might be related to the development of CIMP. Using a mouse model, Carragher et al. demonstrated that the *BRAF* mutation can induce hyperplastic crypts. However, DNA methylation-induced inactivation of genes involved in senescence did not occur immediately after expression of the *BRAF* mutant. This study indicates that the *BRAF* mutation is not a causative factor in generating epigenetic changes and that unidentified factors are required to generate epigenetic changes (Carragher et al., 2010). Similarly, transfection of the *BRAF* mutant to a colonic tumor cell line did not lead to the enhancement of promoter CpG island hypermethylation (Hinoue et al., 2009). Furthermore, in contrast to the strong relationship between the *BRAF* mutation and CIMP in CRCs, no correlation between CIMP and the *BRAF* mutation was observed in serrated polyps (Vaughn et al., 2010).

Conclusion and future directions

CIMP is one of the important molecular pathways involved in colorectal carcinogenesis and contributes to the molecular heterogeneity of CRCs. The past decade has brought us a better understanding of the epigenetic changes occurring in CRCs and associated premalignant lesions. We know that CIMP is involved in the pathogenesis of sessile serrated adenomas/polyps and serrated adenocarcinomas. However, we still do not know the cause of CIMP in serrated polyps and CRCs. To identify the causative factor of CIMP, we need to characterize comprehensive genomic and epigenomic alterations in sessile serrated adenomas/polyps. Through integrative analysis of genomic and epigenomic alterations in sessile serrated polyps/adenomas, we might gain new insights about the cause of CIMP. Through the Laird team's study, we can see changes occurring in the landscape of genome-wide methylation in CRCs and can clearly differentiate four epigenotypes of CRCs. Although we can easily obtain positive control of CIMP-positive CRC via application of the Infinium HM 27K methylation assay, we cannot routinely perform the Infinium methylation assay because it does not work on formalin-fixed, paraffin-embedded tissue samples. Next, we must establish the reference marker panel and methodology to define CIMP, and we should explore whether the reference marker panel and methodology work equally well with fresh tissue and formalin-fixed paraffin-embedded tissue samples. We can then characterize the clinicopathological features of CIMP-positive tumors defined by the reference criteria and determine the prognostic and predictive effect of CIMP through prospective studies. Whether the four molecular subtypes differ in their response to the standard adjuvant chemotherapy (12 cycles of adjuvant chemotherapy with oxaliplatin, 5-FU and leucovorin (FOLFOX)) must be elucidated. Finally, we have to conduct a close evaluation of whether epigenetically acting drugs (inhibitors of DNA methyltransferases and inhibitors of histone deacetylases) are useful for treating CRC of the $CIMP^{+}MSI^{-}$subtype and whether a combinatorial approach utilizing epigenetic therapy along with standard chemotherapy holds promise for the successful treatment of the $CIMP^{+}MSI^{-}$subtype.

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