Aberrant TET1 Methylation Closely Associated with CpG Island Methylator Phenotype in Colorectal Cancer

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Abstract

Inactivation of methylcytosine dioxygenase, ten-eleven translocation (TET) is known to be associated with aberrant DNA methylation in cancers. Tumors with a CpG island methylator phenotype (CIMP), a distinct subgroup with extensive DNA methylation, show characteristic features in the case of colorectal cancer. The relationship between TET inactivation and CIMP in colorectal cancers is not well understood. The expression level of TET family genes was compared between CIMP-positive (CIMP-P) and CIMP-negative (CIMP-N) colorectal cancers. Furthermore, DNA methylation profiling, including assessment of the TET1 gene, was assessed in colorectal cancers, as well as colon polyps. The TET1 was silenced by DNA methylation in a subset of colorectal cancers as well as cell lines, expression of which was reactivated by demethylating agent. TET1 methylation was more frequent in CIMP-P (23/55, 42%) than CIMP-N (2/113, 2%, $P <$ 0.0001) colorectal cancers. This trend was also observed in colon polyps (CIMP-P, 16/40, 40%; CIMP-N, 2/24, 8%; $P = 0.002$), suggesting that TET1 methylation is an early event in CIMP tumorigenesis. TET1 methylation was significantly associated with BRAF mutation but not with hMLH1 methylation in the CIMP-P colorectal cancers. Colorectal cancers with TET1 methylation have a significantly greater number of DNA methylated genes and less pathological metastasis compared to those without TET1 methylation ($P = 0.007$ and 0.045, respectively). Our data suggest that TET1 methylation may contribute to the establishment of a unique pathway in respect to CIMP-mediated tumorigenesis, which may be incidental to hMLH1 methylation. In addition, our findings provide evidence that TET1 methylation may be a good biomarker for the prediction of metastasis in colorectal cancer. Cancer Prev Res; 8(8); 702–11. ©2015 AACR.

Introduction

Colorectal cancer is one of the most frequent malignancies worldwide and is associate with accumulations of genetic and epigenetic alterations (1). Studies of colorectal cancers have indicated the existence of a CpG island methylator phenotype (CIMP) in a subset of cases, in which a high rate of aberrant DNA methylation was present and characteristic clinical features were exhibited, such as high rates of BRAF or KRAS mutations, specific histology (mucinous, poorly differentiated), proximal location, serrated polyp pathway, and better prognosis (2). In addition,

recent studies suggested that CIMP is an early, possibly tumor-initiating event (3). This accumulated evidence indicates that CIMP-positive (CIMP-P) colorectal cancers may have distinct clinicopathological manifestations and develop through a unique pathway (4).

Although many research studies have demonstrated the existence of CIMP in colorectal cancers, a key missing piece of information is the underlying cause of CIMP. DNA methylation is catalyzed by DNA methyltransferase (DNMT) family members, DNMT1, DNMT3A, and DNMT3B. The relationship between expression levels of DNMTs and CIMP status in colorectal cancers has been extensively studied (5–7). However, conclusive observations have not been demonstrated so far, suggesting that accumulation of DNA methylation is not simply explained by over-activation of DNMTs.

Recently, members of the ten-eleven translocation (TET) family, TET1, TET2, and TET3, which catalyze the conversion of 5-methylcytosine (5 mC) into 5-hydroxymethylcytosine (5 hmC) were identified (8). As this conversion is a primary mode of DNA demethylation, impaired TET activity may affect DNA hypermethylation.

Somatic mutations of isocitrate dehydrogenase 1 (IDH1) produces 2-hydroxyglutarate (2-HG), which inhibits the catalytic activity of TET families and consequently induces accumulation of aberrant DNA methylation (9, 10). Indeed, studies demonstrated that IDH1 mutations in glioma may be sufficient to establish CIMP in glioma (10, 11). Another study of acute

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myeloid leukemia (AML) also revealed that *IDH1* or *TET2* mutations induce DNA hypermethylation (12). These studies indicate that the demethylation process mediated by TET may have a causative role in various types of CIMP-P tumors. However, inactivation of TET family members and the corresponding clinical impact of such alterations in CIMP-P colorectal cancers have not been well studied.

In this study, we found that *TET1* is frequently silenced by DNA methylation in CIMP-P colorectal cancers. *TET1* methylation appears to be an early event, even frequently found in precancerous lesions, and is associated with a global effect on DNA methylation status, as well as clinical outcome of colorectal cancers. Our data shed light on the clinical impact of *TET1* methylation during colorectal cancer tumorigenesis.

Materials and Methods

Cell lines and 5-aza-2′-deoxycytidine treatment

Six colorectal cancer cell lines, SW480, SW48, RKO, HT29, LOVO, and LS174T as well as a breast cancer cell line, MCF7 were purchased from the American Type Culture Collection. Although these cell lines were not authenticated, they were relatively young passage cells after being obtained. SW480, RKO, HT29, LOVO, LS174T, and MCF7 cells were maintained in D-MEM medium (Life Technologies). SW48 was maintained in L-15 medium (Life Technologies). Colorectal cancer cell lines were classified as CIMP-P (SW48, RKO, HT29) or CIMP-N (SW480, LOVO, LS174T), as described previously (13). All cell lines were supplemented with 10% fetal bovine serum (Life Technologies) and antibiotic–antimycotic reagent (Life Technologies) at 37°C in a humidified incubator with 5% CO_2. SW48, RKO, and HT29 cells were treated with 1 μmol/L 5-aza-2′-deoxycytidine (DAC; Sigma-Aldrich) as described previously for 72 hours, with the drug and medium replaced every 24 hours (14).

Clinical samples

Samples of colon polyp ($n = 64$), colorectal cancers ($n = 168$) and their corresponding normal colonic mucosal tissues ($n = 63$) were collected from individuals who underwent surgical resection at the Aichi Cancer Center Central Hospital and Sapporo University Hospital, Japan, after appropriate institutional review board approval was received and written informed consent had been obtained. The background information of the patients and associated colorectal cancer tumor specimens used are shown is in Supplementary Table S1. Colon polyps that were assessed consisted of 30 sessile serrated adenoma/polyps (SSA/P), 18 traditional serrated adenomas (TSA), 6 hyperplastic polyps (HP), and 10 tubular or tubulovillous adenomas. Among these, 11 out of the 30 SSA/P and one of the 10 adenomas were mixed lesions containing both adenoma and adenocarcinoma components in the same polyp.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using TRizol (Life Technologies), with 2 μg used for reverse transcription with a SuperScript VILO cDNA Synthesis Kit (Life Technologies). TaqMan PCR and SYBR Green qPCR were carried out for the target genes in duplicate. Oligonucleotide primers used for TaqMan PCR assays (Applied Biosystems) are as follows, *GAPDH* (AB Assay ID Hs00266705_g1) and proliferating cell nuclear antigen (*PCNA*, Hs00952870_g1).

Primer sets for SYBR Green assays are shown in Supplementary Table S2.

Western blot analysis

Cell lysates were extracted from SW480, SW48, RKO, HT29, LOVO, LS174T, and MCF7 cells. Cell lysates were also extracted from 16 clinical colorectal cancer samples. Fifty micrograms of each lysate was run on SDS/PAGE gels, transferred to nitrocellulose membranes, and incubated with the following antibodies: rabbit polyclonal anti-TET1 (GTX 124207; GeneTex), rabbit polyclonal anti-TET2 (ab94580; Abcam), rabbit polyclonal anti-TET3 (GTX 121453; GeneTex), rabbit monoclonal anti-MLH1 (ab92312; Abcam), and rabbit polyclonal anti-β-actin (4967; Cell Signaling Technology).

DNA methylation analysis

We performed bisulfite treatment on extracted genomic DNA as described previously (15). The DNA methylation levels were measured using pyrosequencing technology (Pyrosequencing AB). Primer sequences and PCR conditions are shown in Supplementary Table S2. CIMP status of 168 colorectal cancers and 64 polyps was evaluated by five classical CIMP markers (*hMLH1*, *MINT1*, *MINT2*, *MINT31*, and *p16*). DNA methylation status (positive or negative) of CIMP markers was determined as described previously (16). Samples with simultaneous methylation of at least two of the five classical CIMP markers were considered CIMP-P (17, 18).

Methylated CpG island amplification microarray (MCAM) profiling

Global analysis using methylated CpG island amplification-microarray technology was carried out using DNA from 22 colorectal cancer samples (five CIMP-P with *TET1* methylation, six CIMP-P without *TET1* methylation, and 11 CIMP-N colorectal cancers) as described previously (14, 16, 19).

Mutation analysis

We performed target resequencing of 16 CIMP-P colorectal cancers using the HaloPlex Cancer Research Panel, which consists of 47 cancer-associated genes (Agilent Technologies), according to the manufacturer's protocol. Sixteen samples were randomly selected from CIMP-P colorectal cancers. Briefly, 900 ng of enzymatically digested DNA was hybridized to specific probes for each gene of interest. Hybridized molecules were captured and amplified using indexed primers. Following this, target libraries were purified using an AMPure XP Kit (Beckman Coulter) and sequenced via use of a high-throughput sequencing instrument (MiSeq, Illumina). Data were analyzed using CLC genomic Workbench v6.5.1 (CLC Inc.).

In addition, mutations in *KRAS* (codons 12 and 13) or *BRAF* (codon 600) were determined in all colorectal cancer cases ($n = 168$) by the pyrosequencing analysis, as previously described (16). Mutations in *TET1* and *IDH1* were examined in CIMP-P colorectal cancers ($n = 13$ and 31, respectively) by conventional or high-throughput sequencing analysis. The PCR primer sequences used in both contexts are listed in Supplementary Table S2.

The Cancer Genome Atlas data (TCGA)

We obtained the genetic alterations, DNA methylation, and clinicopathological data for 593 colorectal cancers, which

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Table 1. Relationship between *TET1* methylation and clinicopathological features in our colorectal cancer cohort and TCGA database

	Our cohort				TCGA database			
	Total	**TET1 methylated**	**TET1 unmethylated**		**Total**	**TET1 methylation**	**TET1 unmethylated**	
Characteristic	**$n = 168$**	**$n = 25$**	**$n = 143$**	**P-value**	**$n = 593$**	**$n = 79$**	**$n = 514$**	**P-value**
Age (years)								
65	93 (55)	19 (20)	74 (80)	0.029	356 (60)	58 (16)	298 (84)	0.01
<65	75 (45)	6 (8)	69 (92)		237 (40)	21 (9)	216 (91)	
Gender								
Female	81 (48)	16 (20)	65 (80)	0.128	274 (46)	45 (16)	229 (84)	0.052
Male	87 (52)	9 (10)	78 (90)		319 (54)	34 (11)	285 (89)	
Location[a]								
Proximal	70 (42)	20 (29)	50 (71)	<0.001	254 (43)	62 (24)	192 (76)	<0.001
Distal	98 (58)	5 (5)	93 (95)		332 (57)	16 (5)	316 (95)	
Histological								
Well/moderate	126 (91)	11 (9)	115 (91)	0.005	516 (88)	61 (12)	455 (88)	0.007
Poor/mucinous	12 (9)	5 (42)	7 (58)		69 (12)	17 (25)	52 (75)	
CIMP								
CIMP(+)	55 (33)	23 (42)	32 (58)	<0.001	229 (39)	75 (33)	154 (67)	<0.001
CIMP(−)	113 (67)	2 (2)	111 (98)		364 (61)	4 (1)	360 (99)	

Values in parentheses indicate percentages.
[a]Proximal, cecum, ascending and transverse colon; Distal, descending and sigmoid colon and rectum.

included the DNA methylation status of *TET1*, *hMLH1*, and CIMP markers, from TCGA web site (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). Regarding the methylation analysis using the Illumina Infinium Human DNA Methylation 27 and 450 platforms, a β-value > 0.2 was considered as methylation-positive. As one of the classical five CIMP markers (*MINT31*) was not present in either of these Illumina Infinium platforms, we used an alternative set of five CIMP markers, namely, *CACNA1G* (Probe ID on the Infinium HumanMethylation 27 or 450 array, cg11262815), *IGF2* (cg13756879), *NEUROG1* (cg11248413), *RUNX3* (cg22737001), and *SOCS1* (cg06220235), as reported previously (20, 21). Although CIMP status was determined by a different collection of markers in the TCGA data set, the clinical features of these TCGA CIMP-P colorectal cancers were consistent with CIMP-P colorectal cancers in our analyses as identified by the classical five CIMP markers (Table 1).

Statistical analysis of associations with clinical features

All statistical analyses were performed using GraphPad Prism 5 (Graphpad Software) and JMP statistical software version 5.1 (SAS Institute).

The Fisher's exact test was used to determine nonrandom associations between two categorical variables. Kruskal–Wallis analysis was used to evaluate the extent of differences among more than three groups. All reported *P* values are two sided, with $P < 0.05$ taken as statistically significant.

Results

Analysis of expression and DNA methylation of TET genes in colorectal cancer cell lines

First, expression of TET family members (TET1, TET2, and TET3) was examined by qRT-PCR and Western blot analyses in six colorectal cancer cell lines, SW48 (CIMP-P), RKO (CIMP-P), HT29 (CIMP-P), LS174T (CIMP-N), SW480 (CIMP-N), and LOVO (CIMP-N) along with a breast cancer cell line, MCF7, which was used as a positive control for *TET* gene expression (ref. 22; Fig. 1A). The CIMP status of each colorectal cancer cell line was determined previously (13).

Neither mRNA nor protein expression of TET2 was detected in any of the colorectal cancer cell lines examined, in contrast to MCF7 cells that highly expressed TET2. TET3 was substantially expressed in all colorectal cancer cell lines regardless of CIMP status. Intriguingly, TET1 expression negatively correlated with CIMP status; indeed, significantly lower expression of TET1 was observed in CIMP-P cell lines (SW48, RKO, HT29) than CIMP-N cell lines (LS174T, SW480, LOVO) at both mRNA and protein levels. These results suggest a possible association between TET1 and CIMP status in colorectal cancers.

Next, in order to address the mechanism of *TET1* silencing in colorectal cancer cell lines, we examined the DNA methylation status of the *TET1* gene by bisulfite pyrosequencing analysis (Fig. 1B and Supplementary Fig. S1A). Three colorectal cancer cell lines without *TET1* expression (SW48, RKO, HT29) showed a high level of DNA methylation, whereas the other three cell lines with *TET1* expression (LS174T, SW480, and LOVO) showed low level of DNA methylation (Fig. 1B). Silencing of *TET1* expression was restored by DAC treatment in SW48, RKO, and HT29 cell lines (Fig. 1C). These results indicate that silencing of *TET1* is closely associated with DNA methylation in colorectal cancer cell lines.

Analysis of expression and DNA methylation of TET1 genes in colorectal cancer tissues

We next examined *TET1* expression by qRT-PCR in paired cancerous and corresponding normal tissues from colorectal cancer cases ($n = 63$). *TET1* expression was significantly downregulated in cancerous tissues compared to normal tissues ($P < 0.0001$; Supplementary Fig. S1B). Among them, 51 of 63 colorectal cancers (81%) showed lower levels of *TET1* expression, 11 of 63 colorectal cancers (17%) showed almost equal levels, and 1 of 63 colorectal cancers (2%) showed higher levels, respectively (Supplementary Fig. S1C).

Intriguingly, among 119 colorectal cancer cases, including aforementioned 63 paired colorectal cancer cases, *TET1* expression was significantly lower in CIMP-P ($n = 28$) than CIMP-N ($n = 91$) colorectal cancers ($P = 0.0063$) as was found in cell line analysis (Figs. 1A and 2A). By contrast, no obvious differences in TET1 expression were observed between normal tissues from CIMP-P cases ($n = 16$) and CIMP-N cases ($n = 47$) ($P = 0.66$; Fig. 2A).

The inverse relationship between *TET1* methylation and protein expression was observed in some colorectal cancers (Supplementary Fig. S1D). We further investigated the relationship
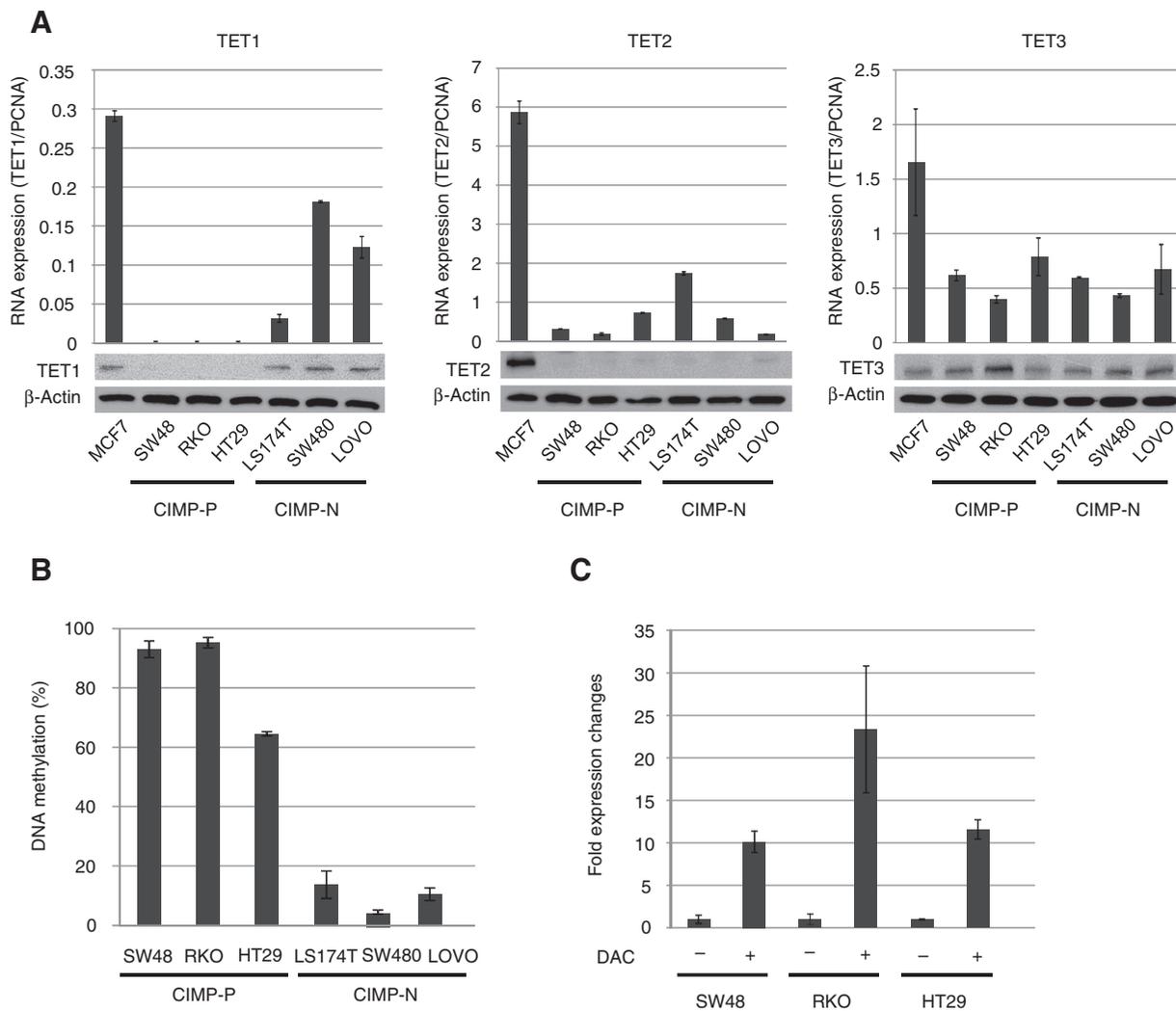
Figure 1.
Expression and DNA methylation status of TET family in colorectal cancer cell lines. A, expression of TET family (TET1, TET2, and TET3) was examined by qRT-PCR and Western blot analysis in colorectal cancer cell lines (SW48, RKO, HT29, LS174T, SW480, and LOVO). The breast cancer cell line, MCF7, was used as a positive control for TET expression. RNA expression was normalized according to *PCNA* mRNA levels. β-Actin was used as a loading control for Western blot analysis. The error bars indicate standard deviation. B, bisulfite-pyrosequencing analysis of *TET1* methylation in colorectal cancer cell lines. C, relative *TET1* expression changes after treatment of DAC (1 μmol/L) for 72 hours in CIMP-P colorectal cancer cell lines (SW48, RKO, HT29) in comparison to without treatment.

between *TET1* methylation and expression levels in colorectal cancers by bisulfite pyrosequencing analysis and qRT-PCR, respectively. A number of colorectal cancers (11 of 119 colorectal cancers, 9%) showed an increased level of DNA methylation (>8%) and significant reduction of TET1 expression (Fig. 2B). Therefore, we defined DNA methylation levels greater than 8% as TET1 DNA methylation-positive hereafter.

We further added 49 colorectal cancers from Sapporo University Hospital in our DNA methylation analysis. Among the 168 colorectal cancers, 23 of 55 (42%) CIMP-P colorectal cancers showed *TET1* methylation, whereas only 2 of 113 (2%) CIMP-N colorectal cancers showed *TET1* methylation ($P < 0.0001$; Fig. 2C). In other words, 23 of the 25 *TET1* methylated colorectal cancers were CIMP-P (92%). Thus, *TET1* silencing by DNA methylation was closely associated with CIMP status during colorectal cancer tumorigenesis.

Analysis of TET1 methylation in colon polyps and cancers

We next endeavored to clarify when *TET1* methylation emerges and whether *TET1* methylation is a cause of CIMP or a downstream target of CIMP-related hypermethylation during colorectal cancer tumorigenesis. Sixty-four colon polyps (a precancerous condition), consisting of 30 SSA/P, 18 TSA, 6 HP, and 10 tubular or tubulovillous adenoma, were examined. *TET1* methylation was significantly higher in CIMP-P polyps than CIMP-N polyps [16/40 (40%) vs. 2/24 (8%), $P = 0.0088$; Fig. 3A]. Thus, the majority of *TET1* methylated polyps (16/18, 89%) were CIMP-P as was found in colorectal cancers.

We found that *TET1* methylation was highly frequent in CIMP-P tumors with *BRAF* mutation (BRAF^{V600E}, $P = 0.005$; Supplementary Table S3). The existence of distinct classes of CIMP tumors has been shown recently. A subset of CIMP-P colon polyps, as well as colorectal cancers, has been demonstrated to

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A

B

C
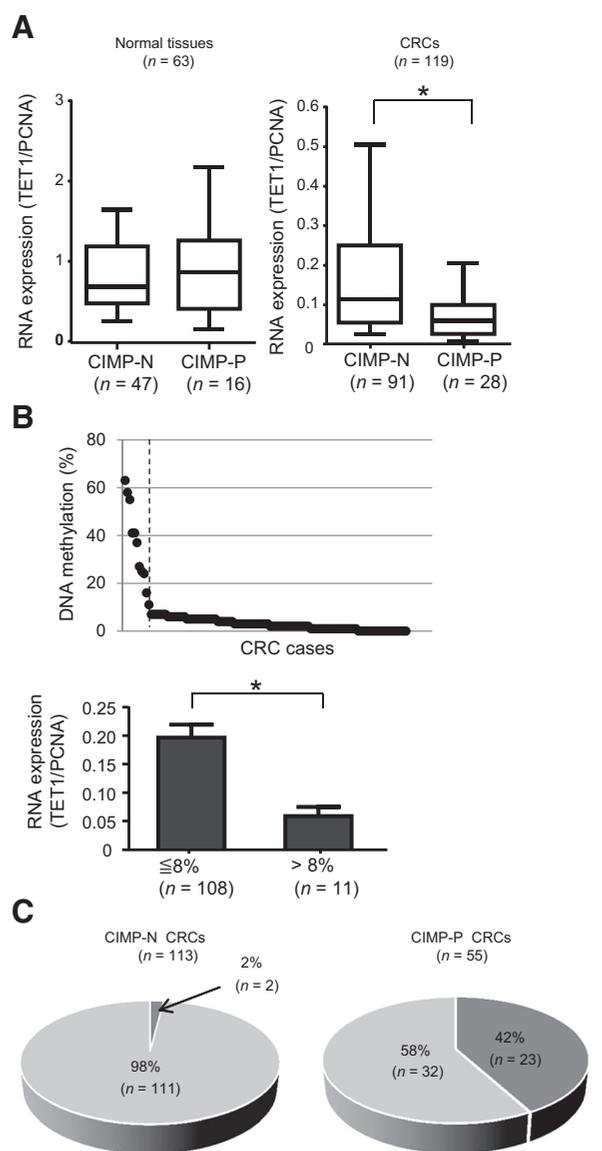
Figure 2.
RNA expression and DNA methylation of *TET1* gene in clinical colorectal cancers. A, *TET1* expression in CIMP-P and CIMP-N colorectal cancers (right, *n* = 119), as well as corresponding normal tissues (left, *n* = 63). *TET1* expression was normalized according to *PCNA* mRNA levels. In a box-and-whisker plot of *TET1* expression, the median is marked by a bold line inside the box whose ends represent the upper and lower quartiles. Error bars denote 10 and 90 percentile values. *, $P = 0.0063$. B, scatter plots of *TET1* methylation level (Y-axis) and its corresponding samples (X-axis) in colorectal cancers (*n* = 119, top). *TET1* expression was significantly different between colorectal cancers with *TET1* DNA methylation level of >8% (*n* = 11) and 8% ≤ (*n* = 108). DNA methylation level of 8% is indicated (dash line). *, $P = 0.002$. C, frequencies of *TET1* methylation in CIMP-N (*n* = 113) and CIMP-P (*n* = 55) colorectal cancers. Dark gray and white color in the pie graph represent *TET1* methylation (+) or (−), respectively.

harbor *BRAF* mutation and exhibit characteristic clinical features, such as more extensive methylation, better prognosis, proximal location, and *hMLH1* methylation, called CIMP1 or CIMP-high (CIMP-H; refs. 18, 23, and 24). We examined *TET1* methylation in CIMP1/CIMP-H colon polyps and colorectal cancers, as defined

by these criteria. A trend toward higher frequency of *TET1* methylation was found in colorectal cancers (15/23, 65%) than polyps (14/32, 44%), although this difference was not statistically significant (Fig. 3B, $P = 0.17$). Intriguingly, DNA methylation of *hMLH1*, which is another characteristic target of CIMP1/CIMP-H tumors, was more cancer-specific [polyps, 3/32 (9%); colorectal cancers, 16/23 (70%); Fig. 3C, $P = 0.001$]. Inverse relationship between *hMLH1* methylation and protein expression was also found in some colorectal cancers (Supplementary Fig. S1D).

Colorectal cancers are generally evolved from a monoclonal cell population. Therefore, molecular dissection of precancerous (adenoma) and cancerous (adenocarcinoma) components within the same tumor may help to understand the onset timing of CIMP during tumorigenesis (25). We analyzed mixed lesions containing both precancerous and cancerous components in CIMP-P tumors and found that CIMP status and/or *BRAF* mutation were frequently observed (10/12, 83% and 9/12, 75%, respectively) in precancerous components, whereas *TET1* methylation was much less common (2/12, 17%, $P = 0.002$; Fig. 3D). In addition, *TET1* methylation was more frequent in cancerous than precancerous components.

Relationship between TET1 methylation and other CIMP markers in CIMP-P colorectal cancers

Next, we analyzed the relationship between *TET1* methylation and other CIMP markers. Levels and/or frequencies of DNA methylation in four CIMP-marker genes (*MINT1*, *MINT2*, *MINT31*, and *p16*) was significantly higher in CIMP-P colorectal cancers with *TET1* methylation than those without *TET1* methylation (Fig. 4A and B and Supplementary Table S3). Of interest, *TET1* methylation status was not correlated with *hMLH1* methylation status in CIMP-P colorectal cancers (Fig. 4A and B and Supplementary Table S3). In addition, around half (12/23, 52%) of CIMP-P colorectal cancers with *TET1* methylation did not show *hMLH1* methylation (Fig. 4D).

Next, genome-wide CpG methylation status was compared between colorectal cancers with and without *TET* methylation using MCAM technology (14, 16). We assessed 22 colorectal cancers in this context: five CIMP-P colorectal cancers with *TET1* methylation, six CIMP-P tumors without *TET1* methylation and 11 CIMP-N tumors without *TET1* methylation. The greatest number of DNA methylated genes were observed in CIMP-P tumors with *TET1* methylation compared with the other two groups ($P = 0.007$; Fig. 4C).

We further analyzed the relationship between genome-wide DNA methylation status, *TET1* methylation, and *hMLH1* methylation in a more extensive cohort (CIMP-P, *n* = 229) from a TCGA data set (Supplementary Fig. S2B). CIMP-P colorectal cancers with either *TET1* or *hMLH1* methylation showed a greater number of DNA methylated genes than those with neither *TET1* nor *hMLH1* methylation. Notably, colorectal cancers with both *TET1* and *hMLH1* methylation showed the greatest number of DNA methylated genes compared to other subgroups of colorectal cancers ($P < 0.0001$).

Analysis of somatic mutations in CIMP-P colorectal cancers

Studies of leukemia and brain tumors showed aberrant accumulation of DNA methylation was caused by genetic alterations, such as *IDH* mutation (10, 12, 26). We first examined mutations in *TET1* in CIMP-P colorectal cancers without *TET1* methylation (*n* = 13) and found no cancer-specific mutation in the *TET1* gene.
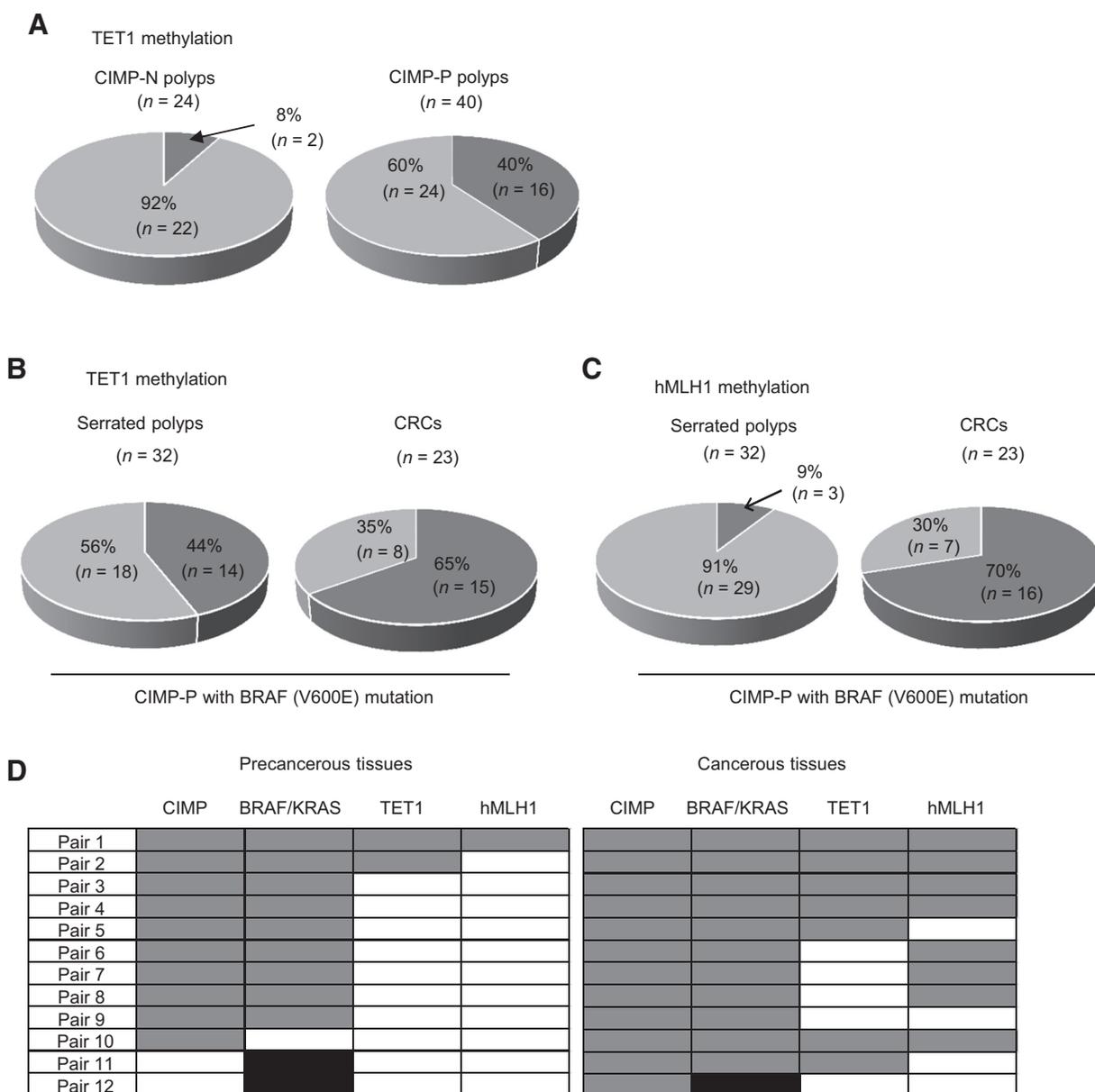
Figure 3.
TET1 methylation in colon polyps. A, frequencies of *TET1* methylation in CIMP-N ($n = 24$, left) and CIMP-P polyps ($n = 40$, right). B, frequencies of *TET1* methylation in the serrated polyps ($n = 32$, left) and colorectal cancers ($n = 23$, right) with CIMP plus *BRAF* V600E mutation. Dark gray and white color in the pie graph represent *TET1* methylation ($+$) or ($-$), respectively (A and B). C, frequencies of *hMLH1* methylation in the serrated polyps ($n = 32$, left) and colorectal cancers ($n = 23$, right) with CIMP plus *BRAF* V600E mutation. Dark gray and white color in the pie graph represent *hMLH1* methylation ($+$) or ($-$), respectively. D, DNA methylation, *BRAF/KRAS* mutation, and CIMP status of mixed lesions containing both precancerous and cancerous components. Each column represents the CIMP status, *hMLH1* and *TET1* methylation, and *BRAF/KRAS* mutations. Gray boxes denote *BRAF* mutation positive, CIMP positive, or DNA methylation positive in the *TET1* and *hMLH1*, respectively. Black boxes denote *KRAS* mutation positive. White boxes denote each these mutations or DNA methylation negative.

We next examined genetic alterations of 47 cancer-associated genes, including *IDH1*, *IDH2*, *BRAF*, *KRAS*, and *TP53* and other cancer-associated genes, in 16 CIMP-P colorectal cancers. Concordant with previous reports, mutations in *BRAF* and *KRAS* were frequently found in CIMP-P colorectal cancers [five cases (31%) and six cases (38%), respectively; refs. 18, 27]. By contrast, *IDH1* and *IDH2* mutations were rare (1 case, 6%; Supplementary Fig. S2A). In addition, we examined data relating to 224 colorectal cancers from TCGA data and found *TET1*, *IDH1*, and *IDH2*

mutations in 10 (4%), 3 (0.5%), and 7 (1%) colorectal cancers, respectively, indicating that *TET1* and *IDH* mutations are rare events in colorectal cancer (1).

Clinical significance of TET1 methylated colorectal cancers

Finally, relationships between *TET1* methylation and clinicopathological features were studied (Table 1). Among 168 colorectal cancers, we found that *TET1* methylation was more frequent in elderly patients ($P = 0.029$), or tumors that were

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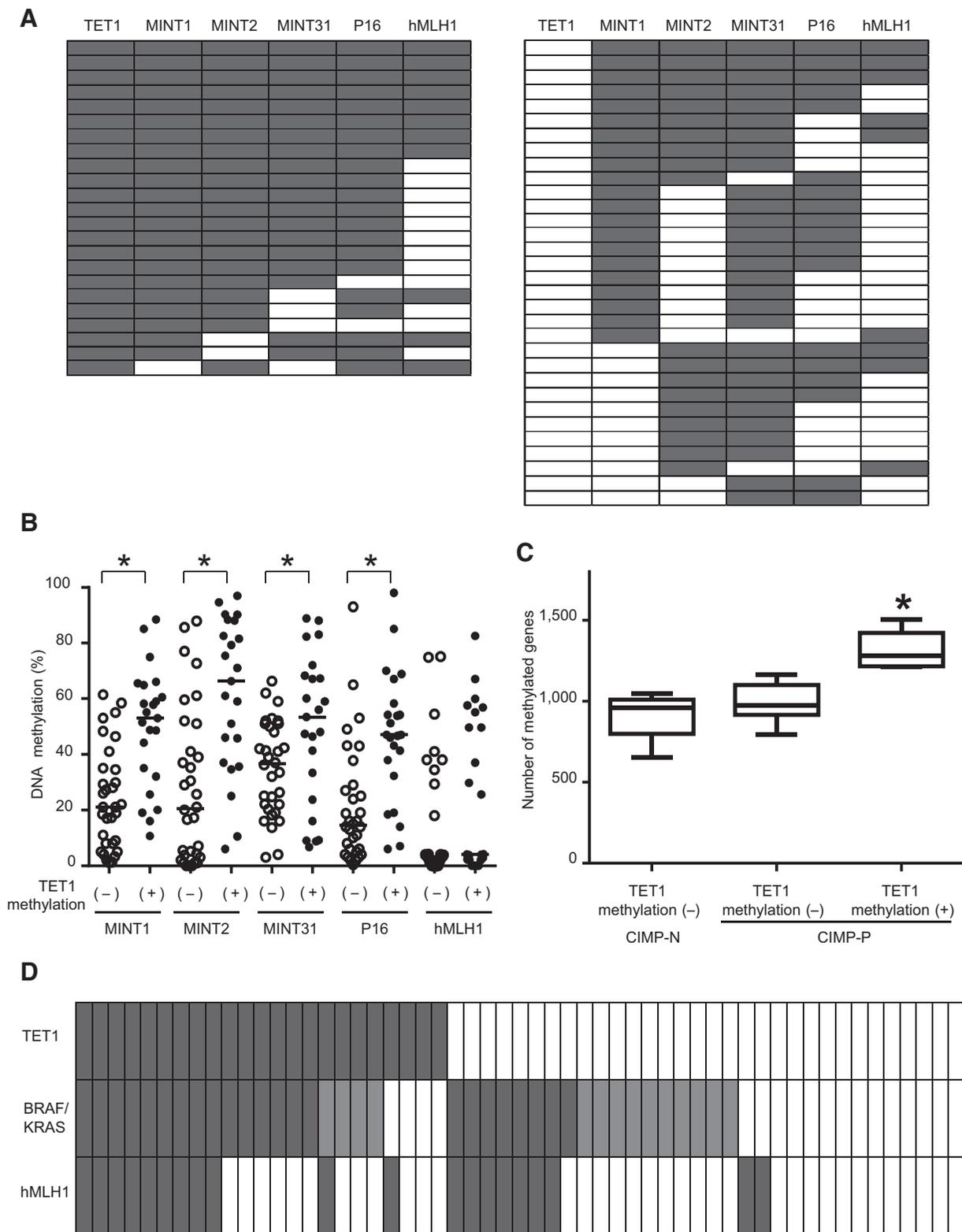
Figure 4.
Analysis of the relationship between *TET1* methylation and other CIMP markers in CIMP-P colorectal cancers. A, each column represents the methylation status of five CIMP markers in CIMP-P colorectal cancers with *TET1* methylation (left) or without *TET1* methylation (right). Black boxes denote DNA methylation positive in *TET1* and CIMP markers, respectively. B, DNA methylation level of five CIMP markers in each CIMP-P colorectal cancer with *TET1* methylation (black dot) or without *TET1* methylation (white dot), respectively. Horizontal lines represent median of DNA methylation levels of each marker. (*Continued on the following page.*)

Table 2. Clinical features of colorectal cancers with and without pathological metastasis

Characteristic	Total *n* = 201	Colorectal cancers with metastasis *n* = 26	Colorectal cancers without metastasis *n* = 175	*P*-value
Age (years)				
65	140 (70)	17 (12)	123 (88)	0.650
<65	61 (30)	9 (15)	52 (85)	
Sex				
Male	106 (53)	14 (13)	92 (87)	1.000
Female	95 (47)	12 (13)	83 (87)	
Location[a]				
Proximal	139 (70)	16 (12)	123 (88)	0.488
Distal	59 (30)	9 (15)	50 (85)	
Histology				
Adenocarcinoma (Well/moderate)	166 (83)	25 (15)	141 (85)	0.086
Mucinous adenocarcinoma	33 (17)	1 (3)	32 (97)	
TET1 methylation				
Positive	67 (33)	4 (6)	63 (94)	0.045
Negative	134 (67)	22 (16)	112 (84)	
hMLH1 methylation				
Positive	76 (38)	6 (8)	70 (92)	0.129
Negative	125 (62)	20 (16)	105 (84)	
TET1 plus hMLH1 methylation				
Positive	43 (21)	0 (0)	43 (100)	0.002
Negative	158 (79)	26 (16)	132 (84)	

Values in parentheses indicate percentages.

[a]Proximal, cecum, ascending and transverse colon; Distal, descending and sigmoid colon and rectum.

proximally located ($P = 0.0001$), poorly or mucinous differentiated ($P = 0.005$), CIMP-P ($P < 0.0001$), and *BRAF* mutated ($P < 0.0001$).

We also extended the analysis of relationship between *TET1* methylation and clinicopathological features in additional colorectal cancer cohorts using the TCGA data set (Table 1; ref. 1). Although CIMP status was determined using different markers (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1*) in the TCGA data set (20), CIMP status was also closely associated with *TET1* methylation status. Among 593 colorectal cancers, 75 of 229 CIMP-P colorectal cancers (33%) showed *TET1* methylation, whereas 4 of 364 (1%) CIMP-N colorectal cancers showed *TET1* methylation ($P < 0.001$; Table 1). In addition, we found that the clinical features of colorectal cancers with *TET1* methylation within the TCGA data set were quite consistent with our data (Table 1).

Next, we further compared the clinical features between CIMP-P colorectal cancers with and without *TET1* methylation in the TCGA data set, which contains TNM (for tumors/nodes/metastases) staging information ($n = 201$). Intriguingly, patients that had colorectal cancers with *TET1* methylation displayed less pathological metastatic lesions than those without *TET1* methylation (Supplementary Table S4). In addition, among this cohort, *TET1* methylation and *hMLH1* methylation plus *TET1* methylation were the only factors that influenced the incidence of metastasis ($P = 0.045$ and 0.002, respectively; Table 2).

Discussion

Recent accumulating evidence has demonstrated that dysregulation of epigenetic modifiers is frequently involved in tumor formation (28). In this study, we focused on the demethylation enzyme TET1 to elucidate the relationships between induction of aberrant DNA methylation and dysregulation of the associated gene in colorectal cancers.

The *TET1* gene was first discovered as a translocation partner of the mixed lineage leukemia (MLL) gene in acute myeloid leukemia (29). Recent studies revealed that TET1 protein has a potential mechanism related to DNA demethylation and may play an important role in transcriptional regulation of genes involved in basic housekeeping processes and those regulating differentiation and development (8, 26, 30). Dysregulation of the *TET1* gene has been found in many types of cancers and appears to be associated oncogenic processes in solid tumor tissues, such as colon, prostate, breast, and hepatocellular cancers (22, 31–33). Suppression of *TET1* promotes tumor cell invasion and correlated with poor survival outcomes in breast cancer patients (22). Even given its putative role in DNA demethylation, no prior study had so far elucidated relationships between *TET1* and accumulation of aberrant DNA methylation in colorectal cancers, especially in CIMP-related cancers.

In this study, we initially found that DNA methylation of the *TET1* gene is associated with loss of its expression in colorectal cancers. The DNA methylation inhibitor DAC restores *TET1*

(*Continued.*) C, box-and-whisker plot of number of DNA methylated genes as determined by MCAM analysis in CIMP-N colorectal cancers without *TET1* methylation ($n = 11$), CIMP-P colorectal cancers without *TET1* methylation ($n = 6$) and CIMP-P colorectal cancers with *TET1* methylation ($n = 5$). The median is marked by a bold line inside the box whose ends represent the upper and lower quartiles. Error bars denote 10% and 90% values. D, DNA methylation status and mutation status of CIMP-P colorectal cancers. Each column represents the CIMP status, *BRAF/KRAS* mutation status, and *hMLH1* and *TET1* methylation status. Black boxes denote CIMP positive, *BRAF* mutation positive, or DNA methylation positive in the *TET1* and *hMLH1*, respectively. Gray boxes denote *KRAS* mutation positive. White boxes denote each these mutations as negative or DNA methylation negative.

expression in colorectal cancer cell lines, indicating that the DNA hypermethylation plays a major role in silencing of this gene in colorectal cancer. Notably, *TET1* methylation is tumor-specific and tightly associated with CIMP-P colorectal cancers, especially those carrying *BRAF* mutation.

The existence of multiple parallel pathways of tumorigenesis (at least three) has been indicated via DNA methylation profiling in colorectal cancers, namely two CIMP-P options (either CIMP1/CIMP-H and CIMP2/CIMP-L) or CIMP-N (18, 23, 24). These distinct oncogenic pathways raise several clinical implications in terms of therapeutic strategy. Among these, the CIMP1/CIMP-H pathway is characterized by serrated precursor lesions, good prognosis, extensive DNA hypermethylation, *BRAF* mutation, and microsatellite instability phenotype with *hMLH1* methylation. Of interest, we found around half of CIMP-P colorectal cancers with *BRAF* mutation showed *TET1* methylation but not *hMLH1* methylation and that *TET1* methylation was significantly associated with *BRAF* mutation but not with *hMLH1* methylation in the CIMP-P colorectal cancers examined. Further, we found that *TET1* methylation was significantly associated with genome-wide DNA hypermethylation status in CIMP-P colorectal cancers (Fig. 4C). Additionally, colorectal cancers with *TET1* methylation plus *hMLH1* methylation were the most extensively methylated (Supplementary Fig. S2B). These data suggest that *TET1* methylation may contribute to the establishment of unique pathway in CIMP-mediated tumorigenesis, which may incidentally led to *hMLH1* methylation. Given the molecular function of TET1, it is reasonable that inactivation of the *TET1* gene may induce the accumulation of DNA methylation during colorectal cancer tumorigenesis, as has been reported in glioma and AML studies (10–12).

Interestingly, a high frequency of *TET1* methylation was found even in precancerous tissue indicating that *TET1* methylation is an early event and may affect global DNA methylation status during tumorigenesis in a subset of tumors, although it remains unclear what triggers the transition to cancer in these polyps. In addition, the occurrence of *TET1* methylation in precancerous tissue enabled us to perform molecular dissection in mixed lesions containing both precancerous and cancerous components in CIMP-P tumors. A higher frequency of CIMP positivity and *BRAF* mutation was found compared to *TET1* methylation, as well as *hMLH1* methylation, in precancerous components: the frequency of both *TET1* methylation and *hMLH1* methylation was increased in cancerous components, although *hMLH1* methylation was more cancer specific. Taken together, these data indicate that *TET1* methylation occurs after acquiring *BRAF* mutation and CIMP status, and that *TET1* methylation might be an earlier event than *hMLH1* methylation in the precancerous condition. In addition, *TET1* may be a frequent target of CIMP-related hypermethylation rather than the cause of CIMP and contribute to the establishment of pathway displaying extensive DNA methylation in tumorigenesis.

Regarding links to clinicopathological variables, colorectal cancers with *TET1* methylation, but not *hMLH1* methylation, showed significantly less metastatic events. Since increased frequency of DNA methylation in a certain set of genes can determine the behavior of these tumors, it may be possible that colorectal cancers with *TET1* methylation may exhibit unique clinicopathological features compared to those without *TET1* methylation.

In conclusion, we demonstrate here, for the first time, the biological and clinical significance of TET1 in colorectal cancers associated with CIMP. The understanding of these multiple pathways towards colorectal cancer has implications for therapy. Recent combined genetic and epigenetic analyses suggest that colorectal cancer is not a uniform disease (1, 24, 34). The distinct clinicopathological features in relation to CIMP raise important implications for prevention, screening, and therapy. We provide new findings here showing a link between *TET1* methylation and enhanced DNA methylation in a subset of tumors, which may be associated with unique clinicopathological features of this disease. Now, individualized therapy for colorectal cancer is required, whereby each tumor may be examined via molecular profiling before therapy can be selected. Our findings provide evidence that *TET1* methylation may be a good biomarker for the prediction of metastasis in colorectal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N. Ichimura, K. Katsushima, Y. Kondo
Development of methodology: N. Ichimura, K. Katsushima, Y. Kondo
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Ichimura, K. Shinjo, B. An, Y. Shimizu, M. Tojo, E. Yamamoto, H. Suzuki
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Ichimura, K. Shinjo, A. Hatanaka, M. Tojo, Y. Kondo
Writing, review, and/or revision of the manuscript: N. Ichimura, Y. Kondo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Ichimura, F. Ohka, Y. Kondo
Study supervision: N. Ichimura, K. Yamao, M. Tojo, E. Yamamoto, H. Suzuki, M. Ueda, Y. Kondo

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