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Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype

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Mucinous differentiation is associated with both CpG island methylator phenotype and microsatellite instability in colorectal cancer. The mucinous phenotype derives from abundant expression of the colonic goblet cell mucin, MUC2, and *de novo* expression of gastric foveolar mucin, MUC5AC. We, therefore, investigated the protein expression levels of MUC2 and MUC5AC, as well as MUC5B and MUC6, in molecular subtypes of colorectal cancer. Seven-hundred and twenty-two incident colorectal carcinomas occurring in 702 participants of the Melbourne Collaborative Cohort Study were characterized for methylator status, *MLH1* methylation, somatic *BRAF* and *KRAS* mutations, microsatellite-instability status, MLH1, MSH2, MSH6, and PMS2 mismatch repair, and p53 protein expression, and their histopathology was reviewed. Protein expression levels of MUC2, MUC5AC, MUC5B, MUC6, and the putative mucin regulator CDX2 were compared with molecular and clinicopathological features of colorectal cancers using odds ratios and corresponding 95% confidence intervals. MUC2 overexpression (>25% positive tumor cells) was observed in 33% colorectal cancers, MUC5B expression in 53%, and *de novo* MUC5AC and MUC6 expression in 50% and 39%, respectively. Co-expression of two or more of the mucins was commonly observed. Expression of MUC2, MUC5AC and MUC6 was strongly associated with features associated with tumorigenesis via the serrated neoplasia pathway, including methylator positivity, somatic *BRAF* p.V600E mutation, and mismatch repair deficiency, as well as proximal location, poor differentiation, lymphocytic response, and increased T stage (all $P<0.001$). Overexpression was observed in tumors with and without mucinous differentiation. There were inverse associations between expression of all four mucins and p53 overexpression. CDX2 expression was inversely associated with MUC2, MUC5AC and MUC6 expression. Our results suggest that, in methylator-positive tumors, mucin genes on chromosome 11p15.5 region undergo increased expression via mechanisms other than direct regulation by CDX2.

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Colorectal cancer displays considerable molecular and biological heterogeneity, which is due to, at least in part, differing pathways of neoplastic progression. The majority of colorectal cancer arises through the adenoma-carcinoma sequence

characterized by chromosomal instability with associated accumulation of genetic alterations in tumor-suppressor genes, such as *APC* and *TP53*.[1] Colorectal carcinoma also arises from atypical serrated polyps, demonstrating little evidence of chromosomal instability. Rather, tumor-suppressor genes are inactivated by widespread epigenetic silencing known as CpG Island Methylator Phenotype (CIMP)[2] often accompanied by the somatic *BRAF* p.V600E mutation.[3–5] CIMP colorectal cancers are associated with clinicopathological features such as proximal location, poor grade, presence of tumor-infiltrating lymphocytes, and mucinous differentiation, as well as frequently demonstrating molecular somatic events including the *BRAF* p.V600E mutation and high levels of microsatellite instability, but with much lower levels of *TP53* mutation than their chromosomal unstable colorectal carcinoma counterparts.[6–12]

Mucins are high-molecular weight proteins characterized by the presence of large amino acid tandem repeat sequences that show allelic size variation. Secreted or gel-forming mucins comprise five known types: MUC2, MUC5AC, MUC5B, MUC6, and MUC19. All but MUC19 are encoded by genes present in a cluster on chromosome 11p15.[13] MUC2 is the predominant secreted mucin synthesized by colonic goblet cells,[14] along with MUC5B.[15] In the normal colon, MUC5AC is rarely expressed, and only by a minority of goblet cells, and there are conflicting reports concerning the expression of MUC6 in the colon.[16,17] MUC5B expression in the lower gastrointestinal tract is relatively unexplored and appears to be restricted to colonic goblet cells.[15] We and others have shown that MUC2 and MUC5AC mucins are expressed at high levels in mucinous colorectal cancers and by tumors exhibiting microsatellite instability.[18–21] CIMP colorectal cancers also exhibit significant mucinous differentiation, suggesting that one or more gel-forming mucins are overexpressed in CIMP. Several regulatory mechanisms governing mucin gene expression have been demonstrated, including promoter CpG island methylation[22,23] and activation of the EGFR-RAS-RAF signal transduction pathway.[24] In addition, the regulation of mucin gene expression by various transcription factors such as Sp1, AP-1, and CDX2 has been explored in a range of tissues (reviewed by Andrianifahanana *et al*[25]). Of these, the homeobox protein, CDX2, reportedly regulates MUC2 expression in gastrointestinal epithelial goblet cells[26,27] and has shown a loss of expression in microsatellite-unstable colorectal cancers[28,29] and serrated polyps of the colorectum.[30]

We report here the results from a large series of colorectal carcinomas demonstrating an association between expression of mucins, MUC2, MUC5AC, MUC5B, and MUC6 and the presence of somatic *BRAF* p.V600E mutation, CIMP, microsatellite instability, and loss of CDX2 expression.

Materials and methods

Patient Cohort

Archival tumor samples were obtained from participants enrolled in The Melbourne Collaborative Cohort Study, a prospective study of 41 514 people (17 045 males and 24 469 females) recruited between 1990 and 1994. Participants were aged between 27 and 81 years at baseline (99.3% were aged 40–69 years). Full details of the design, recruitment, and study procedures have been published previously.[31] All participants gave informed consent.

Between study enrollment and December 2010, 976 participants were diagnosed with incident colorectal carcinoma and were ascertained through linkage to the Victorian Cancer Registry to which notification is a legal requirement. Ethical approval was obtained from relevant participating centers and the Queensland Institute of Medical Research Human Research Ethics Committee under protocol P799.

Histopathology Review

Colorectal carcinomas were reviewed by one of the two specialist gastrointestinal pathologists (Professor Jeremy Jass and Dr Christophe Rosty) with regard to anatomical site, tumor grade, tumor margin, and synchronous colorectal carcinoma. The presence of any minor mucinous component (defined as mucinous differentiation present in $<50\%$ of the tumor). Tumor budding was assessed using the criteria described by Ueno *et al*[32] (≥ 10 foci of isolated tumor cells or clusters of fewer than five tumor cells at the invasive margin within a $\times 25$ microscopic field). Peritumoral lymphocytes (a mantle or cap of lymphoid cells at the deepest point of direct spread), Crohn's-like lymphocytic reaction (at least three nodular lymphoid aggregates deep to the invasive margin in a $\times 4$ field), and tumor-infiltrating lymphocytes (at least four intraepithelial lymphocytes per $\times 40$ field) were scored using the criteria described previously by Young *et al*.[33] Tumors in the ileocecal junction, cecum, ascending colon, hepatic flexure, and transverse colon were grouped as right-sided (proximal) colon cancers (ICD-O-3 codes C180, C182, C183, and C184),[34] whereas those in the splenic flexure (C185), descending colon (C186), sigmoid colon (C187), recto-sigmoid junction (C199) and rectum (C209) were grouped as left-sided (distal).

Immunohistochemistry

Tissues for analysis were fixed for several hours in neutral buffered formalin and then embedded in paraffin. Paraffin sections ($4\,\mu$m) were routinely dewaxed and rehydrated, then subjected to heat-induced epitope retrieval in either High pH Target

Retrieval solution (Dako, Carpinteria, CA) (MUC2 and MUC6) or Reveal Decloaking solution (BioCare Medical, Concord, MA) for 8 min, and then incubated with primary antibody for 90 min. The antibodies used were (a) anti-MUC2 (clone Ccp58, 1/500 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), (b) anti-MUC5AC (clone 45M1, 1/750 dilution) (Neomarkers Inc., Fremont, CA), (c) anti-MUC6 (clone CLH5, 1/250 dilution) (Santa Cruz Biotechnology Inc.), (d) anti-MUC5B (clone EU-MUC5B, 1/500 dilution), (e) anti-p53 (clone DO7, 1/100 dilution) (Dako), and (f) anti-CDX2 (clone CDX2-88, 1/100 dilution) (BioCare Medical) followed by the EnVision Plus Mouse HRP detection system (Dako). Antigenic sites were developed using DAB+ liquid chromogen (Dako), and then the sections were counterstained with hematoxylin before mounting. Expression of the MLH1, MSH2, MSH6, and PMS2 was assessed as described previously.[35]

Stained sections were scored by one observer (Dr Michael Walsh) blinded to clinical and molecular testing results, and a subset of tumors was scored independently by another observer (Professor Jeremy Jass) to assess reproducibility. The proportion of positive cancer cell staining was graded as follows: 0 (negative), <10% (1+), 11–25% (2+), 26–50% (3+), 51–75% (4+), and >75% (5+). The staining intensity of cancer cells was graded as weak (1+), moderate (2+), or strong (3+). Note was also made of the cellular localization of stains for each antibody (cytoplasmic membrane, apical membrane, and extracellular membrane). Cases were classified as positive for MUC2 and MUC5B where staining was observed in >25% tumor cells, and >0% for MUC5AC or MUC6. Histologically normal colonic mucosa served as positive control tissue for MUC2 and MUC5B, and normal stomach as the positive control for MUC5AC and MUC6. Previously demonstrated colorectal cancers were used as positive and negative controls for assessment of p53 overexpression, and normal colonic epithelium served as the CDX2 control.

Molecular Assays

Colorectal carcinoma DNA from formalin-fixed paraffin-embedded tissue was tested for microsatellite instability using a 10-marker microsatellite panel as previously described.[36] Tumors were scored as showing microsatellite instability when instability was detected at >40% of loci tested.[37] Testing for somatic mutations in *KRAS* codons 12 and 13 was performed using direct Sanger sequencing as previously described.[3] Testing for the *BRAF* p.V600E somatic mutation was performed using an allele-specific PCR assay as described previously.[37] Positive controls were run in each experiment, and 10% of samples were replicated with 100% concordance. Methylation analysis of the CIMP markers *RUNX3*, *SOCS1*, *CACNA1G*, *NEUROG1*, and *IGF2* and the *MLH1* promoter was performed on bisulphite-converted tumor DNA using MethyLight as previously described.[3,37] High levels of CIMP (CIMP-positive) were defined when ≥3 of the 5 markers were positively methylated, whereas cancers with <3 positively methylated markers were considered CIMP-negative.

Statistical Analysis

The association between mucin expression and clinicopathological features was assessed using χ^2-tests and quantified using odds ratios (ORs) with 95% confidence intervals (CIs). Differences between mean ages at diagnosis were assessed between groups using *t*-test after assessing the equality of variances. The level of statistical significance was set at $P<0.05$.

Results

Altogether, 976 incident cases of colorectal carcinoma were identified, and for 722 (74%) of these, tissue was available. Ten participants had two and one had three synchronous colorectal cancers, whereas two had two metachronous cancers, and a further two had three metachronous cancers. Patient ($n=702$, 367 males, 335 females) age at colorectal cancer diagnosis ranged from 42.0 to 83.4 years (mean age = 67.9 years, s.d. 7.9). Details of clinicopathological and molecular features of the tumors are summarized in Supplementary Table 1. There was considerable overlap in clinicopathological features associated with both CIMP-positive and *BRAF* mutation, including mucinous differentiation (Supplementary Table 2).

MUC2 Expression

Positive staining for MUC2 core protein using Ccp58 is characterized by intense cytoplasmic reactivity in goblet cells in normal colonic epithelium (Figure 1a). The majority of cancers (67%) exhibited either complete negativity for MUC2 (8%) or rare (<25%) stained epithelial cells (59%) with or without apparent goblet cell differentiation (Figure 1b). Using a threshold of ≥25% of tumor cells stained, 215/655 (33%) colorectal cancers were classified as MUC2-positive. The associations between MUC2 expression and tumor clinicopathological features are presented in Table 1. MUC2-positive carcinomas were twice as likely to be proximal (OR = 2.83, 95% CI = 2.01–3.99), and have mucinous differentiation ($P<0.001$), although strong MUC2 expression could be observed in areas with conventional adenocarcinoma differentiation (Figure 1c). MUC2 expression was also associated with higher T stage (OR = 1.64, 95% CI = 1.09–2.47),

and the presence of tumor-infiltrating lymphocytes (OR = 2.43, 95% CI 1.66–3.58) and Crohn's-like aggregates (OR = 2.30, 95% CI = 1.49–3.56). All signet ring cell tumors and 49/51 (96%) of mucinous tumors showed MUC2 positivity (Figure 1d).

MUC2-positive colorectal cancers were more likely to be CIMP-positive (51/75, 68%) than CIMP-negative (145/539, 27%) (OR = 5.77, 95% CI = 3.43–9.72), more frequently *BRAF* p.V600E-mutated (OR = 3.77, 95% CI = 2.48–5.73), demonstrate *MLH1* promoter methylation (OR = 4.74, 95% CI = 2.73–8.26), show DNA mismatch repair deficiency, that is loss of expression of one or more mismatch repair proteins (OR = 7.15, 95% CI = 4.28–11.95), and were less likely to have p53 overexpression (OR = 0.42, 95% CI = 0.30–0.60).

MUC5AC Expression

MUC5AC was present in the foveolar epithelial cells of the normal stomach control sections both as cytoplasmic staining and also extracellular mucin (Figure 1e). In the normal colon distant from the tumor, MUC5AC was not expressed, but normal epithelium in close proximity to cancers frequently showed staining of a variable proportion of normal goblet cells, predominantly staining of the thecal contents but also extracellular mucin (Figure 1f). Any MUC5AC staining of tumor cells was considered *de novo* expression, and 321/649 (49%) cancers were classified as MUC5AC-positive. Expression ranged from infrequent isolated cells or clusters of cells (Figure 1g) to diffuse positivity (Figure 1h).

Tumor clinicopathological features that are associated with MUC5AC expression are presented in Table 1. As was observed for MUC2 expression, MUC5AC-positive colorectal carcinomas were more likely to be proximal (OR = 1.98, 95% CI = 1.42–2.75), have mucinous differentiation ($P < 0.001$), be of higher T stage (OR = 1.57, 95% CI = 1.08–2.28), and have tumor-infiltrating lymphocytes (OR = 2.21, 95% CI = 1.51–3.24). MUC5AC expression was also associated with higher-grade carcinomas (OR = 2.24, 95% CI = 1.51–3.34) and female sex (OR = 1.48, 95% CI = 1.08–2.01). Positive MUC5AC expression was associated with CIMP positivity (OR = 8.58, 95% CI = 4.18–17.63), *BRAF* p.V600E mutation (OR = 4.08, 95% CI = 2.56–6.50), *MLH1* promoter methylation (OR = 6.36, 95% CI = 3.16–12.79), and DNA mismatch repair deficiency (OR = 6.65, 95% CI = 3.59–12.30) and was less likely to have p53 overexpression (OR = 0.52, 95% CI = 0.38–0.72). The association between MUC2 and MUC5AC expression, and CIMP and *BRAF* status is shown in a 'heat map' (Figure 2).

MUC5B Expression

There was intense goblet cell reactivity for MUC5B in normal colon, which was most prominent in the basal two-thirds of crypts in all specimens, diminishing to little or no MUC5B staining of surface epithelium (Figure 3a). In tumors, staining was localized to the cytoplasm, commonly in the supranuclear region, as well as extracellular mucin. There was often intense reactivity on the apical cell membranes in gland spaces within tumors (Figures 3b–d). The relationship between MUC5B expression and tumor clinicopathological features is summarized in Table 2. MUC5B-expressing carcinomas were associated with female gender (OR = 2.28, 95% CI = 1.07–4.84), mucinous differentiation ($P = 0.006$), and the presence of tumor-infiltrating lymphocytes (OR = 2.62, 95% CI = 1.08–6.36), but showed no statistical evidence of increased prevalence in the proximal colon. In addition, associations with mismatch repair deficiency (OR = 4.17, 95% CI = 1.43–12.16) and lack of p53 expression (OR = 0.24, 95% CI = 0.11–0.51) and MUC5B expression were observed.

MUC6 Expression

MUC6 was present in the gastric gland epithelial cells of the normal stomach control sections as cytoplasmic staining (Figure 3e) and was not seen in normal colon epithelium. Staining in a subset of colorectal cancers was exclusively cytoplasmic in nature, and any staining of tumor cells was considered *de novo* expression. Of the 126 colorectal carcinomas tested, 49 (39%) showed evidence of MUC6 staining. In the majority of MUC6-positive cases, staining was restricted to <10% tumor cells (31/49, 63%) (Figures 3f–h). Tumor clinicopathological features that are associated with MUC6 expression are presented in Table 2. Positive staining for MUC6 was associated with proximal location (OR = 4.83, 95% CI = 2.20–10.56), presence of tumor-infiltrating lymphocytes (OR = 7.71, 95% CI = 3.05–19.46), and mucinous differentiation ($P = 0.029$). As for MUC2 and MUC5AC expression, MUC6 expression was associated with CIMP-positive colorectal carcinomas (OR = 1.33, 95% CI = 1.09–1.63), *BRAF* p.V600E mutation (OR = 7.85,

Figure 1 (**a**) MUC2 expression restricted to the cytoplasm of goblet cells in normal colonic epithelium. (**b**) Tumor scored negative for MUC2, showing only isolated cells (<25%) stained for MUC2. These cells have typical goblet cell differentiation. (**c**) Extensive MUC2 immunoreactivity in an adenocarcinoma without any mucinous differentiation. (**d**) Strong MUC2 staining in a mucinous carcinoma. (**e**) MUC5AC reactivity in the foveolar epithelium of normal stomach. (**f**) Moderate staining of a typical adenocarcinoma with intense apical cytoplasmic reactivity. (**g**) Isolated MUC5AC-positive tumor cells with evidence of staining of extracellular mucin. The transitional epithelium adjacent to the tumor is strongly stained, but more distant colonic epithelium is essentially MUC5AC-negative. (**h**) Intense MUC5AC staining of a colorectal carcinoma including staining of goblet cell contents and extracellular mucin.

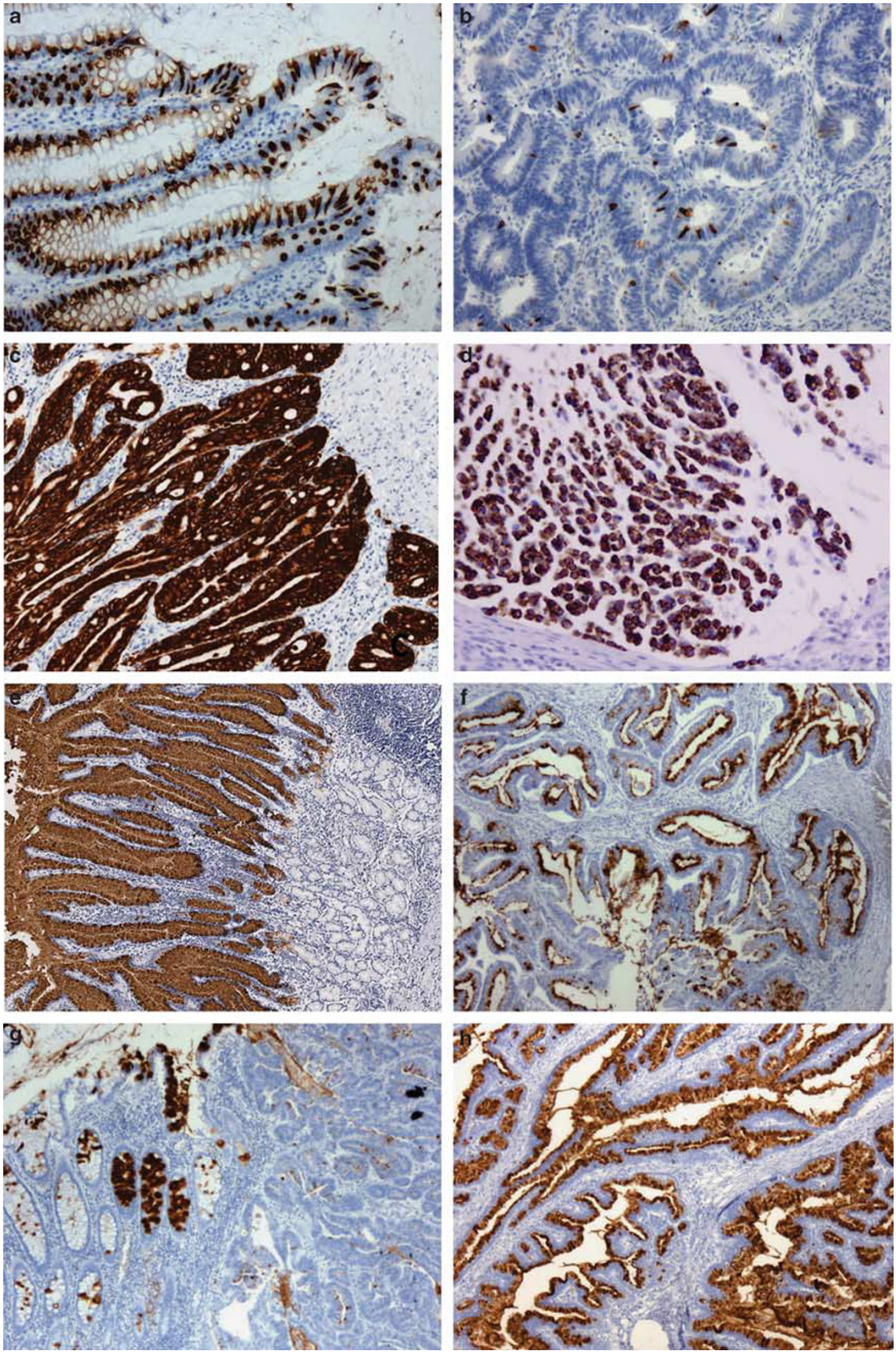

Figure 1 For caption see page 1645.

Table 1 MUC2 and MUC5AC expression related to tumor clinicopathological features

	MUC2			*MUC5AC*		
	MUC2-positive, N *(%)*	P-*value*	*OR (95% CI)*	*MUC5AC-positive,* N *(%)*	P-*value*	*OR (95% CI)*
Gender						
Male	111/351 (32)	0.505	1.12	156/347 (45)	**0.015**	1.48
Female	104/304 (34)		0.81–1.56	165/302 (55)		1.08–2.01
Ethnicity						
Non-mediterranean	169/502 (34)	0.429	0.84	258/501 (51)	**0.073**	0.70
Mediterranean	45/150 (30)		0.57–1.25	62/145 (43)		0.49–1.02
Tumor side						
Left	99/410 (24)	<**0.001**	2.83	180/417 (43)	<**0.001**	1.98
Right	110/232 (47)		2.01–3.99	132/220 (60)		1.42–2.75
Tumor type						
Adenocarcinoma	44/416 (11)	<**0.001**		147/420 (35)	<**0.001**	
Adenocarcinoma 1–49% mucinous component	113/174 (65)			119/167 (71)		
Mucinous >50%	49/51 (96)			46/49 (94)		
Signet ring cell ca.	9/9 (100)			8/8 (100)		
Undifferentiated	0/4 (0)			1/4 (25)		
Tumor grade						
Well/moderate	168/524 (32)	0.407	1.19	235/517 (45)	<**0.001**	2.24
Poor/undifferentiated	47/131 (36)		0.79–1.77	86/132 (65)		1.51–3.34
Tumor T stage						
T1/T2	39/155 (25)	**0.022**	1.64	65/150 (43)	**0.018**	1.57
T3/T4	159/448 (35)		1.09–2.47	243/445 (56)		1.08–2.28
Lymph node metastases						
Absent	105/317 (33)	0.595	1.10	162/316 (51)	0.312	1.18
Present	90/254 (35)		0.78–1.57	140/252 (56)		0.85–1.66
Venous invasion						
Absent	177/527 (34)	0.225	0.78	264/519 (51)	0.277	1.19
Present	21/53 (40)		0.46–1.34	42/76 (55)		0.74–1.94
Margins						
Expanding	142/441 (32)	0.695	1.09	219/432 (51)	0.520	1.14
Infiltrating	55/161 (34)		0.75–1.60	88/163 (54)		0.80–1.64
Budding						
Absent	128/368 (35)	0.061	0.69	191/370 (52)	0.724	0.93
Present	55/205 (27)		0.47–1.00	98/197 (50)		0.76–1.31
Tumor-infiltrating lymphocytes						
Absent	144/510 (28)	<**0.001**	2.43	226/497 (45)	<**0.001**	2.21
Present	68/139 (49)		1.66–3.58	94/145 (65)		1.51–3.24
Peritumoral lymphocytes						
Absent	168/497 (34)	0.738	0.91	253/485 (52)	0.603	0.89
Present	35/110 (32)		0.59–1.42	56/114 (49)		0.59–1.33
Crohn's-like aggregates						
Absent	147/492 (30)	<**0.001**	2.30	242/480 (50)	0.162	1.36
Present	50/101 (50)		1.49–3.56	61/105 (58)		0.89–2.09
p53 Overexpression						
Absent	123/281 (44)	<**0.001**	0.42	158/265 (60)	<**0.001**	0.52
Present	85/343 (25)		0.30–0.60	153/353 (43)		0.38–0.72
BRAF						
WT	140/518 (27)	<**0.001**	3.77	226/512 (44)	<**0.001**	4.08
p.V600E	67/115 (58)		2.48–5.73	87/114 (76)		2.56–6.50

Table 1 (Continued)

	MUC2			*MUC5AC*		
	MUC2-positive, N *(%)*	P-*value*	*OR (95% CI)*	*MUC5AC-positive,* N *(%)*	P-*value*	*OR (95% CI)*
KRAS						
WT	136/453 (30)	**0.011**	1.61	221/456 (48)	0.239	1.26
Codons 12/13 mutation	72/176 (41)		1.12–2.32	90/166 (54)		0.88–1.80
Mismatch repair						
Proficient	154/569 (27)	<**0.001**	7.15	250/563 (44)	<**0.001**	6.65
Deficient	61/84 (73)		4.28–11.95	69/82 (84)		3.59–12.30
CIMP						
Negative	145/539 (27)	<**0.001**	5.77	237/537 (44)	<**0.001**	8.58
Positive	51/75 (68)		3.43–9.72	61/70 (87)		4.18–17.63
MLH1 methylation						
Negative	143/516 (28)	<**0.001**	4.74	229/509 (45)	<**0.001**	6.36
Positive	40/62 (65)		2.73–8.26	52/62 (84)		3.16–12.79

Abbreviations: CI, confidence intervals; CIMP, CpG island methylator phenotype; OR, odds ratio; WT, wild type.
The statistically significant *P*-values have been highlighted in bold.

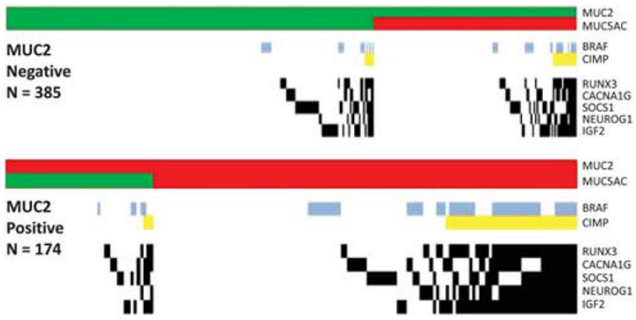

Figure 2 'Heat map' style presentation of the association between MUC2 and MUC5AC positivity, somatic *BRAF* p.V600E mutation, overall CIMP status, and methylation of individual markers. From the top, MUC2 and MUC5AC expression status is indicated either in red (positive) or green (negative). Blue boxes indicate the presence of *BRAF* mutation, yellow indicates CIMP-positive cases, and the individual markers RUNX3, CACNA1G, SOCS1, NEUROG1, or IGF2 are indicated in black where methylated.

95% CI = 2.62–23.53), and DNA mismatch repair deficiency (OR = 27.38, 95% CI = 6.02–124.47), as well as *MLH1* methylation (OR = 10.63, 95% CI = 1.18–96.00), and was inversely associated with p53 overexpression (OR = 0.36, 95% CI = 0.17–0.76).

Co-expression of Mucins

Overall, 117 tumors were stained for all four mucin proteins, and of these, 25 (21.4%) were negative for all four mucins, 29 (24.8%) expressed only one mucin, 21 (17.9%) expressed two mucins, 17 (14.5%) expressed three mucins, and 15 (12.8%) expressed all four mucins. Expression of the four mucins in individual colorectal carcinomas is represented in a heat map (Figure 4). Co-expression of more than two mucins was more common in females (23/47; 48.9%) than males (19/70; 27.1%) (OR = 2.57, 95% CI = 1.18–5.60), in proximal tumors (23/44; 52.3%) compared with distal cancers (17/69; 24.6%) (OR = 3.35, 95% CI = 1.50–7.50), and mismatch repair-deficient cancers (18/22) compared with mismatch repair proficient tumors (24/94) (OR = 13.13, 95% CI = 4.04–42.65). There were also strong associations between expression of three or four mucins and the presence of tumor-infiltrating lymphocytes (OR = 7.22, 95% CI = 2.87–18.18) and p53 negativity (OR = 0.16, 95% CI = 0.07–0.38). High levels of MUC2 or MUC5B expression (>50% tumor cells stained) were each predictive of co-expression of other mucins. Of the 43 high MUC2-expressing tumors, 33 (76.7%) showed immunoreactivity for at least two other mucins compared with only 9 out of 74 MUC2-low/negative cancers (OR = 23.83, 95% CI = 8.83–64.35). Similarly, 37/50 (74%) of high MUC5B-expressing tumors also demonstrated expression for two or more other mucins, whereas only 5/67 (7.5%) of MUC5B-low/negative cancers showed similar co-expression (OR = 35.29, 95% CI = 11.64–106.97). There were also strong associations between co-expression of three or more mucins and the presence of CIMP (OR = 9.00, 95% CI = 1.67–48.41) and *BRAF* mutation (OR = 3.36, 95% CI 1.22–9.24).

Mucin Expression and CDX2 Status

CDX2 immunohistochemistry was performed on a subset of 120 tumors and was scored as absent in 6 cases (5%) and reduced (<50% tumor nuclei stained) in a further 15 cases (12.5%). The associations between reduced or complete loss of expression of CDX2 and expression of MUC2, MUC5AC, MUC5B, and MUC6 are presented in Table 3.

Table 2 MUC5B and MUC6 expression related to tumor clinicopathological features

	MUC5B			*MUC6*		
	MUC5B-positive, N *(%)*	P-*value*	*OR (95% CI)*	*MUC6-positive,* N *(%)*	P-*value*	*OR (95% CI)*
Gender						
Male	32/72 (44)	**0.040**	2.28	26/75 (35)	0.267	1.55
Female	31/48 (65)		1.07–4.84	23/51 (45)		0.75–3.21
Ethnicity						
Non-Mediterranean	49/92 (53)	0.667	0.82	37/98 (38)	0.825	1.13
Mediterranean	13/27 (48)		0.35–1.92	11/27 (41)		0.48–2.70
Tumor side						
Left	34/71 (48)	0.343	1.49	18/73 (25)	<**0.001**	4.83
Right	26/45 (58)		0.70–3.16	30/49 (61)		2.20–10.56
Tumor type						
Adenocarcinoma	27/65 (42)	**0.006**		19/70 (27)	**0.029**	
Adenocarcinoma 1–49% mucinous component	21/33 (64)			17/33 (52)		
Mucinous >50%	13/16 (81)			9/17 (53)		
Signet ring cell ca.	2/2 (100)			2/2 (100)		
Undifferentiated	0/3 (0)			2/3 (67)		
Tumor grade						
Well/moderate	48/85 (56)	0.228	0.58	34/90 (38)	0.691	1.18
Poor/undifferentiated	15/35 (43)		0.26–1.28	15/36 (42)		0.54–2.59
Tumor T stage						
T1/T2	15/27 (56)	0.659	0.78	8/29 (28)	0.189	1.93
T3/T4	40/81 (49)		0.33–1.87	36/85 (42)		0.77–4.84
Lymph node metastases						
Absent	31/57 (54)	0.555	0.74	26/60 (43)	0.697	0.80
Present	22/47 (47)		0.34–1.60	19/50 (38)		0.37–1.72
Venous invasion						
Absent	48/92 (52)	0.359	0.69	37/96 (39)	0.584	0.96
Present	6/14 (43)		0.22–2.14	6/16 (38)		0.32–2.85
Margins						
Expanding	31/60 (52)	0.847	0.90	24/62 (39)	1.000	1.02
Infiltrating	23/47 (49)		0.42–1.92	20/51 (39)		0.48–2.18
Budding						
Absent	35/62 (56)	0.297	0.62	22/64 (34)	0.533	1.33
Present	16/36 (44)		0.27–1.41	16/39 (41)		0.58–3.02
Tumor-infiltrating lymphocytes						
Absent	41/87 (47)	**0.035**	2.62	25/92 (27)	<**0.001**	7.71
Present	21/30 (70)		1.08–6.36	23/31 (74)		3.05–19.46
Peritumoral lymphocytes						
Absent	42/81 (52)	1.000	1.05	32/84 (38)	0.547	1.30
Present	18/34 (53)		0.47–2.33	16/36 (44)		0.59–2.87
Crohn's-like aggregates						
Absent	45/88 (51)	0.797	0.85	32/92 (35)	0.017	3.75
Present	8/17 (47)		0.30–2.40	12/18 (67)		1.29–10.93
p53 Overexpression						
Absent	43/62 (69)	<**0.001**	0.24	31/61 (51)	**0.010**	0.36
Present	20/57 (35)		0.11–0.51	17/63 (28)		0.17–0.76
BRAF						
WT	41/82 (50)	0.805	1.22	26/86 (30)	<**0.001**	7.85
p.V600E	11/20 (55)		0.46–3.26	17/22 (77)		2.62–23.53

Table 2 (Continued)

	MUC5B			*MUC6*		
	MUC5B-positive, N *(%)*	P-*value*	*OR (95% CI)*	*MUC6-positive,* N *(%)*	P-*value*	*OR (95% CI)*
KRAS						
WT	39/78 (50)	0.285	1.64	36/83 (43)	0.391	0.65
Codons 12/13 mutation	18/29 (62)		0.68–3.91	10/30 (33)		0.27–1.57
Mismatch repair						
Proficient	44/95 (46)	**0.009**	4.17	28/101 (28)	<**0.001**	27.38
Deficient	18/23 (78)		1.43–12.16	21/23 (91)		6.02–124.47
CIMP						
Negative	35/77 (45)	0.466	2.00	24/82 (29)	<**0.001**	1.33
Positive	5/8 (63)		0.45–8.96	8/8 (100)		1.09–1.63
MLH1 methylation						
Negative	33/70 (47)	0.200	5.61	24/75 (32)	**0.020**	10.63
Positive	5/6 (83)		062–50.48	5/6 (83)		1.18–96.00

Abbreviations: CI, confidence intervals; CIMP, CpG island methylator phenotype; OR, odds ratio; WT, wild type.
The statistically significant *P*-values have been highlighted in bold.

A reduction in, or loss of, CDX2 staining was associated with the positive expression of MUC2, MUC5AC, and MUC6, whereas no significant association was observed for MUC5B. However, when MUC5B expression was considered in terms of high (>50% tumor cells positive) or low MUC5B expression, there was a stronger inverse effect with reduction in, or loss of, CDX2 staining, although this did not achieve statistical significance (OR = 2.75, 95% CI = 0.97–7.81). Similarly, when MUC2 expression was classified as high expression (>50% positive tumor cells) and low expression, a stronger effect was observed with CDX2 deficiency (OR = 5.10, 95% CI = 1.86–14.08) than with a MUC2-positive staining threshold of 25% (OR = 3.05, 95% CI = 1.13–8.26). Reduced or complete loss of expression of CDX2 was associated with CIMP positivity (OR = 5.16, 95% CI = 1.20–22.4), the *BRAF* p.V600E mutation (OR = 5.05, 95% CI = 1.73–14.73), *MLH1* methylation (OR = 14.55, 95% CI = 2.50–84.56), and mismatch repair deficiency (OR = 5.93, 95% CI = 2.04–17.26), but not *KRAS* mutation (OR = 0.78, 95% CI = 0.26–2.34). An example of a CDX2-deficient colorectal carcinoma with expression of all four mucins is shown in Figure 5.

Discussion

We assessed expression of epithelial mucins MUC2, MUC5AC, MUC5B, and MUC6 in a large series of colorectal carcinomas, and related it to tumor clinicopathological features. We observed ectopic expression of the gastric mucins MUC5AC and MUC6 in 49% and 39% of tumors, respectively, and overexpression of the colonic mucins MUC2 and MUC5B in 33% and 48% of cancers, respectively. There were strong associations between MUC2, MUC5AC, and MUC6 expression and carcinomas that demonstrate CIMP positivity, *BRAF* p.V600E mutation, *MLH1* promoter methylation, mismatch repair deficiency, tumor-infiltrating lymphocytes, and proximal location, all of which are features associated with the CIMP-positive subtype of colorectal carcinoma[3] and the serrated neoplasia tumorigenic pathway. In the most comprehensive study of mucin protein expression in colorectal cancer, we have also shown that overexpression of one mucin protein is frequently accompanied by the co-expression of one or more of the other mucins whose genes reside on chromosome 11p15.5, suggesting that a tumor milieu exists, which favors expression of these mucin genes. We have also shown an inverse association between the reduced or absent expression of a putative mucin gene regulator, CDX2, and positive expression of the MUC2, MUC5AC, and MUC6 proteins in colorectal cancer.

Of the chromosome 11 mucins, MUC2 expression has been most extensively studied in colorectal malignancy where it is commonly downregulated both at the mRNA and protein levels,[16,38,39] except in tumors displaying a mucinous phenotype.[19,20,40] Various immunohistochemical studies have reported MUC2 positivity in colorectal cancers ranging from 21 to 63%.[19,39,41–43] MUC5AC expression has been reported in 13–64% carcinomas,[16,42,44,45] with highest levels of expression in microsatellite-unstable/mismatch repair-deficient tumors[18,19,46] and those showing characteristic mucinous phenotype.[19,47] MUC5B and MUC6 expression in the lower gastrointestinal tract has remained relatively unexplored. MUC5B has been detected in normal colon where expression appears to be restricted to goblet cells,[15,48] and in the only study to date, MUC5B expression in 3/8 colorectal cancers was diffusely and strongly positive.[44]

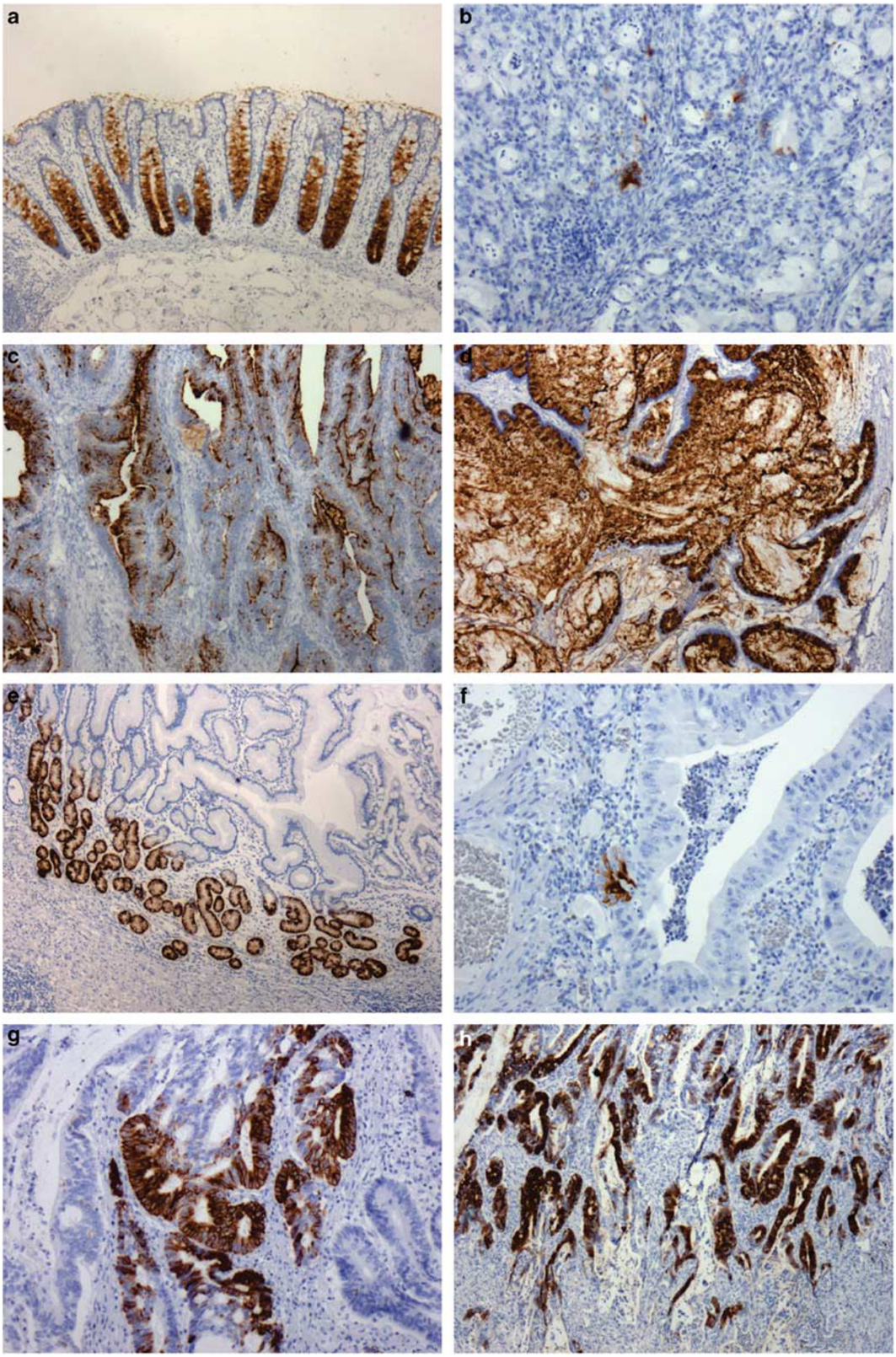

Figure 3 (**a**) MUC5B expression in normal colon shows a gradient along the crypt, being most intense in the crypt bases. Staining is present in both goblet cells and absorptive cells. (**b**) Rare isolated MUC5B-positive tumor cells within a negative colorectal carcinoma. (**c**) Moderate MUC5B staining of a conventional adenocarcinoma without evidence of mucinous differentiation. (**d**) Mucinous carcinoma showing intense MUC5B reactivity in both the epithelial cells and extracellular mucin. (**e**) MUC6 reactivity in the normal stomach is restricted to the gastric glands. (**f**) Rare intensely MUC6-positive tumor cells. (**g**) Approximately 50% of tumor cells stained in a conventional adenocarcinoma without mucinous differentiation. (**h**) Diffuse MUC6 immunoreactivity in a mucinous cancer.

Table 3 Reduced or absent CDX2 expression compared with clinicopathological features and mucin expression

	N *(%)*	P	*OR (95% CI)*
Tumor side			
Right	13/47 (35.4%)		
Left	7/69 (10.1%)	**0.023**	3.39 (1.23–9.29)
BRAF			
WT	12/86 (14.0%)		
p.V600E	9/20 (45.0%)	**0.004**	5.05 (1.73–14.73)
MLH1 methylation			
Unmethylated	11/75 (14.7%)		
Methylated	5/7 (71.4%)	**0.003**	14.55 (2.50–84.56)
KRAS			
WT	16/78 (20.5%)		
Mutant	5/30 (16.7%)	0.789	0.78 (0.26–2.34)
CIMP			
Negative	11/82 (13.4%)		
Positive	4/9 (44.4%)	**0.038**	5.16 (1.20–22.24)
Mismatch repair			
Proficient	12/99 (12.1%)		
Deficient	9/20 (45.0%)	**0.002**	5.93 (2.04–17.26)
MUC2			
Negative	7/65 (10.8%)		
Positive	14/52 (26.9%)	**0.030**	3.05 (1.13–8.26)
MUC2 HIGH			
Negative	7/76 (9.2%)		
Positive	14/41 (28.1%)	**0.002**	5.10 (1.86–14.08)
MUC5AC			
Negative	4/58 (6.9%)		
Positive	17/60 (28.3%)	**0.003**	5.35 (1.67–16.95)
MUC6			
Negative	6/70 (8.6%)		
Positive	14/43 (32.6%)	**0.002**	5.15 (1.80–14.71)
MUC5B			
Negative	7/51 (13.7%)		
Positive	11/55 (20.0%)	0.445	1.57 (0.56–4.24)
MUC5B HIGH			
Negative	7/63 (11.1%)		
Positive	11/43 (25.6%)	0.067	2.75 (0.97–7.81)

Abbreviations: CI, confidence intervals; CIMP, CpG island methylator phenotype; OR, odds ratio; WT, wild type.
The statistically significant *P*-values have been highlighted in bold.

Figure 4 'Heat map' style representation of the co-expression of the four chromosome 11 mucins in 117 colorectal cancers. Positive tumors for each mucin are highlighted in red and negative cases are highlighted in green.

Similarly, MUC6 expression in colorectal tumors has been restricted largely to adenomas and serrated polyps, the latter have been found to express MUC6 commonly,[30,49] whereas several groups have reported MUC6 expression in cancers as rare or absent,[16,50,51] even in mucinous carcinomas.[52]

In 1999, Toyota *et al*[53] described the existence of the CIMP in colorectal carcinoma, in which hypermethylation is induced in a wide variety of genes. Ferracin *et al*[9] found that CIMP-positive colorectal cancers were more likely than CIMP-negative cancers to show areas of mucinous differentiation. Several studies have examined the expression of various mucin genes in relation to the nature and extent of CpG island methylation, and have found that *MUC2* and *MUC5B* in particular, show strong correlations between promoter methylation and suppression of mucin protein synthesis.[23,54–57] There is currently little or no evidence of methylation-induced silencing of either *MUC5AC* or *MUC6*.[23] Furthermore, MUC17, a transmembrane mucin gene, is regulated by promoter CpG island methylation and histone H3-K9 acetylation in pancreatic ductal adenocarcinomas, where promoter hypomethylation is associated with MUC17 expression.[58] In this study, we have shown increased expression of MUC proteins in CIMP-positive colorectal cancers, which suggests the counter intuitive finding of increased protein expression in an environment that methylates (and silences) gene promoters. Interestingly, Ferracin *et al*[9] found similar evidence of upregulation of *MUC17* mRNA in CIMP cancers, supporting the concept of alternate regulatory mechanisms besides promoter methylation as a cause of increased MUC protein expression.

Besides mucin gene promoter methylation, expression of the chromosome 11 mucins is reportedly controlled by a number of other regulatory systems, including the EGFR-RAS-RAF pathway and paracrine exposure to cytokines (reviewed by Adrianifahanana *et al*[25]). Several studies have reported a link between somatic mutations in components of the EGFR-RAS-RAF pathway and evidence of mucinous differentiation in colorectal carcinomas,[59–63] but the inclusion of microsatellite-unstable carcinomas in many of these studies is likely to have confounded the association because of previous observations that microsatellite-unstable tumors are associated with overexpression of MUC2 and MUC5AC[19] and a mucinous phenotype.[11,12,64] *KRAS* mutations have been identified in 65% of mucinous colorectal carcinoma,[65] and associations between mutations in the EGFR-RAS-RAF pathway and other types of malignancy have been made,[66] including mucinous ovarian cancers, half or more of which possess somatic *KRAS* mutations.[67,68] Taken together, these studies implicate activation of the EGFR-RAS-RAF pathway in upregulating mucin synthesis. The results from our current study support the association between expression of MUC2, MUC5AC, and MUC6 and activation

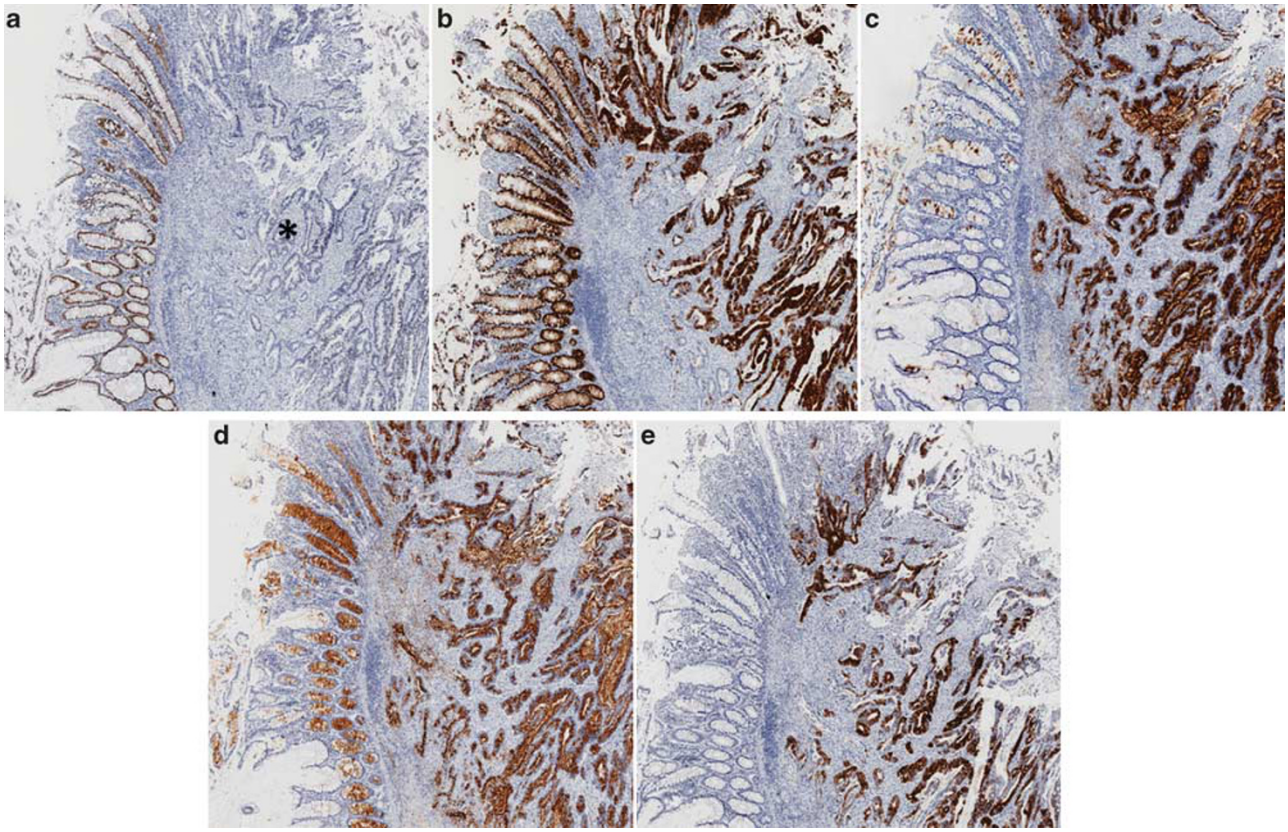

Figure 5 CDX2 loss associated with strong expression of all four chromosome 11 mucins. (**a**) CDX2 expression is present within the normal epithelium (left) but is significantly reduced in the colorectal cancer (*). (**b**) MUC2, (**c**) MUC5AC, (**d**) MUC5B, and (**e**) MUC6 expression in the same tumor.

of the EGFR-RAS-RAF pathway via the presence of somatic *BRAF* p.V600E mutation, with *KRAS* codon 12 and 13 mutations also associated with the overexpression of MUC2.

The mucin genes contain numerous binding sites for transcription factors, including Sp1, SP3, AP-1, various members of the GATA family, NFκB, and CDX2 among others (reviewed in Adrianifahanana *et al*[25]). CDX2 has been identified by Yamamoto *et al*[27] as a protein able to bind to the *MUC2* promoter and initiate transcription, and subsequent studies by Mesquita *et al* have shown two functionally active CDX2-binding sites.[26] Ectopic expression of CDX2 has also been linked to MUC2 expression in gastric intestinal metaplasia,[69] Barrett's esophagus,[70] and cholangiocarcinoma,[71] suggesting an important regulatory role for CDX2 in MUC2 expression. We and others have reported that CDX2 may be somatically lost in some colorectal cancers either due to out-of-frame allelic gains or losses of the microsatellite repeat within the *CDX2*-coding region,[29,72] epigenetic silencing via methylation,[73] or loss of heterozygosity.[74] Interestingly, Mochizuka *et al*[30] reported reduced expression or complete loss of CDX2 in serrated polyps of the colorectum in combination with increased expression of MUC2, MUC5AC, and MUC6.

We have found that CDX2 expression was completely lost in 5% of colorectal carcinoma cases and noticeably reduced in a further 12.5%. There was a strong inverse relationship between loss of expression of CDX2 and the overexpression of mucins MUC2, MUC5AC, and MUC6. Loss of CDX2 was also strongly linked to CIMP, *MLH1* methylation, and *BRAF* mutation, as well as mismatch repair deficiency in these tumors, features that were also associated with MUC2, MUC5AC, and MUC6 overexpression. In our current study design, it is difficult to determine whether the associations observed between MUC protein expression, CDX2 expression and features of the serrated pathway of tumorigenesis (CIMP, *BRAF* mutation, and *MLH1* methylation) are a cause or a consequence of altered MUC protein expression. Serrated colorectal polyps have been shown to have high levels of methylation and somatic *BRAF* mutations, and are thought to be the precursor lesions for many non-familial microsatellite-unstable cancers in which *MLH1* has been epigenetically silenced.[75] Our observations are, thus, in keeping with the findings of Mochizuka *et al*.[30] Although CDX2 might have an important role in regulating MUC2 expression, particularly in the normal colorectum, the findings of increased expression of MUC2 (and other chromosome

11 mucins) in a setting of decreased or lost expression of CDX2 suggest that other regulatory mechanisms are likely to be involved to compensate for the loss of CDX2.

Previous studies have described mucinous differentiation based solely on morphological assessment rather than mucin protein expression, which precludes detecting mucin gene overexpression in tumors lacking a component of characteristic mucinous differentiation. We have shown that microsatellite-unstable colorectal carcinomas lacking mucinous or signet ring cell differentiation overexpress one or more chromosome 11 mucin proteins, and that overexpression is often linked to the presence of the activating *BRAF* p.V600E mutation in these cancers.

In conclusion, we report the expression pattern of chromosome 11 gel-forming mucins, MUC2, MUC5AC, MUC5B, and MUC6 in a large series of colorectal carcinomas and, in doing so, provide the first comprehensive immunohistochemical assessment of MUC5B expression in colorectal tumors. We have demonstrated heterogeneity of expression of these mucins, such that overexpression was strongly associated with features associated with the CIMP subtype of colorectal cancer, including the *BRAF* p.V600E somatic mutation, mismatch repair deficiency, and proximal tumor location. In addition, we provide evidence for the role of the homeobox gene *CDX2* in mucin expression, demonstrating an inverse association with the expression of the chromosome 11 gel-forming mucins, suggesting that additional mucin regulatory mechanisms are altered in carcinomas that overexpress these mucins.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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