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Title: Methyltransferase expression and tumor suppressor gene methylation in sporadic and familial colorectal cancer

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ABSTRACT (GENES CHROM CANCER=250 words)

Molecular mechanisms underlying coordinated hypermethylation of multiple CpG islands in cancer remain unclear. In particular, expressional and other studies of methyltransferase enzymes have arrived at conflicting results. We focused on DNMT1 and DNMT3B, DNA methyltransferases responsible for (*de novo*) methylation, and EZH2, histone (H3K27) methyltransferase, and examined their roles in tumor suppressor gene (TSG) methylation patterns we have previously established in sporadic and familial cancers. Our investigation comprised 165 tumors, stratified by tissue of origin (117 colorectal and 48 endometrial carcinomas) and sporadic vs. familial disease (57 sporadic vs. 60 familial, mainly Lynch syndrome, colorectal carcinomas). By immunohistochemical evaluation, DNMT1, DNMT3B, and EZH2 proteins were expressed at significantly higher levels in tumor vs. normal tissues. DNMT1 and EZH2 expression were positively correlated and higher in microsatellite-unstable vs. microsatellite-stable tumors, whether sporadic or hereditary. The expression of Ki-67, a marker of cell proliferation, mirrored the same pattern. EZH2 expression was associated with a TSG methylator phenotype. Methylation at the promoters of the methyltransferase genes themselves was addressed as a possible cause behind their altered expression. No differential methylation between normal and tumor tissues was evident for DNMT1 or EZH2, whereas the analysis of DNMT3B corroborated the regulatory role of a distal promoter region. Our study shows that methyltransferase expression in cancer depends on the tissue of origin, microsatellite-instability status, cellular proliferation, and – in the case of DNMT3B – promoter methylation of the respective gene. Translation of methyltransferase expression into DNA methylation appears complex as suggested by the fact that except for EZH2, no clear association between methyltransferase protein expression and TSG methylation was observed. (262 words)

INTRODUCTION

Cancer cells often show coordinated hypermethylation of multiple CpG islands, the CpG island methylator phenotype (CIMP), which can result in the silencing of hundreds of genes per cancer cell (Beggs *et al.* 2013). CIMP was originally described for colorectal carcinomas (Toyota *et al.* 1999) and subsequently shown to exist in most human cancers with patterns that are clearly non-random and to certain extent tumor type-specific (Costello *et al.* 2000; Joensuu *et al.* 2008). While a consensus definition for CIMP is still lacking (this is true for non-colonic cancers in particular), CIMP is usually measured by multi-gene panels of methylation markers and tumor suppressor genes (Berg *et al.* 2014). Cancers with CIMP have distinct molecular and clinical features compared to those without CIMP (Issa 2008).

Despite extensive descriptions of CIMP in cancer, the underlying molecular mechanisms remain elusive (McCabe *et al.* 2009). In search of responsible factors, alterations in the epigenetic machinery itself are of obvious interest especially as epigenetic modifiers are commonly affected by structural or expressional changes in cancer (Aumann and Abdel-Wahab 2014; Roy *et al.* 2014). DNA methyltransferase (DNMT) enzymes transfer a methyl group from S-adenosyl-L-methionine to cytosines in CpG nucleotides (Subramaniam *et al.* 2014). DNMT1 is mainly responsible for maintenance methylation and DNMT3A and DNMT3B for *de novo* methylation, although there is significant functional overlap (Egger *et al.* 2006; Hermann *et al.* 2004). Current evidence suggests that DNA methylation and chromatin repressive proteins act together to achieve epigenetic silencing of genes (Deb *et al.* 2014). In particular, Enhancer of Zeste Homologue 2 (EZH2), a component of the Polycomb repressive complex 2 (PRC2) and responsible for methylation of Lys27 of histone H3

(H3K27), has been suggested to serve as a recruitment platform for DNMTs (Vire *et al.* 2006). Polycomb-mediated H3K27 methylation may pre-mark genes for *de novo* methylation in cancer (Schlesinger *et al.* 2007; Widschwendter *et al.* 2007). Combination treatment targeting the dual modification of DNA methylation and H3K27-trimethylation may constitute an efficient approach for cancer cell-specific epigenetic therapy (Takeshima *et al.* 2015).

We have shown that tumor suppressor gene methylator phenotypes in familial/hereditary and sporadic cancers are characteristic of tumor type and family category (Joensuu *et al.* 2008). Familial/hereditary cancers can serve as tools to explore the causes and consequences of CIMP for the following reasons. First, epigenetic mechanisms play a prominent role in the various pathogenetic steps (constitutional susceptibility, tumor initiation, and tumor progression) of Lynch syndrome (LS) associated with hereditary defects in DNA mismatch repair (MMR) (Peltomaki 2014). Hypermethylation of the MMR gene *MLH1*, the most common susceptibility gene for LS (Thompson *et al.* 2014), connects CIMP with microsatellite instability (MSI) in sporadic cancers (Issa 2008). Second, tumors from non-polypotic colorectal cancer families without MMR defects (Familial colorectal cancer type X, FCCX) display different epigenetic phenotypes that may be distinguished by tumor suppressor promoter methylation (Joensuu *et al.* 2008) or alternatively, global hypomethylation (Goel *et al.* 2010; Pavicic *et al.* 2012); the latter may contribute to chromosomal instability in tumors (Issa 2008). While the molecular basis of FCCX is mostly unknown, the above observations imply a potential role for genes controlling epigenetic processes. Third, the CIMP profiles in familial/hereditary cancers mimic those of their sporadic counterparts (Esteller *et al.* 2001; Joensuu *et al.* 2008), suggesting that familial cancers can be used as general CIMP models.

This study was designed to investigate if altered expression of DNMTs (DNMT1 and DNMT3B) or EZH2 might be responsible for the differential tumor suppressor gene methylation patterns observed in familial and sporadic tumors (Joensuu *et al.* 2008). Moreover, promoter methylation of some key epigenetic genes (DNMTs and microRNAs targeting methyltransferase genes) was recently implicated in the regulation of epigenetic patterns (Huidobro *et al.* 2012; Suzuki *et al.* 2013), encouraging us to evaluate such events as possible mechanisms behind altered methyltransferase expression in our sample series.

MATERIALS AND METHODS

Patients and samples

This study included 165 tumor samples from colorectal adenocarcinoma or endometrial cancer patients, one tumor from each individual (CRC and EC, respectively; Table 1). Samples were divided into different categories based on the tissue, microsatellite instability (MSI) status and familial or sporadic background of cancer (Table 1). Familial CRC (Hereditary Non-Polyposis Colorectal Cancer) included DNA mismatch repair (MMR) gene mutation-positive and mutation-negative groups (LS-CRC and FCCX, respectively). All ECs were from LS patients.

In addition, we studied a series of endometrial samples representing different hyperplastic stages; 37 normal endometrium (N), 11 simplex hyperplasia (SH), 9 complex hyperplasia (CH), 18 complex atypical hyperplasia (CAH), and 11 endometrial carcinomas from 34 LS patients (Nieminen *et al.* 2009). MSI was present in 0, 18, 78, 50 and 70% of the sample groups, respectively.

The patients represented either a nationwide registry of hereditary CRC families (LS groups, hyperplasias and FCCX (Mecklin *et al.* 1987)) or a population-based cohort of sporadic CRCs from Mikkeli hospital district in Finland (Joensuu *et al.* 2008).

Tissue sections for immunohistochemical (IHC) slides were cut from paraffin-embedded tissue blocks or tissue microarrays and DNA was extracted by a method modified from Isola *et al*. (1994). Mutation screening, MSI analysis (revised Bethesda panel: Umar *et al.* 2004), IHC analysis for the MMR proteins and part of the tumor suppressor gene (TSG) promoter methylation screening were performed in previous studies (Abdel-Rahman *et al.* 2005; Holmberg *et al.* 1998; Joensuu *et al.* 2008; Nieminen *et al.* 2009; Ollikainen *et al.* 2005; Renkonen *et al.* 2003; Schweizer *et al.* 2001).

This study was approved by the institutional review board of the Helsinki University Central Hospital.

Immunohistochemical analyses

IHC analyses for DNMT1, DNMT3B and EZH2 were performed using mouse or rabbit primary antibodies and the Dako EnVision^{TM}+ System-HRP (DAB) detection by the manufacturer's standard protocol (Agilent Technologies, Glostrup, DK). Rabbit polyclonal DNMT1 antibody (product no ab19905, Abcam, Cambridge, UK) was used in 1:700 dilution with antigen retrieval in EDTA pH 8.0 buffer and with primary antibody incubation for 18-20 hrs. Mouse monoclonal DNMT3B antibody (product no IMG-184A, Imgenex/Novus Biologicals, Littleton, CO) was used in 1:250 dilution in 0.01 M citrate buffer pH 6.0, and primary antibody incubation was 18-20 hrs. Mouse monoclonal EZH2 antibody (product no 612667, clone 11, BD Biosciences, Franklin Lakes, NJ) was used in 1:25 dilution in Tris-EDTA pH 9.0 with primary antibody incubation of 24 hrs.

Ki-67 (Dako, clone MIB-1) staining was done by Ventana/Roche's ultraVIEW Universal DAB Detection Kit with RTU preparation buffer CC1 and RTU Hematoxylin II. Antibody dilution was 1:100 and incubation time 32 min.

To quantify nuclear expression of DNMT1, DNMT3B and EZH2, a staining index (SI) was determined by the product of intensity of the staining (range 0-3) multiplied by the percentage of stained nuclei (0=0, <10=1, 10-50=2, >50=3) similar to published studies (Collett *et al.* 2006; Fluge *et al.* 2009; Foulkes *et al.* 2003). Ki-67 expression was measured by the percentage of stained nuclei.

Custom MS-MLPA for *DNMT1*, *DNMT3B* and *EZH2* promoter methylation

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay was designed to study DNA methylation of selected sites in *DNMT1*, *DNMT3B* and *EZH2* promoter areas. We screened genomic sequences 5000 bp upstream from the transcription start site (TSS) and the downstream sequences including the first exon and intron of *DNMT1*, *DNMT3B* and *EZH2* for CpG islands. EMBOSS Cpgplot and TFSEARCH softwares were used to locate the CpG islands and common transcription factor binding sites. Methylation status of the CpG islands was determined by bisulfite sequencing with methylation-unbiased primers (Supporting Information Table S1) and *HhaI* sites from representative areas were chosen for the design of MS-MLPA probes (1 probe for *DNMT1*, 2 for *DNMT3B* and 4 for *EZH2*, Supporting Information Table S1). Custom probes were added to the SALSA MLPA P300 Human DNA reference-2 probemix with FAM-labeled SALSA MLPA EK1 reagent kit (MRC-Holland, Amsterdam, NL) and the assays performed following the manufacturer's protocol. The studied CpG sites were the following: *DNMT1* +31 bp, *DNMT3B* -444 and -104 bp and *EZH2* -1217, -56/-87, +619 and +22770 bp from TSS (www.ensembl.org). The methylation dosage ratios (Dm) were calculated as described (Gylling *et al.* 2008). MS-MLPA results

were validated against bisulfite sequencing in 3 commercial normal tissue samples and 7 cancer cell lines by a protocol outlined in Pavicic *et al*. (2011).

Bisulfite sequencing

Bisulfite sequencing was performed to validate the custom MS-MLPA methylation results. We used altogether 10 commercial samples or cell lines to study the methylation status of *DNMT1*, *DNMT3B* and *EZH2* promoter areas (specific sites described above). DNA bisulfite conversion was done by CpGenome DNA Modification Kit (Chemicon/Merck Millipore, Billerica, MA) according to the manufacturer's instructions and bisulfite sequencing was performed by using HotstarTaq (Qiagen, Hilden, DE) with specifically designed primers amplifying both methylated and unmethylated DNA by manufacturer's standard cycling protocol (for primer sequences see Supporting Information Table S1).

mRNA expression microarray before and after DNA demethylating treatment

RMA pre-processed mRNA microarray expression data, before and after DNA demethylating treatment, were retrieved for DNMT1, DNMT3B and EZH2 from Niskakoski *et al*. (2014). Expression data were available for RKO, HCT116 and HCT15 colon cancer cell lines. Cells were treated with 5 µM 5-aza-2'deoxycytidine (A3656, Sigma-Aldrich, St. Louis, MO) for 96 hours and 300 nM TSA (trichostatin, T1952, Sigma-Aldrich) for 18 hours. The mRNA levels were studied by Affymetrix GeneChip Human Genome U133 Plus 2.0 Arrays.

Statistical analyses

Statistical analyses were conducted by using PASW Statistics 18 software (SPSS Inc.). Shapiro-Wilk test was used to test the normality of the data. Correlations between DNMT1, DNMT3B, EZH2 and Ki-67 expressions and 24 tumor suppressor genes or LINE-1 methylation were tested by Spearman's rank correlation for non-parametric data. Mann-Whitney U test was used to test the differences in expression between groups (e.g., CIMP-low/CIMP-high or Ki-67-low/Ki-67-high) and Wilcoxon Signed-Rank to test the differences between tumors and paired normal samples. Clinicopathological characteristics (location of tumor, age of diagnosis, gender, grade and stage) or Dm values from custom MT promoter MS-MLPA or MT targeting miRNAs MS-MLPA (Walter 2011) vs. protein expressions tested either with Kruskal-Wallis, Mann-Whitney U, or Spearman correlation analysis as appropriate. All presented *P*-values (sig.) are 2-tailed ($P < 0.05$ were considered significant).

RESULTS

In an attempt to clarify underlying causes for aberrant DNA methylation in tumors (Joensuu *et al.* 2008), DNMT1, DNMT3B and EZH2 protein expression was studied by IHC in 203 samples of formalin-fixed paraffin-embedded colorectal or endometrial tumors or endometrial hyperplasia and paired normal tissues (Materials and Methods and Table 1). In addition, we used MS-MLPA to investigate if methyltransferase genes are regulated by promoter methylation and if specific methyltransferase targeting microRNAs (miR-152, miR-148a and miR-124a) show any association with the expression levels of the methyltransferases under study.

DNMT1, DNMT3B, EZH2 and Ki-67 protein expression in colorectal and endometrial tumors vs. normal tissues

Examples of different staining patterns are depicted in Supporting Information Fig. S1 and Fig. S2. In all patient groups with CRC, DNMT1, DNMT3B and EZH2 proteins were expressed at significantly higher levels in tumors than in normal mucosa (Fig. 1 and Supporting Information Table S2). DNMT1 in FCCX-CRC constituted the only exception (with borderline significance of $P = 0.067$). The average group-specific staining indices were 2.65–5.35 for DNMT1, 6.52–8.00 for DNMT3B, and 1.79–6.58 for EZH2 in CRC tumors and 1.13–2.56, 0–2.11, and 0–1.33 in paired normal mucosa, respectively. DNMT3B showed a significantly higher expression in LS-EC vs. normal endometrium, whereas DNMT1 and EZH2 protein expressions were relatively low in endometrial tumors and normal endometrium samples with no statistically significant differences (Fig. 1, Supporting Information Table S2). The results are shown case by case in Supporting Information Fig. S3.

Ki-67 was investigated to address cellular proliferation. Ki-67 expression was consistently higher in tumor than normal tissues (Fig. 1). Statistical significance was reached in sporadic MSS-CRC and LS-CRC groups (average staining 43.3% vs. 16.8%, $P = 0.001$, and 48.8% vs. 12.6%, $P < 0.001$, in tumors vs. normal tissues, respectively).

Relationship between different methyltransferases and comparison of tumors from different patient groups

The patterns of DNMT1 and EZH2 expression in tumors mirrored each other (Fig. 1). By Spearman correlation analysis, EZH2 was significantly correlated with DNMT1 expression in the combined groups of all CRCs ($P = 0.001$) and sporadic CRCs ($P = 0.003$).

To evaluate expression differences between CRC tumors representing different patient groups, in addition to the group averages shown in Fig. 1, box plots for expression distributions in tumors were established (Supporting Information Fig. S4). The expression of DNMT1 and EZH2 were higher in MSI than MSS tumors, whether sporadic (sporadic MSI vs. sporadic MSS) or hereditary (LS-CRC vs. FCCX). The difference was statistically significant between LS-CRC and FCCX for DNMT1 (P = 0.002), and between sporadic MSI-CRC and sporadic MSS-CRC for EZH2 (P = 0.026). Furthermore, Ki-67 expression showed a MSI-status-dependent pattern similar to DNMT1 and EZH2 (Supporting Information Fig. S4). DNMT3B revealed high levels of protein expression in all CRC groups and no clear group-specific patterns.

For colorectal vs. endometrial comparisons, the four CRC subgroups were combined ("all CRC" in Supporting Information Table S2) and the single LS-EC group represented ECs. EZH2 and DNMT1 were expressed at significantly higher levels in colorectal than endometrial carcinomas (mean SI 4.20 vs. 0.89 for EZH2 and 4.34 vs. 1.19 for DNMT1 in CRC and EC, respectively; $P < 0.001$ for both comparisons, Supporting Information Table S2). A similar trend was seen already in the respective normal tissue samples. DNMT3B was expressed at high levels in both CRCs and ECs (mean SI 6.94 in CRC and 6.50 in EC).

Ki-67 expression vs methyltransferase expression in tumors

We divided the tumors into two subgroups based on their Ki-67 expression values: Ki-67-high with expression greater than or equal to the median (47.5% of stained nuclei for CRC and 15% for EC) and Ki-67-low with expression lower than the median. Mann-Whitney U test was used to test differences in methyltransferase expression between the two subgroups (Fig. 2 upper panel, Supporting Information Table S3). EZH2 expression was significantly associated with the high Ki-67

subgroup in sporadic MSS-CRCs (SI 2.94 in Ki-67 high vs. 0.76 in Ki-67 low subgroup; *P* = 0.017) and in the combined sporadic CRCs (SI 3.95 in Ki-67 high vs. 0.88 in Ki-67 low subgroup; *P* = 0.001). However, this association was not evident in all patients (Supporting Information Fig. S2). DNMT1 expression was associated with Ki-67-high in sporadic MSI CRCs (SI 5.65 in Ki-67 high vs. 2.00 in Ki-67 low subgroup; *P* = 0.014) and LS-EC (SI 1.50 in Ki-67 high vs. 0.12 in Ki-67 low subgroup; *P* = 0.030). For DNMT3B expression, stratification by Ki-67 status had little effect (Supporting Information Table S3).

Methyltransferase expression vs aberrant TSG promoter methylation in tumors

Tumor suppressor gene methylation status differed significantly between individual patient groups (*P* < 0.001) with sporadic MSI-CRC having clearly the highest number of methylated TSG promoters (mean 7.24 out of 24 genes) compared to other CRC groups (3.39–4.03; Supporting Information Table S2). We defined high TSG methylator phenotype (CIMP+ in Fig. 2) as at least 5 methylated promoters out of 24 and low TSG methylator phenotype (CIMP-) as less than 5 methylated promoters out of 24 (Joensuu *et al.* 2008). EZH2 expression was significantly associated with CIMP+ in sporadic MSS-CRC (average SI 3.88 in CIMP+ vs. 1.35 in CIMP- tumors; *P* = 0.028) and in the combined sporadic (MSS + MSI) group (average SI 4.62 in CIMP+ vs. 1.65 in CIMP- tumors, *P* = 0.003; Fig. 2 lower panel and Supporting Information Table S3). Stratification by CIMP status did not reveal significant differences in DNMT1 or DNMT3B expression (Fig. 2 and Supporting Information Table S3).

Methyltransferase expression in the endometrial hyperplasia series

A progressive trend from normal endometrium to SH to CH to CAH was evident for DNMT1, DNMT3B and EZH2 expression (Fig. 3, Supporting Information Fig. 5 and Table S2). Statistical significance could not be tested due to small sample sizes (see Materials and Methods). The results are broadly concordant with our previous findings of TSG promoter methylation in hyperplastic tissues with normal endometrium and SH clustering into a low-methylator group and CH, CAH, and EC clustering into a high-methylator group; (Nieminen *et al.* 2009).

Possible causes behind aberrant methyltransferase expression

To investigate if expression of the methyltransferases in question is controlled by methylation of their own promoters, a custom MS-MLPA assay was designed that included probes targeting the promoter areas of *DNMT1*, *DNMT3B* and *EZH2* (Materials and Methods). Only one *DNMT3B* site, monitored by the *DNMT3B* probe 1, showed a statistically significant ($P < 0.001$) differential methylation between CRC tumors and normal mucosa (Table 2). This *DNMT3B* probe scanned the same distal promoter area as that described in Huidobro *et al*. (2012), our CpG site being located 444 bp upstream of TSS. In individual tumors, the methylation dosage ratio (Dm) varied between 0 and 0.46 (indicating 0–46% methylation) and in the paired normal mucosae between 0 and 0.24 (data not shown). Among the different patient groups, the *DNMT3B* distal promoter showed a significantly higher methylation relative to normal mucosa in sporadic MSS-CRC and LS-CRC tumors ($P = 0.010$ and $P = 0.033$, respectively; Table 2). The highest degrees of promoter methylation were seen in samples from LS-CRC (up to Dm 0.46) and sporadic MSI-CRC groups (up to Dm 0.40).

LS-CRC was the only patient group with some variation in DNMT3B protein expression between the individual tumors (Supporting Information Fig. S4). Therefore, we focused on LS-CRCs to study

if methylation of the distal promoter was associated with protein expression. DNMT3B expression was stratified into low (SI $\leq$ 4) and high (SI > 4) based on the box plot in Supporting Information Fig. S4. Methylation of the *DNMT3B* distal promoter was negatively associated with protein expression with borderline significance (P = 0.080). The median Dm was 0.35 in DNMT3B-low expression 0.15 in DNMT3B-high expression group.

Demethylation treatments of colorectal cancer cell lines, followed by array-based profiling, revealed a 2.4-fold increase in DNMT3B mRNA expression in HCT15 and a 1.6-fold increase in RKO after treatment (P < 0.05), in agreement with the results from Huidobro *et al*. (2012). In HCT116, the distal promoter was unmethylated and consequently, no treatment effect was either expected or seen.

We also analyzed if methyltransferase protein expression correlated with the methylation status of specific miRNA loci (Pavicic *et al.* 2011) known to target the respective methyltransferase genes. MiR-152 targets *DNMT1* (Braconi *et al.* 2010), miR-148a *DNMT3B* (Duursma *et al.* 2008) and miR-124a(1-3) *EZH2* (Zheng *et al.* 2012). No significant positive correlations were observed (see Discussion).

Methyltransferase expression vs. clinical characteristics

There was no significant correlation between the protein expression of DNMT1, DNMT3B or EZH2 and different clinical characteristics (gender, age of diagnosis, stage, grade or location of tumor). This probably reflected sample size and the selective nature of the samples.

DISCUSSION

DNMTs and EZH2 control multiple cancer-relevant biological processes, such as cell proliferation, DNA damage response, DNA repair, and stemness, which in turn can influence malignant transformation, invasion, cancer progression, and metastasis (Campbell *et al.* 2013; Jin and Robertson 2013; Yamaguchi and Hung 2014). These activities may be mediated by altered DNA or histone methylation e.g., inactivation of genes involved in DNA damage repair by promoter methylation, (Jin and Robertson 2013) or be independent of methylation (several functions known, Jin and Robertson 2013; Yamaguchi and Hung 2014). Prompted by discordant data between DNMT expression and CIMP in the available literature (Eads *et al.* 1999; Kanai *et al.* 2001) and the virtual lack of published data concerning the relationship between EZH2 expression and CIMP, we undertook an immunohistochemical investigation of DNMT1, DNMT3B, and EZH2 expression in CRC and EC from sporadic and familial cases. Consistent patterns of expression emerged, showing significant variation between the individual patient groups. Moreover, elevated expression of EZH2, rather than DNMT1 or DNMT3B, was associated with TSG methylator phenotype in tumors. Implications of the findings will be discussed below.

Results of DNMT expression and relationship with CIMP in cancer vary a lot in part because of different methodologies used to assess expression (real-time PCR analysis of mRNA vs. IHC analysis of protein) and CIMP (no consensus definition exists). Overexpression of one or several DNMTs compared to adjacent normal tissue is seen from below 10% to over 70% of various cancers (Etoh *et al.* 2004; Girault *et al.* 2003; Kanai *et al.* 2001; Nosho *et al.* 2009; Xiong *et al.* 2005). Even studies employing the same methodology have arrived at conflicting results. Focusing on CRC, Eads *et al.* (1999) found that mRNAs of DNMT1, DNMT3A, or DNMT3B were not upregulated when normalized with proliferation-associated genes, and expression showed no correlation with CIMP, whereas Kanai *et al*. (2001) reported that mRNAs of DNMT1 and DNMT3B were significantly

elevated after normalization with proliferation-associated markers, and overexpression of DNMT1 was significantly associated with CIMP. Overexpression of EZH2 (typically determined by IHC and defined as staining indices above the median) has been reported to occur in 17% of CRC (Fluge *et al.* 2009), 18% of EC (Bachmann *et al.* 2006), and up to 51% of breast carcinomas (Collett *et al.* 2006). While we made no attempt to determine cut-off-based fractions of tumors overexpressing DNMT1, DNMT3B, or EZH2 among our CRCs and ECs, the SI values in tumor tissues significantly exceeded those in paired normal tissues in virtually all patient groups (Fig. 1). In studies mentioned above (Bachmann *et al.* 2006; Collett *et al.* 2006; Fluge *et al.* 2009), EZH2 was significantly associated with Ki-67 expression, which broadly agrees with our data (Supporting Information Fig. S2). None of the investigations examined CIMP. Compatible with our findings, Hoffman *et al*. (2007), who studied prostate cancer, observed elevated expression of EZH2, but not DNMT1 or DNMT3B, in association with increased DNA methylation (of the same TSGs we analyzed). The association of EZH2 expression with TSG promoter methylation is consistent with the idea that EZH2 is required for the establishment of DNA methylation patterns (Deb *et al.* 2014).

Our observation of higher DNMT1 and EZH2 protein expression in MSI vs. MSS CRCs is particularly interesting and novel. The finding applied to both sporadic and hereditary cancers (P = 0.026 for EZH2 in sporadic MSI-CRC vs. sporadic MSS-CRC, and P = 0.002 for DNMT1 in LS-CRC vs. FCCX, Supporting Information Fig. S4). Previously, overexpression of DNMT3B protein was reported to be significantly associated with CIMP-high and MSI-high phenotypes in CRC (Nosho *et al.* 2009), whereas CRCs from our study showed uniformly high levels of DNMT3B expression irrespective of MSI status (Supporting Information Fig. S4). Mechanisms accounting for the observed associations between DNMT1 or EZH2 expression and MSI are unknown. Studies have found no evidence of physical interaction between MMR proteins and DNMTs or EZH2, although altered

methyltransferase expression can indirectly influence MMR gene expression (Loughery *et al.* 2011; Wang *et al.* 2013).

Altered expression and/or function of DNMTs and EZH2 in tumors may result from several alternative mechanisms. These include constitutional polymorphisms (Crea *et al.* 2012; Shen *et al.* 2002) and somatic mutations (Kanai *et al.* 2003; Roy *et al.* 2014) of the *DNMT* and *EZH2* genes. Alternative splicing can influence the expression levels of DNMT3B (Veeck and Esteller 2010). We focused on miRNAs targeting DNMTs or EZH2 (so called "epi-miRNAs") whose roles as methyltransferase regulators are well established (Suzuki *et al.* 2013), and promoter methylation of the *DNMT* and *EZH2* genes themselves, for which little information is available (Huidobro *et al.* 2012; Li *et al.* 2014). It has been hypothesized that inactivation of an epi-miRNA (e.g., miR-152) by methylation leads to increased expression of its target epigenetic gene (e.g., *DNMT1*), thereby creating a self-amplified loop (Valeri *et al.* 2009). We found no significant positive correlations between methylation of CpG islands associated with miR-152, miR-148, or miR-124 (targeting *DNMT1*, *DNMT3B*, and *EZH2*, respectively) vs. DNMT or EZH2 expression, which could reflect a variety of reasons, including the likely scenario that the independent effects of the miRNAs were not strong enough to control the expression of the respective genes alone. Evaluation of *DNMT1*, *DNMT3B*, and *EZH2* promoters for methylation in patient specimens revealed a consistent difference between normal and tumor tissues for *DNMT3B* only (Table 2), and DNMT3B – unlike DNMT1 and EZH2 – also showed upregulation after demethylation treatment of cell lines. Our data as a whole supported the findings by Huidobro *et al*. (2012) of an area outside the CpG island ("distal region") regulating DNMT3B expression.

The mechanistic basis of CIMP remains unsettled. The CIMP phenotype and altered methyltransferase expression often co-exist in tumorigenesis, sharing early appearance (Ding *et al.* 2006; Ibrahim *et al.* 2011), tissue-specificity (Joensuu *et al.* 2008, this study), involvement in tumor progression (Ibrahim *et al.* 2011, this study in regard to endometrial hyperplasias), and contribution to the prognosis of CRC and EC (Bachmann *et al.* 2006; Benard *et al.* 2014; Fluge *et al.* 2009; Issa 2008). Such observations imply that the processes are likely to be linked, although their relationship may not be straightforward. In our investigation, EZH2 in particular came up through associations with the TSG methylator phenotype (Fig. 2) and a role in the multistep endometrial tumorigenesis (Fig. 3). While our study was in progress, Jia N *et al*. (2014) reported that EZH2 overexpression stratified endometrial lesions into high expression (CH, CAH, and EC) and low expression groups (normal endometrium and SH), a division identical to that we have previously shown to apply to the distribution of high vs. low TSG methylator phenotypes among endometrial lesions (Nieminen *et al.* 2009). Our EZH2 expression results indicated a progressively increasing trend from normal endometrium to SH to CH to CAH to EC (Fig. 3), but no statistical differences between types of lesions were obtained due to the small number of cases. Given that H3K27 methylation may pre-mark genes for *de novo* methylation in cancer (Schlesinger *et al.* 2007; Widschwendter *et al.* 2007), EZH2 may contribute to some of the selective aspects of CIMP mentioned above. In this capacity, EZH2 may also constitute a promising therapeutic target that could allow for efficient anti-cancer treatment without affecting global methylation status and gene silencing beyond cancer cells (Deb *et al.* 2014; Roy *et al.* 2014; Yamaguchi and Hung 2014).

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