Promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing targets the deleted in colon cancer (DCC) gene in colorectal carcinogenesis without affecting neighboring genes on chromosomal region 18q21

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Chromosomal loss of 18q21 is a frequent event in colorectal cancer (CRC) development, suggesting that this region harbors tumor suppressor genes (TSGs). Several candidate TSGs, among which methyl-CpG-binding domain protein 1 (MBD1), CpG-binding protein CXXC1, Sma- and Mad-related protein 4 (SMAD4), deleted in colon cancer (DCC) and methyl-CpG-binding domain protein 2 (MBD2) are closely linked on a 4-Mb DNA region on chromosome 18q21. As TSGs can be epigenetically silenced, this study investigates whether MBD1, CXXC1, SMAD4, DCC and MBD2 are subject to epigenetic silencing in CRC. Methylation-specific polymerase chain reaction and sodium bisulfite sequencing of these genes show that DCC, but not MBD1, CXXC1, SMAD4 and MBD2, has promoter CpG island methylation in CRC cell lines and tissues (normal mucosa [29.5% (18/61)], adenomas [81.0% (47/58)] and carcinomas [82.7% (62/75)] (P = 8.6 × 10⁻⁵)) that is associated with reduced DCC expression, independent of 18q21 loss analyzed by multiplex ligation-dependent probe amplification. Reduced gene expression of CXXC1, SMAD4 and MBD2 correlates with 18q21 loss in CRC cell lines (P = 0.04, 0.02 and 0.02, respectively). Treatment with the demethylating agent 5-aza-2′-deoxycytidine, but not with the histone deacetylase inhibitor trichostatin A exclusively restored DCC expression in CRC cell lines. Chromatin immunoprecipitation studies reveal that the DCC promoter is marked with repressive histone-tail marks H3K9me3 and H3K27me3, whereas activity related H3K4me3 was absent. Only active epigenetic marks were detected for MBD1, CXXC1, SMAD4 and MBD2. This study demonstrates specific epigenetic silencing of DCC in CRC as a focal process not affecting neighboring genes on chromosomal region 18q21.

Introduction
Colorectal cancer (CRC) development is characterized by the growth of a benign precursor lesion from which a small percentage will progress into a carcinoma. Genetic alterations underlying the adenoma to carcinoma transition have been extensively studied over the past two decades. Chromosomal loss of 18q has been reported to occur in 60–70% of CRCs, suggesting this region harbors tumor suppressor genes (TSGs) involved in colorectal carcinogenesis (1–3). Although chromosomal loss of 18q often affects a large section of the chromosomal arm, a minimal loss region on 18q21 including deleted in colon cancer (DCC) has been identified (2,4). Near to DCC, within the same 4 Mb chromosomal region, lie several other candidate TSGs, including Sma- and Mad-related protein 4 (SMAD4) (5), methyl-CpG-binding domain protein 1 (MBD1), CpG-binding protein CXXC1 and methyl-CpG-binding domain protein 2 (MBD2) (6,7).

Since loss of function for TSGs requires biallelic inactivation, these genes have been examined for the presence of mutations in the remaining allele. For DCC (8), MBD1, CXXC1 and MBD2 (6) mutations are relatively rare events (<5%) in CRC. SMAD4 has some reports of mutations (5%) (9), which may be higher in tumors with distant metastasis (35%) (10), but even this mutational rate does not match the frequency of loss (60–70%) at 18q21. While haplo-insufficiency might provide another explanation for the observed low mutation frequencies (11), this raises the question of whether alternative mechanisms might account for inactivation of these TSGs. In addition to genetic changes in cancer, epigenetic modifications including promoter CpG island methylation are associated with gene silencing and serve as mechanism to inactivate TSGs and DNA repair genes (12). Promoter CpG island methylation is often associated with histone modifications (13). Histone H3 trimethyl lysine 9 (H3K9me3) or lysine 27 (H3K27me3) marks are associated with methylated DNA and transcriptional silencing (14). In contrast, acetylation of histone H3 (15) and trimethylation of histone H3 at lysine 4 (H3K4me3) are associated with unmethylated DNA and gene expression and can be considered as marks of active chromatin (13).

While chromosomal region 18q21 deletion occurs frequently in colorectal carcinogenesis and has been extensively studied for genetic mechanisms potentially leading to gene inactivation (16), little is known about epigenetic silencing of genes in this region. Therefore, in this study, we investigate whether epigenetic mechanisms serve as second hit in inactivating genes on chromosome 18q21 in CRC.

Materials and methods
Cell lines, study population and tissues
CRC cell lines (Caco2, Colo205, HT29, SW480, HCT116, RKO and LS174T and SW48) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal calf serum (HyClone Perbio Science, Erembodegem-Aalst, Belgium). MBD1, CXXC1, SMAD4, DCC and MBD2 promoter CpG island methylation was investigated in a well-characterized series of sporadic colorectal carcinomas. The series consists of formalin-fixed, paraffin-embedded CRC tissues (n = 75) of patients >50 years of age, 37 males and 38 females (mean age 71.0, range 50–88, at time of diagnosis) that were retrospectively collected from the tissue archive of the department of Pathology of the Maastricht University Medical Center. When present, also normal (n = 61) and adenoma (n = 58) tissue was collected from these patients. Histological normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n = 48) was selected (23 males, 25 females, age 63.9, range 50–87 years at time of diagnosis). Clinicopathologic characteristic are listed in supplementary Table 1 (available at Carcinogenesis Online). This study was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

DNA isolation and bisulfite treatment
A 5-μm section of each tissue block was stained with hematoxylin and eosin and reviewed by pathologist (A.P.D.B.). Five sections of 20 μm thickness were collected from these patients. Histological normal biopsy material from patients undergoing endoscopy for non-specific abdominal complaints (n = 48) was selected (23 males, 25 females, age 63.9, range 50–87 years at time of diagnosis). Clinicopathologic characteristic are listed in supplementary Table 1 (available at Carcinogenesis Online). This study was approved by the Medical Ethical Committee of the Maastricht University Medical Center.

Abbreviations: Aza, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; CRC, colorectal cancer; DCC, deleted in colon cancer; MBD1, methyl-CpG-binding domain protein 1; MBD2, methyl-CpG-binding domain protein 2; MLPA, multiplex ligation-dependent probe amplification; MSP, methylation-specific polymerase chain reaction; PCR, polymerase chain reaction; SMAD4, Sma- and Mad-related protein 4; TSA, trichostatin A; TSG, tumor suppressor gene; TSS, transcription start site.

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DNA isolation kit (Genta systems, Minneapolis, MN) according to the manufacturer’s instructions. Sodium bisulfite modification of 500 ng genomic DNA was performed using the EZ DNA methylation kit (ZYMO research Co., Orange, CA) according to the manufacturer’s instructions.

Promoter CpG island methylation analyses: sodium bisulfite sequencing and methylation-specific PCR

For sequencing of sodium bisulfite-converted DNA, polymerase chain reaction (PCR) products were amplified and cloned using the TOPO-TA cloning kit (Invitrogen). Single colonies were sequenced using an automated sequencer (Applied Biosystems, Foster City, CA).

Methylation-specific polymerase chain reaction (MSP) analysis on bisulfite-treated DNA were performed as described in detail elsewhere (17,18). To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that was used as a template for the MSP reaction. All primer sequences and conditions are shown in supplementary Table 2 (available at Carcinogenesis Online).

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) was performed as described before (19,20). In brief, an oligonucleotide MLPA probe set was designed for 45 genes on chromosomes 3, 5, 8p, 9, 11q, 15q, 18q and 19q. This mix contained probes for eight genes located on chromosome 18q, including probes for MB1 and SMAD4. Additionally, 11 reference probes (two probes on chromosome 2p, three probes on chromosome 4q, two probes on chromosome 12 and four probes on chromosome 16) were included for quality control and normalization purposes. Data analysis was performed using excel-based software developed at MRC-Holland, which provides a reliable and robust normalization for MLPA fragment data files (21). The data were normalized using the median of the reference probes. As reference DNA, a pool of DNA extracted from normal paraffin-embedded tissue samples of different organs (10 colon, 10 stomach, four kidneys, four liver and four spleen) was used. DNA copy number ratios were obtained by dividing the median area under the peak for each probe in the sample by the median area under the peak of at least three reference DNA samples. Each sample was run at least three times, and the median of the different runs was used for analysis. Finally, all DNA copy number ratios were normalized by setting the median copy number ratio of the reference probes to 1.0. A ratio <0.8 was considered as a deletion and a ratio >1.2 as a gain.

Quantitative reverse transcription–PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) following the manufacturer’s instructions. Possible genomic DNA contaminations were removed by DNase treatment with the RNase-free DNase set (Qiagen). Complementary DNA synthesis was performed using the Iscript complementary DNA synthesis kit (Bio-Rad, Richmond, CA). Quantitative reverse transcription–PCR was performed as described previously (22) using SYBR Green PCR master mix (Applied Biosystems, Nieuwkerk a/d IJssel, The Netherlands). MB1, CXXC1, SMAD4, DCC and MB2D expression levels were normalized to CYCLOPHILIN and average expression levels in normal colon tissues adjacent to carcinoma tissues (n = 10) for each tissue sample using the following equation: relative expression = 2−(ΔΔCt), where ΔΔCt = average Ct (tissue sample)−average Ct (CYCLOPHILIN)−average Ct (normal colon tissues). Since alternative splicing of DCC exons 6–7 and 18–23 has been reported (23,24), DCC reverse transcriptase primers are located in exon 2–3.

To investigate re-expression of MB1, CXXC1, SMAD4, DCC and MB2D following inhibition of DNA methyltransferases or inhibition of histone deacetylation, HCT116, RKO cells and HT29 cells were treated with 5 μM 5-aza-2’-deoxycytidine (Aza) (Sigma, Nieuwegein, The Netherlands) and with 300 nM trichostatin A (TSA) (Sigma) for 48 hours, respectively.

As a positive control for gene expression following inhibition of DNA methyltransferases, re-expression of GATA4 and GATA5 was used. As a positive control for gene expression following treatment with TSA, previously described control gene FABP4 (15,25) was analyzed.

Chromatin immunoprecipitation assays

Chromatin immunoprecipitations (ChIPs) were performed for cell lines HCT116 and HT29 cells and analyzed essentially as described previously (26). Briefly, HCT116 and HT29 cells were fixed in 1% formaldehyde. Samples were immunoprecipitated with antibodies for either hemagglutinin (HA) or mouse anti-SMAD4 (ab5850, Abcam). Immunoprecipitated DNA was quantified by real-time PCR. Enrichment was calculated as percentage of input DNA. HA was used as negative control and subtracted from enrichment values. For MB1, CXXC1, SMAD4 and MB2D, three primer pairs were designed covering −600 bp to the transcription start site (TSS). For DCC, six additional primer pairs were designed to investigate the region −597 to +467 bp relative to the TSS. Control PCR for each antibody immunoprecipitation were performed using primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PglyneoA [mononuclear leukocyte in HCT116 and biallelic methylation in HT29 (data not shown)] as negative and positive control dependent on the antibody used.

Data analysis

We used the Pearson’s χ2 or Fisher’s Exact test, students t-test, Kruskal-Wallis, Mann–Witney or Wilcoxon rank test where appropriate to compare categorical and continuous data, respectively. Since a significant difference in age was observed between CRC cases and controls (P = 1.1 × 10−5), logistic regression analyses were used to adjust for age. All quoted P values are two sided, and a P value <0.05 was considered statistically significant. Where appropriate, the Bonferroni method was used to correct for multiple comparisons. Data analysis was done using SPSS software (version 12.0.1).

Results

DCC promoter is methylated in CRC cell lines, whereas MBD1, CXXC1, SMAD4 and MB2D are unmethylated

We first analyzed promoter Cpg island methylation of five TSGs genes within a 4-Mb region in proximity to DCC (46.0–49.9 Mb) including MB1, CXXC1, SMAD4, DCC and MB2D. Using public ENCODE, and EMBoss CpG plot/Cpg software, we observed that MB1, CXXC1, SMAD4, DCC and MB2D each have a Cpg island-associated promoter region [GC content >60%, ratio of observed CpG:expected CpG >0.6 and minimum length 200 bp (27)]. For an initial assessment of the methylation status of these promoter CpG islands, MSP was performed on CRC cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Since DCC has two CpG-rich regions, region 1 located −95 to +170 bp and region 2 located between +608 and +962 bp relative to the TSS, both regions were analyzed. MB1, CXXC1, SMAD4 and MB2D promoter CpG island methylation status was analyzed using primers for the CpG islands spanning the TSS [primers, locations and PCR conditions are provided in supplementary Table 2 (available at Carcinogenesis Online)]. All CRC cell lines have complete (CACO2, COLO205, HT29, SW480, HCT116, RKO and SW48) or partial (LS174T) DCC DNA methylation in region 1, whereas region 2 showed complete DCC methylation for all cell lines. In contrast, CpG-rich regions within MB1, CXXC1, SMAD4 and MB2D promoters were unmethylated in all cell lines analyzed (data not shown).

To study in more detail about the DNA methylation of these promoter regions, sodium bisulfite sequencing of individual clones of CRC cell lines HCT116 and RKO and three paired normal and adjacent CRC tissues showed that both DCC CpG islands are densely hypermethylated in CRC tissues, whereas normal tissues showed less methylated CpG dinucleotides (Figure 1). This difference is most apparent for the promoter region of DCC (region 1), in which normal tissues 1, 4 and 8 have, respectively, 7.3, 1 and 3.1% of sequenced CpG dinucleotides methylated, whereas in the more 3’ region (region 2), 40.3, 22.2 and 25% of sequenced CpG dinucleotides were methylated. Since the promoter region (region 1) demonstrated the most cancer-specific methylation and epigenetic changes are most tightly associated with transcription near the TSS, we continued this analysis for DCC in region 1.

In contrast with DCC, the MB1, CXXC1, SMAD4 and MB2D gene promoter regions are almost completely unmethylated at all CpG dinucleotides.

Reduced CXXC1, SMAD4 and MB2D gene expression associates with 18q21 loss

To determine the effect of promoter CpG island methylation and loss of 18q21 on gene expression, we analyzed DNA copy number changes of eight loci on region 18q harboring SMAD4 (46.8 Mb) and DCC (48.1 Mb) in detail by MLPA (28) in CRC cell lines (Figure 2A). These analyses revealed that cell lines CACO2, COL0205, HT29 and SW480 had loss of the complete region tested, including the DCC locus. This was not observed for cell lines HCT116, RKO, LS174T and SW48 that had no loss of 18q21.

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Next, we studied MBD1, CXXC1, SMAD4, DCC and MBD2 gene expression and the relation with 18q21 deletion in CRC cell lines CACO2, COLO205, HT29, SW480, HCT116, RKO, LS174T and SW48. Expression of CXXC1, SMAD4 and MBD2 was significantly lower in cell lines with 18q21 deletion when compared with cell lines without 18q21 deletion. Data represent mean and standard error of mean of two or three independent experiments. Mann–Whitney, *P = 0.04. Mann–Whitney **P = 0.02.

Fig. 1. Sodium bisulfite sequencing results of MBD1, CXXC1, SMAD4, DCC and MBD2 gene promoters on cell lines HCT116 and RKO and normal and adjacent tumor tissues of three patients (1, 4 and 8) show that the DCC promoter is densely methylated. Each row represents an individual cloned allele and each square indicates a CpG dinucleotide. Black square, methylated CpG site; white square, unmethylated CpG site; gray square, not determined.

Fig. 2. (A) Heatmap representation of MLPA that shows loss of eight loci on chromosomal region 18q in cell lines CACO2, COLO205, HT29 and SW480. A ratio <0.8 (indicated by blue) was considered as a deletion, a ratio >1.2 (indicated by yellow) as a gain. (B) Quantitative reverse transcription–PCR of MBD1, CXXC1, SMAD4, DCC and MBD2 on CRC cell lines. Gene expression levels are relative to the mean expression level of the normal colon tissues (n = 10), which is set to equal 1. Figure shows that DCC transcript levels are nearly undetectable in all CRC cell lines, whereas high expression was present in human brain (HB). Expression of MBD1, CXXC1, SMAD4 and MBD2 is lower in CRC cell lines when compared with normal colon–adjacent to carcinoma tissues (n = 10). Expression of CXXC1, SMAD4 and MBD2 was significant lower in cell lines with 18q21 deletion when compared with cell lines without 18q21 deletion. Data represent mean and standard error of mean of two or three independent experiments. Mann–Whitney, *P = 0.04. Mann–Whitney **P = 0.02.
methylation observed in these cell lines correlated with absent expression.

**DCC promoter methylation is a frequent and early event in colorectal carcinogenesis**

Next, promoter CpG island methylation of these 18q21 genes was studied in a well-characterized series of formalin-fixed, paraffin-embedded primary CRCs (n = 75). Where available, adenoma- (n = 58) and normal tissue (n = 61) of the same patients was also analyzed. This analysis showed that DCC promoter methylation is a common event in CRC, which occurred in 82.7% (62/75) of CRCs, 81% (47/58) of adenomas and 29.5% (16/61) of normal colon mucosa of CRC patients (P = 8.6 × 10⁻⁹) (Table I).

For 50 patients, normal-, adenoma- and carcinoma tissue was available allowing a detailed analysis of DCC promoter methylation in colorectal carcinogenesis (Table II). These analyses showed that for 13 patients, carcinoma-, adenoma- and normal tissues were synchronously methylated indicating that DCC promoter methylation occurs early in CRC development. DCC DNA promoter methylation was also studied in age-matched normal colorectal mucosa tissues of 48 non-cancer patients of which 20.8% (10/48) was methylated.

**MBD1, CXXC1, SMAD4 and MBD2** gene promoters were unmethylated in the vast majority of CRCs (32/33, 27/32, 28/29, 31/32, respectively), adenoma (32/33, 33/34, 30/32, 31/32, respectively) and normal tissues (31/31, 32/32, 32/32, 31/31, respectively) (Table I). Remarkably, in one carcinoma, CXXC1, SMAD4, DCC and MBD2 were synchronously methylated.

**18q21 copy number change complements DCC promoter methylation**

Next, we studied how 18q21 deletion and DNA promoter methylation collaborate in transcriptional gene silencing in a second series of normal and adjacent CRC tissues of patients (n = 20) of which frozen tissue was available. In this series, DCC promoter methylation was detected in 75% (15/20) of CRCs (Figure 3A). In the paired normal tissues only 5% (1/20) was methylated. MBD1, CXXC1, SMAD4 and MBD2 promoters were all unmethylated in these tumors (data not shown). Deletion of 18q21 was detected in 40% (8/20) of cancer tissues and in none of the normal tissues. In all detectable 18q21 deletions, the DCC locus was included and in seven of these eight CRCs, all probes on 18q showed DNA copy number loss. Thereby we show that DCC is affected by promoter CpG island methylation and/or loss of the DCC locus in 90% of CRCs. No specific correlation between DNA promoter methylation and 18q21 copy number loss was observed.

The effect of DCC promoter methylation and 18q21 loss on gene expression was studied in 10 of 20 patient samples for which RNA was available (Figure 3B). In this subset, DCC promoter methylation was observed for all CRCs and one normal tissue (patient 16). Although inter-individual differences in gene expression levels were observed, quantitative reverse transcription-PCR analyses showed that DCC expression was significantly reduced in all CRCs when compared with matching normal tissues. Very low DCC transcript levels were detected in both normal and carcinoma tissue of patient 16 that corresponds to the methylated status of both tissues. Apart from tumor T11, in which DCC expression was more selectively targeted, most of the carcinoma tissues showed reduced expression of MBD1, CXXC1, SMAD4 and MBD2 as well. In T20, characterized by loss of 18q21, a strong reduction in expression of all genes was observed. However, in contrast to the correlations observed in cell lines, reduced gene expression of CXXC1, SMAD4 and MBD2 was not restricted to tumors with loss of 18q21, indicating the presence of other transcriptional silencing mechanisms for MBD1, CXXC1, SMAD4 and MBD2 in CRC.

**Demethylating agents, but not histone deacetylase inhibitors, restore DCC expression**

In order to directly investigate the roles of DNA promoter methylation or histone deacetylation in transcriptional silencing of 18q21 genes,
HCT116, RKO and HT29 cells were treated with the DNA methyltransferase inhibitor Aza and the histone deacetylase inhibitor TSA. Aza restored DCC expression in HCT116, RKO and HT29 cells (Figure 4A). Of the unmethylated genes, MBD1 showed a modest increase in expression after treatment with Aza, whereas no change in expression was observed for CXXC1, SMAD4 and MBD2 (Figure 4B). TSA was unable to reactivate DCC or increase the expressions of MBD1, CXXC1, SMAD4 and MBD2. These results indicate that promoter CpG island methylation, but not histone deacetylation alone, accounts for transcriptional silencing of DCC. MBD1, CXXC1, SMAD4 and MBD2 expressions are not dependent upon these processes.

DCC promoter CpG island methylation is associated with inactive chromatin marks

We then analyzed the presence of inactive (H3K9me3 and H3K27me3) and active (H3K4me3) chromatin marks on MBD1, CXXC1, SMAD4, DCC and MBD2 promoter regions with ChIP assay for cell line HCT116 (18q21 retention) and cell line HT29 (18q21 copy number loss). ChIP in HCT116 demonstrated that the entire DCC promoter and adjacent 3' region have enrichment of the repressive histone-tail marks H3K9me3 and H3K27me3 and depletion of the active chromatin mark H3K4me3 (Figure 5A). As a control, HCT116 had enrichment of H3K9me3 and H3K4me3 but not H3K27me3 on the P16INK4A promoter that is consistent with a recent report by Kondo et al. (29) and with the known active and silent alleles in this cell line (30). Conversely, the MBD1, CXXC1, SMAD4 and MBD2 promoters have enrichment of the active H3K4me3 mark, but not of the repressive histone-tail marks H3K9me3 and H3K27me3. In cell line HT29, a similar pattern was observed (Figure 4B), with active marks for all genes, except DCC, and repressive marks at DCC, although the pattern was slightly different with enrichment for only H3K27me3 and not H3K9me3 at DCC. Thus, ChIP analyses showed a clear distinction for the DCC gene, with the DCC promoter surrounded by inactive chromatin, whereas MBD1, CXXC1, SMAD4 and MBD2 have enrichment for active histone-tail marks and no signs of epigenetic gene silencing.

Discussion

In this study, we investigated epigenetic silencing of five genes, MBD1, CXXC1, SMAD4, DCC and MBD2, located on a 4-Mb region on chromosome 18q21; a chromosomal region that becomes frequently lost during colorectal carcinogenesis (1). Although a variety of studies have investigated genetic silencing of genes on this...
chromosomal region, the contribution of epigenetic modifications is still unclear.

With promoter CpG island methylation analysis, we showed for the first time that the DCC promoter is densely methylated in CRC cell lines and primary CRC tissues. DCC promoter CpG island methylation has been described in oral squamous cell carcinomas (31), head and neck squamous cell carcinomas (32), breast cancer (33), gastric cancer (34) and esophageal squamous cell carcinoma (35,36). In CRC, research on inactivation of DCC has mainly focused on loss of heterozygosity and gene mutation. DCC is affected by DNA promoter methylation and/or loss of the DCC locus in 90% of CRCs. While 18q21 loss of heterozygosity mainly occurs in advanced lesions (3), DCC promoter methylation occurs early in CRC development, being present in 80% of adenoma- and carcinoma tissues and 29% of normal tissues.

To the contrary, MBD1, CXXC1, SMAD4 and MBD2 promoter CpG island methylation is rare in CRC. However, all five 18q21 genes show reduced gene expression in CRC cell lines and primary tissues.
compared with normal colon. CXXC1, SMAD4 and MBD2 reduced expression in cell lines was associated with 18q21 copy number loss, but in primary cancer tissues low messenger RNA levels were not restricted to CRCs with 18q21 deletion, indicating the presence of other targeting mechanisms. However, none of the genes responded to treatment with histone deacetylase inhibitor TSA and only DCC expression was restored by the demethylating agent AzA in CRC cell lines. ChIP studies revealed that only the DCC promoter CpG island methylation is associated with repressive histone-tail marks H3K9me3 and H3K27me3 and not with active chromatin mark H3K4me3. The opposite was observed for unmethylated MBD1, CXXC1, SMAD4 and MBD2 promoters that are enriched with active histone-tail mark H3K4me3 and not with H3K9me3 and H3K27me3. Therefore, we conclude that promoter CpG island methylation and histone modifications specifically target DCC on chromosomal region 18q21, whereas neighboring genes MBD1, CXXC1, SMAD4 and MBD2 remain unaffected.

In general, epigenetic gene inactivation is considered a gene- and tissue-specific process (37). However, Frigola et al. (38) recently showed that epigenetic modifications can cover large chromosomal regions in CRC as well, a mechanism termed long-range epigenetic silencing. In this study, genes located on a 4-Mb domain on chromosomal region 2q14 are coordinately epigenetically suppressed by H3K9 methylation, which occurs independently of promoter CpG island methylation. Recently, the same mechanism was observed for chromosomal region 3p22 that flanks the mH1L gene (39) and chromosomal region 5q32.2 (40). Here, we show for chromosomal region 18q21 that this type of long-range epigenetic silencing was not observed in cell lines HCT116 (18q21 retention) and HT29 (18q21 loss). Interestingly, in one primary CRC, the CXXC1, SMAD4, DCC and MBD2 were synchronously methylated; however, this was not observed for the other tumors (n = 31) or in any CRC cell line.

The observation that only DCC is inactivated by promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing, whereas MBD1, CXXC1, SMAD4 and MBD2 are not, raises questions about which underlying mechanism leads to this gene specificity. Recent studies have shown that genes that become de novo methylated in cancer are often bivalently marked by both active (H3K4me3) and repressive (H3K27me3) histone marks in human embryonic stem cells (14,41). In adult cancer cells, H3K4me3 is diminished and TSGs generate a fully repressive chromatin state (14). H3K27me3-marked loci are often occupied by polycomb group repressive complexes. Interestingly, in a genome-wide ChIP-ChIP assay that identified target genes of the human polycomb group protein SUZ12 in human embryonic stem cells (42), the DCC locus was found enriched for H3K27me3 and SUZ12 in its promoter region, whereas MBD1, CXXC1, SMAD4 and MBD2 were not. As it is clear for other cancer-associated hypermethylated genes (14), these findings seem to suggest an inherent susceptibility of DCC for aberrant methylation during cancer progression.

In summary, this study shows how genetic and epigenetic alterations collaborate in transcriptional silencing of DCC. While chromosomal loss of 18q21 frequently involves the entire chromosomal region, promoter CpG island hypermethylation- and H3K9me3 and H3K27me3-mediated epigenetic silencing specifically targets the DCC gene. Long-range epigenetic gene silencing has been observed on chromosomal regions 2q14, 3p22 and 5q32.2, but on 18q21 a rather fine-tuning role for epigenetic gene silencing is observed.

Supplementary material
Supplementary Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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