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3 ***MUC5AC* hypomethylation is a predictor of microsatellite instability independently of**
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5 **clinical factors associated with colorectal cancer**
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10 **Running Title**

11 *MUC5AC* hypomethylation in colorectal cancer
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27 **Abbreviations used in this paper:** CI, confidence interval; CRC, colorectal cancer; IHC,
28 immunohistochemistry; LOH, loss of heterozygosity; MMR, mismatch repair; MSI,
29 microsatellite instability; MSS, microsatellite stable; PCR, polymerase chain reaction
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38 **Appropriate article category:** Cancer Genetics
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40 **What’s new ?**

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42 This is the first study investigating the methylation profile of mucin genes in different CRC
43 subgroups. Our data demonstrate that MUC2 and MUC5AC expression in CRC is partially
44 regulated by DNA methylation and that the regulation of MUC5AC expression by DNA
45 methylation is specific of MSI tumors. Moreover, *MUC5AC* hypomethylation is a strong
46 predictor of MSI in CRC independently of other clinicopathological characteristics. *MUC5AC*
47 hypomethylation may serve as a specific marker of the serrated pathway.
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Abstract

Colorectal cancers (CRC) with microsatellite instability (MSI) display unique clinicopathologic features including a mucinous pattern with frequent expression of the secreted mucins MUC2 and MUC5AC. The mechanisms responsible for this altered pattern of expression remain largely unknown. We quantified DNA methylation of mucin genes (*MUC2*, *MUC5AC*, *MUC4*) in colonic cancers and examined the association with clinicopathological characteristics and molecular (MSI, *KRAS*, *BRAF* and *TP53* mutations) features. A control cohort was used for validation. We detected frequent hypomethylation of *MUC2* and *MUC5AC* in CRC. *MUC2* and *MUC5AC* hypomethylation was associated with *MUC2* and *MUC5AC* protein expression ($P = 0.004$ and $P < 0.001$, respectively), poor differentiation ($P = 0.001$ and $P = 0.007$, respectively) and MSI status ($P < 0.01$ and $P < 0.001$, respectively). Interestingly, *MUC5AC* hypomethylation was specific to MSI cancers. Moreover, it was significantly associated with *BRAF* mutation ($P < 0.001$). All these results were confirmed in the control cohort. In the multivariate analysis, *MUC5AC* hypomethylation was a highly predictive biomarker for MSI cancers. *MUC5AC* demethylation appears to be a hallmark of MSI in CRC. Determination of *MUC5AC* methylation status may be useful for understanding and predicting the natural history of CRC.

Key Words: Hypomethylation; epigenetics; mutator pathway; mucinous cancer

INTRODUCTION

Colorectal cancer (CRC) is a major public health problem with over one million new cases diagnosed worldwide every year¹. Colorectal carcinogenesis results from a multi-step process associated with accumulation of genetic and epigenetic alterations in epithelial cells². CRC can be subdivided into two main groups defined by the genetic pathways involved. The ‘suppressor’ pathway accounts for 70-80% of CRC, characterized by chromosomal instability (CIN) with loss of heterozygosity of tumor suppressor genes and frequent mutations in *APC*, *KRAS*, and *TP53*. The ‘mutator’ pathway, on the other hand, accounts for 12-15% of CRC and is characterized by extensive microsatellite instability (MSI) as a result from defective DNA mismatch repair (MMR) system². Most of MSI cancers are sporadic as a result from somatic epigenetic silencing of the *MMR* gene *MLH1*³. MSI is also a hallmark feature of Lynch syndrome, as the consequence of a germ line mutation in a *MMR* gene⁴. MSI cancers differ from MSS (microsatellite stable) cancers by a number of clinicopathologic features including their location which is more frequently in the proximal colon, poor differentiation, mucinous pattern, stromal lymphoid infiltrate and favorable prognosis⁵. Moreover, MSI cancers commonly express the secreted mucins MUC2 and MUC5AC⁶.

Mucins are a large family of high molecular weight glycoproteins widely expressed by epithelial surfaces⁷. MUC2 is the major mucin in the intestine, expressed by mucus-secreting goblet cells. The transmembrane mucin MUC4 is also expressed within the intestinal tract, by both goblet cells and absorptive columnar cells. The pattern of mucin expression is altered during colorectal carcinogenesis, with a loss of MUC2 expression in most CRC, and variable *de novo* expression of the gastric-secreted mucin MUC5AC^{6,8-12}. Increased expression of MUC2 and neo-expression of MUC5AC are frequent occurrences in mucinous and MSI carcinomas^{6,12}. MUC4 expression in CRC is variable and has been suggested as a predictor of

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3 poor prognosis¹³. The mechanisms responsible for these altered expression patterns remain
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5 unknown.

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7 *MUC2*, *MUC5B* and *MUC4* mucin genes have been shown *in vitro* to be regulated by
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9 DNA methylation and histone modifications in epithelial cancer cells, including colonic
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11 cancer cells. This regulation is highly cell-specific and related to cell differentiation status^{14,15}.
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13 To date, the methylation status of mucin genes has not been investigated in CRC tissues,
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15 except for *MUC2* that has been shown to be hypermethylated in *MUC2*-negative CRC¹⁶⁻¹⁸.

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18 We hypothesized that abnormal mucin gene expression in CRC may be related to an
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20 altered methylation profile of their promoters. To investigate this, we developed quantitative
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22 pyrosequencing assays to assess the methylation profile of *MUC2*, *MUC5AC*, and *MUC4*
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24 promoters in colonic cancers stratified by MSI status, and we analyzed the relationship
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26 between methylation levels and mucin expression. Further, we studied the relationship
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28 between methylation and expression profile, as well as clinical, pathological and molecular
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30 features of CRC.
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MATERIALS AND METHODS

Patients and tissues

This study was conducted on two distinct populations with the approval of the Institutional Review Board. A first study cohort of 36 patients was selected from a series of 136 patients with primary colonic adenocarcinoma, surgically treated at Lille University Hospital from 2003 to 2006. All carcinomas were MSI tested using the pentaplex mononucleotide panel as previously described¹⁹. Retrieved clinical and pathological data included age, sex, tumor site, differentiation, mucinous pattern, lymph node metastasis and tumor stage according to the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis staging system for colorectal tumors²⁰. Seventeen patients with MSI-high CRC were matched for age (<60 vs ≥60), sex (male vs female), tumor site (proximal vs distal), and stage (II vs III) with 19 MSS CRC patients. A second control cohort was retrospectively collected and included 34 patients with colonic cancers surgically treated at Lille University Hospital from 2002 to 2010 and for whom full clinical data and MSI status were available. The details of the clinicopathologic features and MSI status for both cohorts are presented in Table 1. MSI cancers included both sporadic MSI-H CRCs and Lynch syndrome-related MSI-H CRCs, as ascertained by IHC for MMR proteins, *MLH1* promoter methylation testing, and germ line MMR mutation testing (Supplemental Table 1).

Tissue specimens and matched adjacent non tumor colonic tissues were obtained through the tumor bank and the collection of the Department of Pathology. Frozen or paraffin-embedded sections were performed for immunohistochemistry and molecular analyses.

Cell lines and culture

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3 Colonic carcinoma cell lines HCT-116, LS174T, and HT-29 were cultured in DMEM
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5 supplemented with 10% (v/v) fetal bovine serum at 37°C in a 10 % CO₂ jacketed incubator.
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7 All cell lines were purchased from the American Type Culture Collection.
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10 11 **Histopathological examination and immunohistochemistry**

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14 Haematoxylin and eosin colored sections were examined to characterize tumor features and to
15
16 evaluate the percentage of tumor cells. Tumor features were established according to the
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18 World Health Organisation classification²¹. Colonic cancer was defined as ‘mucinous’ when
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20 extracellular mucin was present in more than 50% of the neoplasm. Immunohistochemistry
21
22 analysis was performed on frozen and paraffin-embedded sections as previously
23
24 described^{14,22}. The average percentage of stained tumor cells and intensity of staining were
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26 calculated using a semiquantitative histological scoring method (Hscore)²³. A blind semi-
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28 quantitative analysis of immunostainings was independently performed by two pathologists
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30 (FR and EL). Hscore below the threshold of 20 was considered as negative expression.
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32 Hscore above or equal this threshold was considered as positive expression.
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40 **Quantitative DNA methylation analyses by pyrosequencing**

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42 The methylation status of *MUC2*, *MUC5AC* and *MUC4* promoters was determined by
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44 pyrosequencing on bisulfite-treated genomic DNA. Genomic DNA was isolated from 30-µm
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46 frozen whole tissue sections or cell lines using BioRobot[®] EZ1 with EZ1 DNA Tissue kit
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48 (Qiagen, Courtaboeuf, France) or from 10-µm paraffin-embedded sections according to the
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50 manufacturer’s protocol for the Qiagen FFPE Tissue DNA extraction kit (Qiagen) and 100 ng
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52 were treated with sodium bisulfite using the EZ DNA Methylation Kit (Zymo Research,
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54 Proteogene, Saint Marcel, France) according to the manufacturer’s instructions.
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3 CpG-rich regions of mucin genes promoters were selected in areas previously
4 demonstrated to contain relevant CpG sites as ascertained by bisulfite sequencing on cancer
5 cell lines^{14,15,17}. For *MUC5AC*, a proximal CpG-rich region located in the 5'-flanking region
6 and another far distal region, so called 'remote-CpG island', were investigated. PCR primer
7 pairs were designed using the PSQ-Assay Design software (Qiagen). Location of primers is
8 indicated in Supplemental Figure 1. The *MUC5AC* assays analyzed the methylation of CpG
9 sites in the region -189 to -126 (*MUC5AC-I*) and -4500 to -4429 (*MUC5AC-II*) relative to the
10 transcription start site (GenBank accession number AF015521). The *MUC2* assays targeted
11 the CpG sites located in the region -6 to +10 (*MUC2-I*) and at position -160 (*MUC2-II*)
12 (NM_002457.2). The *MUC4* assays analyzed the CpG sites in the region +341 to +389
13 (*MUC4-I*) and +292 to +302 (*MUC4-II*) (NM_018406.5). PCR and pyrosequencing primers
14 sequences are detailed in Supplemental Table 2. PCR amplifications were performed in a
15 25 µl reaction mixture containing 1.5 mM HotStar buffer (Qiagen), 1.5 mM MgCl₂, 0.2 mM
16 deoxyribonucleotides, 5 pmoles each of forward primer and reverse primer, 0.8 U
17 HotStarTaq[®] (Qiagen), and 1 µl of bisulfite-treated DNA. A touchdown PCR cycling
18 approach was used for all amplicons.
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38 Pyrosequencing was performed according to the manufacturer's instructions, using the
39 PyroGold kit in conjunction with the PyroMark[™] MD instrument (Qiagen). Successful
40 bisulfite conversion was ensured by complete conversion of cytosine at a non-CpG site.
41 Pyrograms were analyzed by the methylation software pyroQ-CpG[™] (Qiagen). The
42 percentage of methylation was calculated as the mean of all CpG sites methylation levels
43 analyzed in a gene promoter region. Unmethylated DNA synthesized by a whole genome
44 amplification system (GenomiPhi, Amersham biosciences, Courtaboeuf, France) and
45 commercial methylated DNA (CpGenome[™] Universal Methylated DNA, Chemicon
46 International) were used as controls. Between-assays variations of methylation levels in
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3 repeated measurements were very low (standard deviation <5%) for each gene and for each
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5 region, showing the robustness of the assays. Mean methylation levels, defined as the mean
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7 value of methylation levels at all CpG sites in one region, were used for comparisons.
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10 11 **Mutation analysis of *KRAS*, *BRAF*, and *TP53***

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13 Mutation analyses of *KRAS* codons 12 and 13, and *BRAF* codon 600 were performed by
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15 pyrosequencing. Genomic DNA was amplified by PCR and sequenced with the PyroMark™
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17 *KRAS* kit and the PyroMark™ *BRAF* kit according to the manufacturer's instructions
18
19 (Qiagen). In the study cohort, *TP53* exons 5-9 were screened for the presence of point
20
21 mutations. Genomic DNA was amplified by PCR using the *AmpliTaq* Gold kit (Applied
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23 Biosystems, Courtaboeuf, France). PCR products were purified and submitted to direct
24
25 sequencing using BigDye™ Terminator version 3.1 and analyzed with a 3130XL automated
26
27 sequencer (Applied Biosystems). In *TP53* mutated tumors, LOH analysis was performed
28
29 using polymorphic microsatellite markers *TP53* and *D17S1353*. The tumor was considered
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31 with LOH when the score for informative markers was <0.6 or >1.67 (24). All alterations
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33 were confirmed on a second independent PCR product.
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41 **5-aza-2'-deoxycytidine treatment**

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43 Colonic carcinoma cell lines (HCT-116 and HT-29) were seeded at low density and allowed
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45 to grow 24 h before treatment. Exponentially growing cells were treated with 2 μM of 5-aza-
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47 2'-deoxycytidine (5-aza-dC) (Sigma, Saint Quentin Fallavier, France) for 48 h or 72 h. Cells
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49 were harvested and washed with 1X PBS before genomic DNA or RNA extraction.
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54 **Quantitative *MUC5AC* mRNA expression analysis**

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3 Total RNA was extracted from cells with the NucleoSpin[®] RNA II kit (Macherey-Nagel,
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5 Hoerd, France). RNA (1 µg) was reverse transcribed with the SuperScript[®]VILO[™] cDNA
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7 Synthesis kit (Invitrogen, Cergy-Pontoise, France) according to the manufacturer's
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9 instructions. Expression of *MUC5AC* mRNA was investigated by quantitative real time RT-
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11 PCR (qRT-PCR) on 7900 HT Fast Real Time System (Applied Biosystems) using the
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13 following primers and probe: AGAGTGGGAGCTGGGAGAGAG (sense) with
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15 AGCTCAGAGGACATATGGGAGGT (antisense), and
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17 CCAGTGTCCCCCATGCACTGACC (probe). The 18S ribosomal RNA gene was used for
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19 normalization. Assays were performed in triplicate. Relative expression of *MUC5AC* in cells
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21 treated or not treated with 5-aza-dC was calculated using the $2^{-\Delta\Delta C_t}$ method.
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27 **Statistical methods**

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29 Statistical analysis was performed using SPSS version 15.0 software (SPSS, Chicago, IL,
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31 USA). Data are shown as prevalence, mean (standard deviation), or median (range). Discrete
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33 variables were compared using the Chi square test or the Fischer exact test as appropriate.
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35 Continuous variables were compared using the Mann-Whitney *U* test. ROC (Receiver
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37 Operating Characteristic) curve analysis was performed to determine the optimal percentage
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39 cut-off value for defining hypomethylation with a threshold set at <70% for *MUC5AC* and
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41 *MUC4* and <60% for *MUC2*. A stepwise binary logistic regression model was built to identify
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43 predictors of MSI status, including all patients from the study and control cohorts. All clinical
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45 factors were included in the model, as well as biological parameters to be tested *i.e.* *MUC2*-I
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47 methylation and *MUC5AC*-II methylation. All statistical tests were two-sided, with the
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49 threshold for significance set at $P < 0.05$.
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RESULTS

Frequent hypomethylation of *MUC2* and *MUC5AC* in CRC

We analyzed the methylation profile of *MUC2*, *MUC5AC*, and *MUC4* in colonic cancers and in their non tumor counterpart by pyrosequencing. In non tumor colonic tissues, the results showed a partial methylation for all genes and regions studied, with low inter-individual variability. Mean methylation levels were 67% ($\sigma\pm 8$) and 65% ($\sigma\pm 14$) for *MUC2*-I and *MUC2*-II, respectively, 65% ($\sigma\pm 11$) and 86% ($\sigma\pm 2$) for *MUC5AC*-I and *MUC5AC*-II, respectively, and 30% ($\sigma\pm 9$) and 23% ($\sigma\pm 25$) for *MUC4*-I and *MUC4*-II, respectively. There was no significant difference according to the age, sex or tumor site, regardless of the gene and the region examined.

In colonic cancers, methylation levels appeared more variable. Mean methylation levels were 54% ($\sigma\pm 17$) and 48% ($\sigma\pm 20$) for *MUC2*-I and *MUC2*-II, respectively, 50% ($\sigma\pm 16$) and 76% ($\sigma\pm 11$) for *MUC5AC*-I and *MUC5AC*-II, respectively, 29% ($\sigma\pm 28$) and 20% ($\sigma\pm 16$) for *MUC4*-I and *MUC4*-II, respectively. Moreover, methylation levels were significantly lower for *MUC2*-I, *MUC5AC*-I and *MUC5AC*-II in tumors, compared to their non tumor counterpart ($P<0.001$, $P = 0.001$, and $P<0.001$, respectively), indicating frequent hypomethylation of these regions in CRC.

Mucin expression pattern in colonic cancers and association with the methylation profile

Immunohistochemistry analysis in colonic cancers showed expression of *MUC2*, *MUC5AC*, and *MUC4* in 24 of 36 (67%) cases, 12 of 36 (33%) cases, and 25 of 36 (69%) cases, respectively. *MUC2* expression in cancers was significantly associated with *MUC2* hypomethylation in *MUC2*-I ($P = 0.004$) (Table 2). Most of the *MUC2*-positive cancers (17 of 24, 71%) were hypomethylated in *MUC2*-I compared to their non tumor counterpart. Non

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3 tumor tissues expressed MUC2 and were partially methylated in *MUC2-I* (67% ($\sigma\pm 8$))
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5 (Figure 1).
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8 MUC5AC expression in cancers was significantly associated with *MUC5AC-II*
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10 hypomethylation ($P < 0.001$) (Table 2 and Figure 2). MUC5AC-positive tumors were
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12 frequently hypomethylated (10 of 12, 83%; mean methylation level 63%, $\sigma\pm 10$), whereas all
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14 the MUC5AC-negative tumors were highly methylated in *MUC5AC-II*, with levels similar to
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16 non tumor tissues (82%, $\sigma\pm 8$) (Figure 2A-D). Non tumor tissues were all negative for
17
18 MUC5AC expression and almost fully methylated in *MUC5AC-II* (86% ($\sigma\pm 2$)).
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21 MUC2, MUC5AC and MUC4 expression were not associated with *MUC2-II*, *MUC5AC-I*,
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23 *MUC4-I* and *-II* methylation, respectively.
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26 27 28 **Association between expression of MUC2 and MUC5AC and clinicopathological and** 29 30 **molecular features** 31

32 Mucin expression analysis showed that MUC2 expression was predominant in poorly
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34 differentiated and mucinous cancers ($P = 0.002$ and $P = 0.033$) and MUC5AC expression was
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36 predominant in poorly differentiated cancers ($P = 0.007$) (Supplemental Table 3). Moreover,
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38 MUC2 and MUC5AC expression were significantly associated with MSI cancers ($P = 0.001$
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40 and $P = 0.004$, respectively), irrespective of their origin (sporadic or Lynch syndrome).
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42 MUC5AC expression was more frequent in *BRAF* mutated cancers ($P = 0.002$) and *TP53*
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44 wild-type cancers ($P = 0.033$).
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50 51 **Association between methylation of MUC2 and MUC5AC and clinicopathological and** 52 53 **molecular features indicates a specific MUC5AC hypomethylation in MSI colonic cancer** 54

55 *MUC2-I* hypomethylation and *MUC5AC-II* hypomethylation in colonic cancers were
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57 significantly associated with poorly differentiated tumors ($P = 0.001$ and $P = 0.007$,
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3 respectively) and MSI status ($P < 0.01$ and $P < 0.001$, respectively) (Table 3). Moreover,
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5 *MUC5AC*-II hypomethylation was closely associated to *MUC5AC* expression in MSI cancers
6
7 ($P < 0.001$). All MSS cancers were highly methylated in the *MUC5AC*-II region, with
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9 methylation levels similar to non tumor tissues (*i.e.* nearly full methylation), regardless of
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11 *MUC5AC* expression. On the contrary, in MSI cancers, only *MUC5AC*-negative cancers
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13 were highly methylated in *MUC5AC*-II. All *MUC5AC*-positive MSI cancers were
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15 hypomethylated in *MUC5AC*-II (Figure 2E). Moreover, *MUC5AC*-II hypomethylation was
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17 associated with *BRAF* mutation and *KRAS* wild-type ($P < 0.001$ and $P = 0.03$, respectively).
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21 *MUC2*-I and *MUC5AC*-II methylation were not associated with other clinicopathological
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23 or molecular features.
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26 27 **Validation of specific *MUC5AC* hypomethylation in MSI colonic cancer in an** 28 29 **independent cohort** 30

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32 Clinicopathologic and molecular features in the control cohort were similar to those of the
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34 study cohort (Table 1). All associations observed in the study cohort were confirmed in this
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36 second cohort (Supplemental Table 4). In particular, *MUC2* expression and *MUC5AC*
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38 expression were associated with *MUC2*-I hypomethylation and *MUC5AC*-II hypomethylation,
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40 respectively, and *MUC2* hypomethylation and *MUC5AC* hypomethylation were associated
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42 with MSI status. Furthermore, *MUC5AC*-II hypomethylation remained a predictor of MSI in
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44 colonic cancers after adjustment for other confounding variables (OR = 1.34; 95% CI: 1.08-
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46 1.67; $P = 0.009$) (Table 4).
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51 52 **5-aza-dC induction of *MUC5AC* expression in colonic cancer cell lines** 53

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55 To validate the role of DNA methylation in transcriptional regulation of *MUC5AC* remote
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57 CpG rich region (*MUC5AC*-II), we compared its methylation status with *MUC5AC* gene
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3 expression in three colonic carcinoma cell lines: LS174T (MSI, high MUC5AC expression),
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5 HCT116 (MSI, MUC5AC-negative), and HT-29 (MSS, low MUC5AC expression).
6
7 Methylation analysis revealed nearly full methylation of *MUC5AC-II* in HCT-116 (92%) and
8
9 HT-29 (89%) and very low methylation level in LS174T (38%) (Supplemental Figure 2). This
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11 result is in accordance with our observations in colonic tumor tissues (Figure 2). Treatment of
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13 HCT-116 and HT-29 cells with the DNA demethylating agent 5-aza-dC led to significant
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15 activation of *MUC5AC* expression, indicating a role for *MUC5AC-II* methylation in the
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17 regulation of MUC5AC expression in colonic tumor cells (Supplemental Figure 2).
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DISCUSSION

We have analyzed the methylation status of *MUC2*, *MUC5AC*, and *MUC4* promoters in colonic carcinoma tissues and have investigated potential associations with expression, clinicopathological and molecular features. We have investigated in particular whether abnormal mucin genes expression in CRC may be related to an altered DNA methylation. We confirm previous data showing that *MUC2* expression is regulated by DNA methylation in colonic cancer. Moreover, we show for the first time that *MUC5AC* expression is also regulated by DNA methylation in colonic cancer and that this mechanism of regulation is specific of MSI cancer. Our data, validated in an independent cohort, show that *MUC5AC* hypomethylation may serve as a specific predictor of the serrated pathway.

CRC are characterized by an altered mucin gene expression pattern, with frequent loss of *MUC2* expression, occasional *de novo* expression of *MUC5AC* and variable expression of *MUC4*^{6,8-12}. In our series, correlations between mucin expression and clinicopathological data showed a significant association between *MUC2* expression and mucinous pattern. In particular, *MUC2* and *MUC5AC* expression were associated with poor differentiation and MSI status and *MUC5AC* expression was associated with the presence of *BRAF* mutation and with the absence of *TP53* mutation. These results are in line with previous studies^{6,9,10,16,25,26}.

We have developed pyrosequencing assays for quantitative measurement of DNA methylation at the promoters of *MUC2*, *MUC5AC*, and *MUC4* genes. Pyrosequencing was particularly suitable here, allowing detection of subtle variations in methylation level without requiring microdissection. Primers were designed in areas that we previously have

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3 demonstrated to contain relevant CpG sites^{14,15}. Additional CpG for *MUC2* and *MUC4* were
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5 targeted according to previous data highlighting important cytosines in these regions^{17,27-29}.
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10 Non tumor colonic tissues were characterized by a high but partial methylation of *MUC2*,
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12 *MUC5AC*, and *MUC4*. Methylation levels were homogeneous in all patient tissues
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14 irrespective of the age, sex, or tumor site. Of note, *MUC2* methylation analysis showed a
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16 mean value of about 67% in non tumor tissues and this percentage allowed the protein
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18 expression. This methylation level probably reflects the relative proportion of goblet cells in
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20 the samples. Indeed, using laser capture microdissection, Gratchev *et al.* showed a mean
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22 methylation level of 51% in goblet cells and 100% in columnar cells, leading to an
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24 intermediate methylation level for the mixed population in normal crypts¹⁷.
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30 Colonic cancers were characterized by frequent *MUC2* and *MUC5AC* hypomethylation.
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32 *MUC2* expression in cancer was linked to the hypomethylation of the proximal part of the
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34 promoter region (*MUC2-I*). The importance of the cytosine at position -6 in *MUC2* site-
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36 specific methylation was previously shown in several cancer cell lines^{14,17,28}, and in gastric
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38 tissues (27). Results remain unclear with regards to the cytosine at position -160^{14,17,18}. In our
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40 study, its methylation was not associated with *MUC2* expression, discarding the hypothesis of
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42 a role of this region in the regulation of *MUC2* expression in this organ. Of note, additional
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44 cytosines located in the distal part of the promoter may also be involved in the regulation of
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46 *MUC2* expression, as suggested previously^{14,18}.
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50 We have shown that hypomethylation of the proximal region of the *MUC5AC* promoter
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52 (*MUC5AC-I*) is not associated with *MUC5AC* protein expression. Our results are in line with
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54 previous studies^{14,30}. Strikingly, we have shown here that *MUC5AC* expression in human
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56 tissues is at least partially regulated by methylation of the remote CpG island located in the
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3 distal part of the 5'-flanking region (cytosines -4500 to -4429). A study from Yamada *et al.*
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5 showed a potential role of DNA methylation in another region including cytosines -3718 to
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7 -3670 of the *MUC5AC* promoter in cell lines³¹.
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10 Interestingly, we have shown that DNA methylation-mediated regulation of *MUC5AC*
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12 expression is specific to MSI cancers. Hypomethylation of the *MUC5AC* remote CpG island
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14 was observed only in MSI and *MUC5AC*-positive colonic tumors. This profile was observed
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16 in both sporadic and Lynch syndrome-related MSI tumors. In contrast, it was constitutively
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18 methylated in MSS tumors regardless of *MUC5AC* expression, suggesting another
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20 mechanism of regulation in this subtype of tumors. These results were confirmed in MSI and
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22 MSS colonic cell lines. The reason for this specific mode of regulation remains to be
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24 elucidated. Altogether, our results suggest that *MUC5AC* hypomethylation may serve as a
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26 specific marker of the 'mutator' pathway. In addition, considering that the MSI phenotype is
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28 largely associated with *MLH1* hypermethylation in sporadic MSI cancers³ and that MSI
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30 sporadic tumors exhibit frequent simultaneous hypermethylation of multiple genes^{32,33} it is
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32 expected that *MUC5AC* hypomethylation may also identify CpG island methylator phenotype
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34 (CIMP) tumors. Interestingly, in a recent study, Walsh *et al.* showed a strong association of
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36 *MUC5AC* and *MUC2* expression with CIMP positivity, supporting our hypothesis²⁶.
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41 Our results argue once again for region and CpG site-specific regulation by methylation of
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43 *MUC2* and *MUC5AC* gene expression^{14,17,27}. We can hypothesize that demethylation of
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45 certain regions may favor the binding of transcription factors regulating *MUC2* and *MUC5AC*
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47 expression and thereafter their activation. We have previously shown that *MUC2* and *MUC5B*
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49 repression by methylation dramatically impairs their activation by the transcription factor
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51 Sp1¹⁴. Sp1 is also known as a strong activator of *MUC5AC* transcription³⁴. The access to
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53 binding sites for other important activators of mucin genes, such as AP-2 (Activating-Protein-
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55 2), HATH1 or SOX9 for *MUC2*^{28,35-37} and SOX2 for *MUC5AC*³⁸ may be facilitated by
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3 demethylation. Around enrichment of particular transcription factors, tissue-specific
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5 hypomethylation has been recently reported to correlate with gene expression profiles that
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7 underlie tissue phenotypes³⁹. A combination of tissue-specific hypomethylation and selective
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9 binding of transcription factors is intimately involved in targeting specific genes during
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11 terminal differentiation. In this regard, secreted mucin genes *MUC2* and *MUC5AC* constitute
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13 targets of choice. MSI tumors have distinct phenotypic features including frequent poor
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15 differentiation and mucinous histology⁵. Demethylation of *MUC2* and *MUC5AC* are likely to
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17 play a role in acquisition of this particular phenotype.
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23 In addition, MSI tumors are largely described to have a different clinical behavior with a
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25 better stage-adjusted prognosis compared with MSS tumors. MSI also predicts a negative
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27 response to 5-fluorouracil-based adjuvant chemotherapy^{40,41}. Interestingly, *MUC5AC*
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29 hypomethylation was highly predictive of MSI in CRC independently of other confounding
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31 clinical or biological parameters. Since DNA methylation is an early event in carcinogenesis,
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33 preceding genetic mutations, abnormally methylated genes appear as potential attractive
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35 biomarkers for diagnosis, prognosis or theragnosis. It would be of interest to determine
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37 whether demethylation of *MUC2* and *MUC5AC* can be detectable in precursor lesions specific
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39 to the MSI carcinomas, *i.e.* sessile serrated adenomas, and therefore aid strategies for early
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41 diagnosis, prevention and management.
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47 In conclusion, we have developed quantitative pyrosequencing assays allowing
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49 determination of mucin gene methylation status in colonic cancer. We have shown that
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51 abnormal expression of *MUC2* and *MUC5AC* in these tumors is closely related to both altered
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53 methylation of their promoter and remote CpG island. Our study clarifies the mechanisms
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55 involved in the abnormal expression of secreted mucins *MUC2* and *MUC5AC* in CRC. The
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3 regulation of MUC5AC expression by DNA methylation is specific of MSI tumors. The
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5 reason for this specific mode of regulation remains to be elucidated. Finally, *MUC5AC*
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7 demethylation appears to be a surrogate predictor of MSI in CRC. Further studies on
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9 preneoplastic lesions will confirm whether this demethylation is an early event and therefore
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11 if it may be useful for understanding and predicting the natural history of CRC and for
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13 guiding clinical management strategies.
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60

REFERENCES

1. Center MM, Jemal A, Ward E. International trends in colorectal cancer incidence rates. *Cancer Epidemiol Biomarkers Prev* 2009;**18**:1688-94.
2. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008;**135**:1079-99.
3. Deng G, Chen A, Hong J, Chae H, Kim Y. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res* 1999;**59**:2029-33.
4. Lynch HT, Lynch PM, Lanspa SJ, Snyder CL, Lynch JF, Boland CR. Review of the Lynch syndrome: history, molecular genetics, screening, differential diagnosis, and medicolegal ramifications. *Clin Genet* 2009;**76**:1-18.
5. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007;**50**:113-30.
6. Biemer-Hüttmann AE, Walsh MD, McGuckin MA, Simms LA, Young J, Leggett BA, Jass JR. Mucin core protein expression in colorectal cancers with high levels of microsatellite instability indicates a novel pathway of morphogenesis. *Clin Cancer Res* 2000;**6**:1909-16.
7. Hollingsworth MA, Swanson BL. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* 2004;**1**:45-60.

- 1
2
3
4
5 8. Bara J, Nardelli J, Gadenne C, Prade M, Burtin P. Differences in the expression of mucus-
6 associated antigens between proximal and distal human colon adenocarcinomas. *Br J Cancer*
7 1984;**49**:495-501.
8
9
10
11
12
13
14 9. Blank M, Klussmann E, Krüger-Krasagakes S, Schmitt-Gräff A, Stolte M, Bornhoeft G,
15 Stein H, Xing PX, McKenzie IF, Verstijnen CP, *et al.* Expression of MUC2-mucin in
16 colorectal adenomas and carcinomas of different histological types. *Int J Cancer*
17 1994;**59**:301-306.
18
19
20
21
22
23
24
25 10. Chang SK, Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim
26 YS. Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in
27 human normal intestine and colon cancer. *Gastroenterology* 1994;**107**:28-36.
28
29
30
31
32
33
34 11. Buisine MP, Janin A, Maunoury V, Audié JP, Delescaut MP, Copin MC, Colombel JF,
35 Degand P, Aubert JP, Porchet N. Aberrant expression of a human mucin gene (MUC5AC) in
36 rectosigmoid villous adenoma. *Gastroenterology* 1996;**110**:84-91.
37
38
39
40
41
42
43 12. Kim DH, Kim JW, Cho JH, Baek SH, Kakar S, Kim GE, Sleisenger MH, Kim YS.
44 Expression of mucin core proteins, trefoil factors, APC and p21 in subsets of colorectal
45 polyps and cancers suggests a distinct pathway of pathogenesis of mucinous carcinoma of the
46 colorectum. *Int J Oncol* 2005;**27**:957-64.
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 13. Shanmugam C, Jhala NC, Katkoori VR, Wan W, Meleth S, Grizzle WE, Manne U.
4
5 Prognostic value of mucin 4 expression in colorectal adenocarcinomas. *Cancer*
6
7 2010;**116**:3577-86.
8
9

10
11 14. Vincent A, Perrais M, Desseyn JL, Aubert JP, Pigny P, Van Seuningen I. Epigenetic
12
13 regulation (DNA methylation, histone modifications) of the 11p15 mucin genes (MUC2,
14
15 MUC5AC, MUC5B, MUC6) in epithelial cancer cells. *Oncogene* 2007;**26**:6566-76.
16
17

18
19
20 15. Vincent A, Ducourouble MP, Van Seuningen I. Epigenetic regulation of the human mucin
21
22 gene MUC4 in epithelial cancer cell lines involves both DNA methylation and histone
23
24 modifications mediated by DNA methyltransferases and histone deacetylases. *FASEB J*
25
26 2008;**22**:3035-45.
27
28

29
30
31 16. Hanski C, Riede E, Gratchev A, Foss HD, Böhm C, Klussmann E, Hummel M, Mann B,
32
33 Buhr HJ, Stein H, Kim YS, Gum J, Riecken EO. MUC2 gene suppression in human colorectal
34
35 carcinomas and their metastases: in vitro evidence of the modulatory role of DNA
36
37 methylation. *Lab Invest* 1997;**77**:685-95.
38
39

40
41
42 17. Gratchev A, Siedow A, Bumke-Vogt C, Hummel M, Foss HD, Hanski ML, Kobalz U,
43
44 Mann B, Lammert H, Mansmann U, Stein H, Riecken EO, Hanski C. Regulation of the
45
46 intestinal mucin MUC2 gene expression in vivo: evidence for the role of promoter
47
48 methylation. *Cancer Lett* 2001;**168**:71-80.
49
50

- 1
2
3 18. Okudaira K, Kakar S, Cun L, Choi E, Wu Decamillis R, Miura S, Sleisenger MH, Kim
4
5 YS, Deng G. MUC2 gene promoter methylation in mucinous and non-mucinous colorectal
6
7 cancer tissues. *Int J Oncol* 2010;**36**:765-75.
8
9
10
11
12 19. Suraweera N, Duval A, Reperant M, Vaury C, Furlan D, Leroy K, Seruca R, Iacopetta B,
13
14 Hamelin R. Evaluation of tumor microsatellite instability using five quasimonomorphic
15
16 mononucleotide repeats and pentaplex PCR. *Gastroenterology* 2002;**123**:1804-11.
17
18
19
20
21 20. Edge SB, Byrd DR, Carducci MA, et al. AJCC Hindgut Taskforce: Colon and rectum.
22
23 In:AJCC Cancer Staging Manual. 7th Ed. New York: Springer 2009:143-46.
24
25
26
27 21. Bosman F, Carneiro F, Hruban R, et al. WHO classification of tumors of the digestive
28
29 system. Lyon, France: IARC Press, 2010:131-182.
30
31
32
33
34 22. Buisine MP, Desreumaux P, Leteurtre E, Copin MC, Colombel JF, Porchet N, Aubert JP.
35
36 Mucin gene expression in intestinal epithelial cells in Crohn's disease. *Gut* 2001;**49**:544-51.
37
38
39
40
41 23. Cappia S, Righi L, Mirabelli D, Ceppi P, Bacillo E, Ardisson F, Molinaro L, Scagliotti
42
43 GV, Papotti M. Prognostic role of osteopontin expression in malignant pleural mesothelioma.
44
45 *Am J Clin Pathol* 2008;**130**:58-64.
46
47
48
49
50 24. Canzian F, Salovaara R, Hemminki A, Kristo P, Chadwick RB, Aaltonen LA, de la
51
52 Chapelle A. Semiautomated assessment of loss of heterozygosity and replication error in
53
54 tumors. *Cancer Res* 1996;**56**:3331-37.
55
56
57
58
59
60

- 1
2
3 25. You JF, Hsieh LL, Changchien CR, Chen JS, Chen JR, Chiang JM, Yeh CY, Hsieh PS,
4
5 Fan CW, Liu CT, Tang R. Inverse effects of mucin on survival of matched hereditary
6
7 nonpolyposis colorectal cancer and sporadic colorectal cancer patients. *Clin Cancer Res*
8
9 2006;**12**:4244-50.
10
11
12
13
14 26. Walsh MD, Clendenning M, Williamson E, Pearson SA, Walters RJ, Nagler B, Packenas
15
16 D, Win AK, Hopper JL, Jenkins MA, Haydon AM, Rosty C, English DR, Giles GG,
17
18 McGuckin MA, Young JP, Buchanan DD. Expression of MUC2, MUC5AC, MUC5B, and
19
20 MUC6 mucins in colorectal cancers and their association with the CpG island methylator
21
22 phenotype. *Mod Pathol* 2013;**26**:1642-56.
23
24
25
26
27 27. Mesquita P, Peixoto AJ, Seruca R, Hanski C, Almeida R, Silva F, Reis C, David L. Role
28
29 of site-specific promoter hypomethylation in aberrant MUC2 mucin expression in mucinous
30
31 gastric carcinomas. *Cancer Lett* 2003;**189**:129-36.
32
33
34
35
36 28. Hamada T, Goto M, Tsutsumida H, Nomoto M, Higashi M, Sugai T, Nakamura S,
37
38 Yonezawa S. Mapping of the methylation pattern of the MUC2 promoter in pancreatic cancer
39
40 cell lines, using bisulfite genomic sequencing. *Cancer Lett* 2005;**227**:175-84.
41
42
43
44
45 29. Yamada N, Nishida Y, Tsutsumida H, Goto M, Higashi M, Nomoto M, Yonezawa S.
46
47 Promoter CpG methylation in cancer cells contributes to the regulation of MUC4. *Br J*
48
49 *Cancer* 2009;**100**:344-51.
50
51
52
53
54
55
56
57
58
59
60

1
2
3 30. Ho JJ, Han SW, Pan PL, Deng G, Kuan SF, Kim YS. Methylation status of promoters and
4 expression of MUC2 and MUC5AC mucins in pancreatic cancer cells. *Int J Oncol*
5 2003;**22**:273-79.
6
7
8

9
10
11 31. Yamada N, Nishida Y, Yokoyama S, Tsutsumida H, Houjou I, Kitamoto S, Goto M,
12 Higashi M, Yonezawa S. Expression of MUC5AC, an early marker of pancreatobiliary
13 cancer, is regulated by DNA methylation in the distal promoter region in cancer cells. *J*
14 *Hepatobiliary Pancreat Sci* 2010;**17**:844-54.
15
16
17
18
19

20
21
22 32. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP. Distinct genetic profiles in colorectal tumors
23 with or without the CpG island methylator phenotype. *Proc Natl Acad Sci U S A*.
24 2000;**97**:710-15.
25
26
27
28
29

30
31 33. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH,
32 Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine
33 J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator
34 phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF
35 mutation in colorectal cancer. *Nat Genet* 2006;**38**:787-93.
36
37
38
39
40
41
42
43
44

45 34. Perrais M, Pigny P, Copin MC, Aubert JP, Van Seuning I. Induction of MUC2 and
46 MUC5AC mucins by factors of the epidermal growth factor (EGF) family is mediated by
47 EGF receptor/Ras/Raf/extracellular signal-regulated kinase cascade and Sp1. *J Biol Chem*
48 2002;**277**:32258-67.
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 35. Leow CC, Romero MS, Ross S, Polakis P, Gao WQ. HATH1, down-regulated in colon
4 adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells. *Cancer Res*
5 2004;**64**:6050-57.
6
7
8

9
10
11 36. Park ET, Oh HK, Gum JR Jr, Crawley SC, Kakar S, Engel J, Leow CC, Gao WQ, Kim
12 YS. HATH1 expression in mucinous cancers of the colorectum and related lesions. *Clin*
13 *Cancer Res* 2006;**12**:5403-10.
14
15
16

17
18
19
20 37. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, Jay P.
21 SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses
22 the CDX2 and MUC2 genes. *J Cell Biol* 2004;**166**:37-47.
23
24
25

26
27
28
29 38. Park ET, Gum JR, Kakar S, Kwon SW, Deng G, Kim YS. Aberrant expression of SOX2
30 upregulates MUC5AC gastric foveolar mucin in mucinous cancers of the colorectum and
31 related lesions. *Int J Cancer* 2008;**122**:1253-60.
32
33
34

35
36
37
38 39. Nagae G, Isagawa T, Shiraki N, Fujita T, Yamamoto S, Tsutsumi S, Nonaka A, Yoshiba
39 S, Matsusaka K, Midorikawa Y, Ishikawa S, Soejima H, Fukayama M, Suemori H, Nakatsuji
40 N, Kume S, Aburatani H. Tissue-specific demethylation in CpG-poor promoters during
41 cellular differentiation. *Hum Mol Genet* 2011;**20**:2710-21.
42
43
44

45
46
47
48 40. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton
49 SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M, Gallinger S. Tumor
50 microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant
51 chemotherapy for colon cancer. *N Engl J Med* 2003;**349**:247-57.
52
53
54
55
56
57
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41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
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41. Sinicrope FA, Sargent DJ. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. *Clin Cancer Res* 2012;18:1506-12.

For Peer Review

Table 1. Clinicopathologic and molecular characteristics of colonic cancers analyzed

Characteristics	Study cohort (n=36)	Control cohort (n=34)
Age at time of diagnosis (yrs), mean (range)	74 (47-95)	64 (34-90)
Sex, n (%)		
Male	17 (47%)	14 (41%)
Female	19 (53%)	20 (59%)
Tumor site, n (%)		
Proximal	26 (72%)	16 (47%)
Distal	10 (28%)	18 (53%)
Differentiation, n (%)		
Well or moderate	25 (69%)	18 (53%)
Poor	11 (31%)	16 (47%)
Mucinous, n (%)		
Presence	8 (22%)	14 (41%)
Absence	28 (78%)	20 (59%)
Lymph node metastasis, n (%)		
Presence	10 (28%)	14 (41%)
Absence	26 (72%)	20 (59%)
Stage, n (%)		
II	26 (72%)	19 (56%)
III	10 (28%)	15 (44%)
MSI status		
MSI	17 (47%)	17 (50%)
MSS	19 (53%)	17 (50%)

MSI, microsatellite instability; MSS, microsatellite stable.

Table 2. MUC2, MUC5AC, and MUC4 expression and mucin genes methylation in colonic cancers (n=36)

	MUC2 expression ^b			MUC5AC expression ^b			MUC4 expression ^b		
	Positive (n=24)	Negative (n=12)	<i>P</i> value	Positive (n=12)	Negative (n=24)	<i>P</i> value	Positive (n=25)	Negative (n=11)	<i>P</i> value
<i>MUC5AC</i> -I methylation ^a			0.378			0.640			0.343
Positive (n=6)	3	3		1	5		3	3	
Negative (n=30)	21	9		11	19		22	8	
<i>MUC5AC</i> -II methylation ^a			<0.001			<0.001			0.128
Positive (n=26)	15	11		2	24		16	10	
Negative (n=10)	9	1		10	0		9	1	
<i>MUC2</i> -I methylation ^a			0.004			0.01			0.070
Positive (n=17)	7	10		2	15		9	8	
Negative (n=19)	17	2		10	9		16	3	
<i>MUC2</i> -II methylation ^a			0.157			0.709			0.715
Positive (n=12)	6	6		3	9		9	3	
Negative (n=24)	18	6		9	15		16	8	
<i>MUC4</i> -I methylation ^a			0.333			1			0.306
Positive (n=1)	0	1		0	1		0	1	
Negative (n=35)	24	11		12	23		25	10	
<i>MUC4</i> -II methylation ^a			0.098			0.588			1
Positive (n=4)	1	3		2	2		3	1	
Negative (n=32)	23	9		10	22		22	10	

^aThreshold for *MUC5AC* and *MUC4* hypomethylation is 70%, threshold for *MUC2* is 60%. Methylation levels strictly below the threshold are considered as hypomethylation and indicated as negative methylation. Methylation levels above or equal the threshold are indicated as positive methylation.

^bThreshold for mucin expression is Hscore 20. Hscore strictly below the threshold are indicated as negative expression. Hscore above or equal the threshold are indicated as positive expression.

NOTE. The statistically significant *P* values (<0.05) are highlighted in bold.

Table 3. *MUC2*-I and *MUC5AC*-II methylation in relation to clinicopathogenetic features in colonic cancers (n=36)

	<i>MUC2</i> -I methylation ^a			<i>MUC5AC</i> -II methylation ^a		
	Positive (n=17)	Negative (n=19)	<i>P</i> value	Positive (n=26)	Negative (n=10)	<i>P</i> value
Sex			0.739			1
Male (n=17)	9	8		12	5	
Female (n=19)	8	11		14	5	
Age (yrs)			0.605			0.559
≤60 (n=4)	1	3		4	0	
>60 (n=32)	16	16		22	10	
Site			0.139			0.223
Proximal (n=26)	10	16		17	9	
Distal (n=10)	7	3		9	1	
Differentiation			0.001			0.007
Well or moderate (n=24)	16	8		21	3	
Poor (n=12)	1	11		5	7	
Mucinous			0.003			0.658
Presence (n=8)	0	8		5	3	
Absence (n=28)	17	11		21	7	
LN metastasis			0.274			0.689
Presence (n=10)	3	7		8	2	
Absence (n=26)	14	12		18	8	
Stage			0.056			0.689
II (n=26)	7	19		18	8	
III (n=10)	10	0		8	2	
MSI status			<0.01			<0.001
MSI (n=17)	4	13		7	10	
MSS (n=19)	13	6		19	0	
<i>KRAS</i>			1			0.03
Mutated (n=10)	5	5		10	0	
Wild type (n=26)	12	14		16	10	
<i>BRAF</i>			0.128			<0.001
Mutated (n=9)	2	7		2	7	
Wild type (n=27)	15	12		24	3	
<i>TP53</i>			0.434			0.08
Mutated (n=8)	4	4		8	0	
Wild type (n=28)	13	15		18	10	

LN, lymph node; MSI, microsatellite instability; MSS, microsatellite stable.

^aThreshold for *MUC5AC* hypomethylation is 70%, threshold for *MUC2* is 60%. Methylation levels strictly below the threshold are considered as hypomethylation and indicated as negative methylation. Methylation levels above or equal the threshold are indicated as positive methylation.

NOTE. The statistically significant *P* values (<0.05) are highlighted in bold.

Table 4. Multivariate analysis of factors predictive of MSI according to clinicopathogenetic features in colonic cancers (n=70)

Variables	χ^2	OR	95%CI	P value
Sex	0.63	0.42	0.05-5.56	0.427
Age	7.69	432.92	5.94-31548.73	0.006
Site	0.26	0.52	0.11-10.68	0.610
Differentiation	0.17	1.88	0.22-5.44	0.681
Mucinous	3.18	0.29	0.03-3.8	0.075
LN metastasis	0	0	0	0.998
Stage	0	0	0	0.999
<i>MUC5AC</i> methylation	6.87	1.34	1.08-1.67	0.009
<i>MUC2</i> methylation	4.31	1.09	1.00-1.18	0.038

CI, confidence interval; LN, lymph node; OR, odds ratio.

NOTE. The statistically significant P values (<0.05) and their 95%CI are highlighted in bold

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TITLES AND LEGENDS TO FIGURES

Figure 1. Representative examples of *MUC2* methylation and expression in colonic cancer

A, B. Analysis of a *MUC2*-negative colonic adenocarcinoma. A, pyrogram showing high *MUC2*-I methylation at the three CpG sites analyzed (grey shading) in both non tumor (N) and tumor (T) tissues. The percentages of methylation are indicated at the top. Full bisulfite DNA conversion is confirmed by the absence of cytosine at the internal control (yellow shading). B, absence of *MUC2* expression by immunohistochemistry (IHC) on the same cancer (non tumor mucosa, x 25; adenocarcinoma, x 100). C, D. Analysis of a *MUC2*-positive adenocarcinoma. C, pyrogram showing *MUC2*-I hypomethylation in the tumor compared to its non tumor counterpart. D, high *MUC2* expression by IHC on the same cancer (non tumor mucosa, x 25; adenocarcinoma, x 50).

Figure 2. Representative examples of *MUC5AC* methylation and expression in colonic cancer

A, B. Analysis of a *MUC5AC*-negative colonic adenocarcinoma. A, pyrogram showing near full *MUC5AC*-II methylation at the ten CpG sites analyzed (grey shading) in both non tumor (N) and tumor (T) tissues. B, absence of *MUC5AC* expression by immunohistochemistry (IHC) on the same tissues (non tumor mucosa, x 50; adenocarcinoma, x 25). C, D. Analysis of a *MUC5AC*-positive adenocarcinoma. C, pyrogram showing *MUC5AC*-II hypomethylation in the tumor compared non tumor tissue. D, high *MUC5AC* expression by IHC in adenocarcinoma (x 25). E, *MUC5AC*-II mean methylation level in MSS and MSI cancer in correlation with protein expression in non tumor (N) and tumor (T) tissues; dark boxplots refer to *MUC5AC* expressing tumors and matched non tumor tissues (dark blue for MSS and

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3 dark red for MSI) and light boxplots refer to MUC5AC negative tumors and matched non
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5 tumor tissues (light blue for MSS and light red for MSI).
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8 9 **Supplemental Digital Content**

10 **Supplemental Figure 1.** Pyrosequencing assays for MUC2, MUC4 and MUC5AC promoter
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12 methylation analysis
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15 Schematic representation of 5' sequences with the transcription start site (brised arrows) and
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17 beginning of coding sequence (grey boxes). Prediction of CpG sites by the UCSC software is
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19 represented below the gene. Amplified regions are expanded, with positions to the TSS
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21 (GenBank accession numbers: MUC2, NM_002457.2; MUC4, NM_018406.5; MUC5AC,
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23 AF015521), CpG studied (black circles) and other CpG present in the amplicons (white
24
25 circles). Arrows indicate positions of PCR primers (full arrows, circled for biotine) and
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27 sequencing primers (plain arrows).
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33 **Supplemental Figure 2.** Hypomethylation and induction of MUC5AC in 5-aza-dC-treated
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35 colonic cell lines
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38 A. Results of bisulfite pyrosequencing in colonic cancer cell lines HT-29 (MSS, weak
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40 MUC5AC expression), HCT-116 (MSI, weak MUC5AC expression), and LS174T (MSI, high
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42 MUC5AC expression). The MUC5AC-II region is nearly fully methylated in both HT-29 and
43
44 HCT-116 cells, whereas it is hypomethylated in LS174T cells.
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47 B. Results of bisulfite pyrosequencing in colonic cancer cell lines HT-29 and HCT-116 after
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49 treatment with 5-aza-dC (2 μ M during 72 h). The MUC5AC-II region evolved from nearly
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51 full methylation to partial methylation in both HT-29 and HCT-116 cells after 5-aza-dC
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53 treatment.
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3 C. Expression of MUC5AC by qRT-PCR in colonic cancer cell lines HT-29 and HCT-116
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5 after treatment with 5-aza-dC (2 μ M during 48h and 72 h). MUC5AC is overexpressed in
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7 both HT-29 and HCT-116 cells after 5-aza-dC treatment.
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Figure 1.

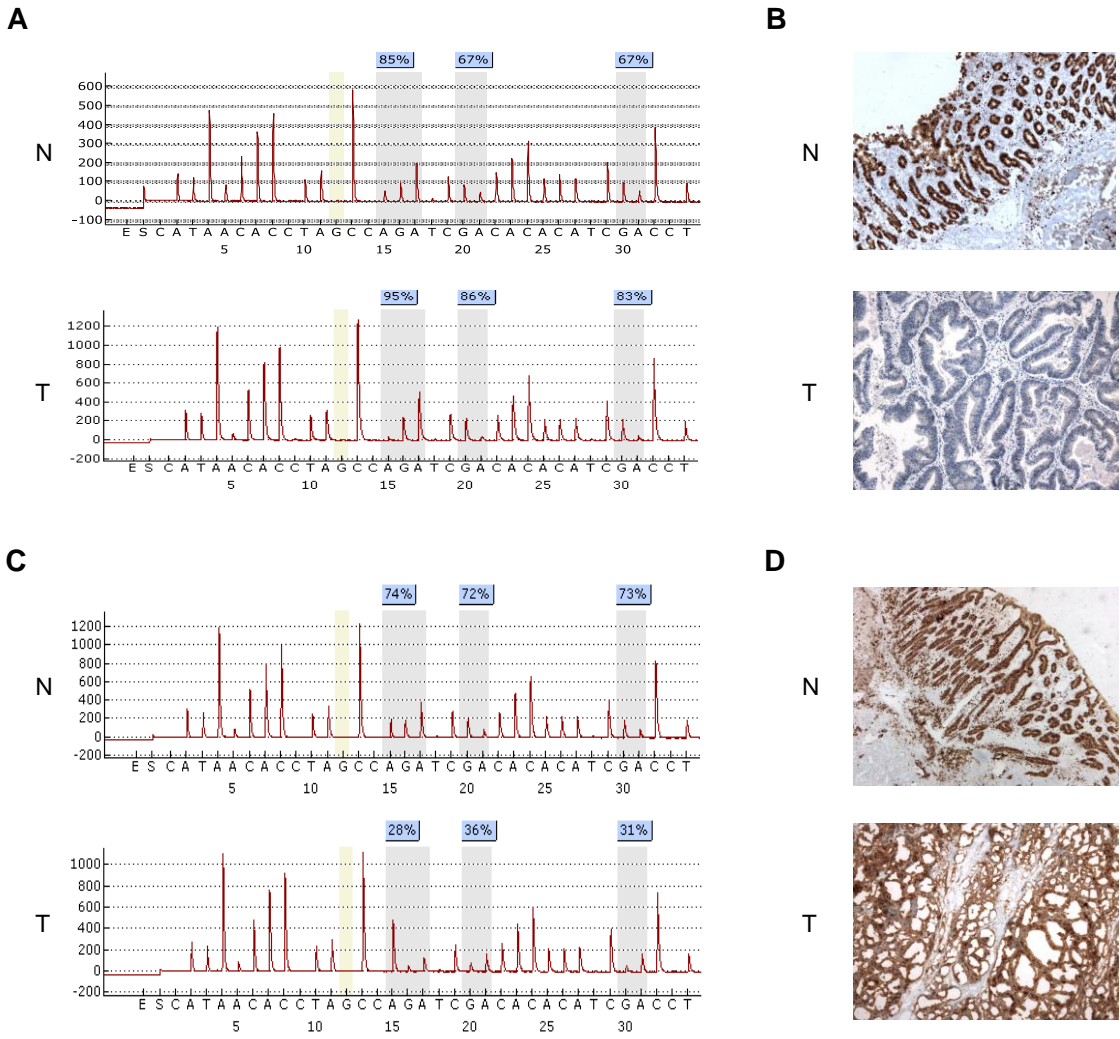


Figure 2.

