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Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component

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Signet ring cell carcinoma and mucinous carcinoma are distinct subtypes of colorectal adenocarcinoma. The morphologic and molecular spectra of colorectal carcinomas with various signet ring cell components and colorectal carcinomas with various mucinous components, compared to non-mucinous adenocarcinomas, have not been examined. The study groups consisted of 39 carcinomas with various signet ring cell components ('the signet group'), 167 carcinomas with various mucinous components ('the mucinous group'), and 457 nonmucinous adenocarcinoma. We visually estimated the amounts of signet ring cell and mucinous components in tumors, and subclassified the signet and mucinous groups according to the amount of each component ($\leq$19, 20–49, and $\geq$50%). We sequenced *BRAF* and *KRAS*, analyzed for microsatellite instability (MSI) and 18q loss of heterozygosity (LOH), and performed immunohistochemistry for TP53, cyclooxygenase-2 (COX2), MLH1, *O*-6-methylguanine DNA methyltransferase (MGMT), p16 (CDKN2A), and fatty acid synthase (FASN). Signet ring cell carcinoma ($\geq$50% signet ring cell tumors) and $\leq$49% signet ring cell tumors showed similar molecular features. Except for MSI and MGMT, $\geq$50% mucinous tumors and $\leq$49% mucinous tumors also showed similar molecular features. *BRAF* mutations, MSI, and MLH1 loss were more frequent in both the signet and mucinous groups than nonmucinous carcinoma. More frequent *KRAS* mutations and less frequent p16 loss and TP53 positivity were observed in the mucinous group than nonmucinous carcinoma. 18q LOH and COX2 overexpression were less common in the signet group than nonmucinous carcinoma. FASN levels were highest in the mucinous group, followed by nonmucinous carcinoma, and lowest in the signet group. In conclusion, a minor ($\leq$49%) signet ring cell or mucinous component in colorectal carcinoma suggests molecular features similar to $\geq$50% signet ring cell or mucinous carcinoma, respectively. Signet ring cell carcinoma and mucinous carcinoma are related subtypes of colorectal adenocarcinoma, but have molecular features distinct from each other.
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Signet ring cell colorectal carcinoma and mucinous colorectal carcinoma are subtypes of colorectal adenocarcinoma with prominent mucin secretion. A unique pathologic feature of signet ring cell carcinoma is the presence of signet ring cells, which are single tumor cells with intracytoplasmic mucin displacing their nuclei aside. In contrast, mucinous colorectal carcinoma is characterized by abundant extracellular mucin produced by tumor cells. By definition, a 50% or greater signet ring cell component is required for the designation of signet ring cell colorectal carcinoma. Mucinous colorectal carcinoma has also 50% or more mucinous components. Signet ring cell colorectal carcinoma has been associated with poor clinical outcomes.[1–6] A number of studies have examined the molecular features of signet ring cell colorectal carcinoma and mucinous

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colorectal carcinoma.[3–5,7] However, the molecular markers these studies examined were few. Furthermore, the biological significance of a minor signet ring cell or mucinous component ($\leq$49% of the tumor) in otherwise conventional colorectal adenocarcinoma has not been studied. In the current practice of surgical pathology, there is no definitive rule on how to report a minor component of signet ring cells or mucinous features in colorectal adenocarcinoma. We hypothesized that carcinomas with a $\leq$49% signet ring cell or mucinous component might have molecular features similar, if not identical, to colorectal carcinoma, with a $\geq$50% signet ring cell or mucinous component, respectively.

A number of genes and pathways have been implicated in colorectal carcinogenesis, and in some cases these genes show differential rates of alteration among different morphologic types of carcinoma. RAS and RAF proteins participate in the RAS–RAF–MEK–ERK–MAP kinase pathway, which mediates cellular responses to growth signals.[8] Somatic mutations of *KRAS* are common in various human cancers including colorectal carcinoma. Activating mutation of the *BRAF* gene is common in malignant melanoma, but less frequent in colorectal carcinoma.[9] *BRAF* mutations in colorectal carcinoma were reported to occur more commonly in those cases with high degree of microsatellite instability (MSI-H),[10] but less frequently in colorectal carcinoma in patients with germline mutation in one of mismatch repair genes than in sporadic MSI-H tumors.[11] Mutations of *BRAF* are associated with *MLH1* promoter methylation in sporadic colorectal carcinoma,[12] and mucinous colorectal carcinoma.[4] Both signet ring cell colorectal carcinoma and mucinous colorectal carcinoma are associated with MSI-H.[3–5] In addition, frequent *BRAF* mutation and infrequent *KRAS* mutation have been reported in mucinous colorectal carcinoma, compared to nonmucinous adenocarcinoma.[4]

Cyclooxygenase-2 (COX2) has been shown to be overexpressed in colorectal cancer,[13] and a high level of COX2 expression is associated with poor prognosis.[14] Recently, COX2 has been shown to be a target of mutant *KRAS*.[15] The new COX2-specific inhibitor celecoxib has been shown to inhibit the growth of colorectal cancer cells.[16]

Fatty acid synthase (FASN) is involved in *de novo* lipogenesis, catalyzing the reaction steps in the conversion of acetyl-CoA and malonyl-CoA to long-chain saturated fatty acid.[17] FASN overexpression is commonly observed in human cancers,[18–22] including colorectal cancer.[23–25] FASN overexpression has been associated with poor prognosis in breast, ovarian, and prostate cancers, and soft tissue sarcomas.[18,20–22,26,27] FASN inhibitor C75 has antitumor activity,[28] and causes apoptosis of p53-deficient colon cancer cells.[29] The FASN inhibitor, Orlistat, used to treat obesity, may serve as a potential anticancer drug.[30] FASN overexpression may be beneficial for tumor cells to retain more energy source and survive.

In this study, we characterize the molecular features of colorectal carcinoma with signet ring cell component, and colorectal carcinoma with mucinous component, but no signet ring cell component, and compared them to those of nonmucinous non-signet ring cell colorectal adenocarcinoma. This study is the first to comprehensively examine the morphologic and molecular spectra of signet ring cell and mucinous differentiations in colorectal carcinoma. We analyzed for a number of molecular abnormalities, including *BRAF*, *KRAS*, MSI, 18q loss of heterozygosity (LOH), and expression of p53 (TP53), COX2, p16 (CDKN2A, also known as INK4a), *O*-6-methylguanine DNA methyltransferase (MGMT), MLH1, and FASN. Our results indicate that a minor signet ring cell or mucinous component in colorectal carcinoma implies molecular features similar to colorectal carcinoma, with a $\geq$50% signet ring cell or mucinous component, respectively.

Materials and methods

Tissue Specimens and Histopathologic Evaluations

Tissue collection and analysis in this study have been approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board. Colorectal adenocarcinoma resection specimens were collected from participants of the Nurses' Health Study and Health Professional Follow-up Study cohorts.[31,32] Informed consents from all study participants have been obtained prior to this study. Tumors were randomly selected from these cohorts, based on availability of tumor tissue samples and assay results at the time of this study. Hematoxylin and eosin-stained slides of the tumors were reviewed, and the percentages of signet ring cell component and mucinous component were estimated under a light microscope. Tumors with signet ring cells (consisting 'the signet group') were classified according to the amount of their signet ring cell component: $\leq$19, 20–49, and $\geq$50%. After excluding tumors with signet ring cells, tumors with any mucinous component (consisting 'the mucinous group') were classified according to the amount of their mucinous components: $\leq$19, 20–49, and $\geq$50%. The colorectal adenocarcinomas without any mucinous or signet ring cell component were designated as nonmucinous non-signet ring cell adenocarcinoma (also referred to as 'nonmucinous carcinoma'). There were totals of 39 carcinomas with any signet ring cell component (the signet group), 167 carcinomas with any mucinous component (the mucinous group), and 457 nonmucinous carcinomas.

Genomic DNA Extraction, and Sequencing of *KRAS* and *BRAF*

For DNA extraction, tumor tissue on glass slides was manually dissected excluding pure normal tissue to

enrich tumor DNA. For tumors with a signet ring cell or mucinous component, we did not separate tumor DNA of the signet ring cell or mucinous areas from non-signet or nonmucinous areas. Instead, we analyzed pooled DNA from representative areas of the tumor. Normal DNA was obtained from normal colorectal tissue at resection margins. Genomic DNA was extracted using QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). For whole genome amplification (WGA), genomic DNA was PCR-amplified using random 15-mer primers.[33] Procedures of WGA has been validated as previously described.[34] Methods of PCR and sequencing targeted for *KRAS* codons 12 and 13, and *BRAF* codon 600, have been previously described.[35] All forward sequencing results were confirmed by reverse sequencing. *KRAS* sequencing was validated by the pyrosequencing technology as described previously.[34]

Analyses for MSI and 18q LOH

The status of MSI was determined by analyzing variability in the length of the microsatellite markers from tumor DNA compared to normal DNA. In addition to the recommended MSI panel consisting of D2S123, D5S346, D17S250, BAT25, and BAT26,[36] we used BAT40, D18S55, D18S56, D18S67, and D18S487 (ie, 10-marker panel). Primers were as follows: BAT40-F, 5′-agc caa gat taa ctt cct aca cca caa c-3′; BAT40-R, 5′-gta gag caa gac cac ctt gtc tc-3′; D18S55-F, 5′-gtg tct tca ata ttg att ctc tat tct agc ct-3′; D18S55-R, 5′-agc ttc tga gta atc tta tgc tgt g-3′; D18S56-F, 5′-gtg tct tcc tga agg acc tgc ctg aga ta-3′; D18S56-R, 5′-cta tac ttt tta ttg tta ggg tgt g-3′; D18S67-F, 5′-ctt ggg ttc cat ctt cag gaa a-3′; D18S67-R, 5′-gtg tct tat gag ata ggc cca aag cat c-3′; D18S487-F, 5′-gtg tct tgc caa att aaa aga atg tat att att gc-3′; D18S487-R, 5′-gat ttt cct cgt gcg tgc tt-3′. Either forward or reverse primer for each marker was labeled with fluorescence, and PCR products were electrophoresed and analyzed by ABI 3730 (Applied Biosystems, Foster City, CA, USA). PCR and DNA fragment analysis for all of the markers except for D2S123, D5S346, and D17S250 was performed in duplicate. 'High degree of MSI' (MSI-H) was defined as having instability in 30% or more of the markers when results of seven or more markers were available.

LOH at each locus (D18S55, D18S56, D18S67, or D18S487) was defined as 40% or greater reduction of one of two allele peaks in duplicated runs in tumor DNA when compared to normal DNA. A tumor was defined as 18q LOH positive when any informative marker showed LOH. A tumor was defined as 18q LOH negative when at least two markers were informative and no informative marker showed LOH.

Immunohistochemistry for p53 (TP53), FASN, p16 (CDKN2A), MLH1, MGMT, and COX2

Methods of immunohistochemistry for TP53, p16, MLH1, and MGMT were described previously.[37–40] We have described methods of COX2 immunohistochemistry.[35]

For FASN immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in 10 mM citrate buffer (BioGenex, San Ramon, CA, USA) by a microwave for 15 min. Tissue sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase, and then incubated with 10% normal goat serum in phosphate-buffered saline (10 min). Primary antibody against FASN (BD Biosciences, Mississauga, ON, Canada) (dilution 1:100) was applied for 60 min at room temperature. Then, Multilink secondary antibody (BioGenex) (20 min) and then streptavidin horseradish peroxidase (BioGenex) were applied (20 min). Sections were visualized by diaminobenzidine (DAB) (5 min) and methyl-green counterstain. FASN expression was interpreted as negative, weak (1+), positive (2+), and strongly positive (3+), using normal colonic epithelial cells and adipose tissue as reference.

Statistical Analysis

Statistical analysis was performed using the SAS program (version 9.1, SAS Institute, Cary, NC, USA). χ^2 test and Fisher's exact test (when a number of any category is less than 10) were utilized for the analysis on categorical data. In Tables 1–4, we ranked *P*-values as follows: between 0.05 and 0.025 ([a]), between 0.025 and 0.01 ([b]), between 0.01 and 0.005 ([c]), between 0.005 and 0.001 ([d]), between 0.001 and 0.0001 ([e]), and 0.0001 or less ([f]).

Results

MSI, MLH1 Loss, and 18q LOH

MSI-H tumors were more frequent in the signet group (25% or above) and the mucinous group (16–38%) than in nonmucinous carcinoma (11%) (Table 1). There was a statistically significant difference in frequencies of MSI-H between $\geq$50% mucinous tumors (38%) and $\leq$19% mucinous tumors (16%; $P<0.01$), and between 20–49% mucinous tumors (34%) and $\leq$19% mucinous tumors (16%; $P<0.025$). Consistent with the MSI results, MLH1 loss was more common in both the signet group (29–40%) and the mucinous group (13–30%) than in nonmucinous carcinoma (10%) (Table 1). 18q LOH was less common in $\leq$19% signet ring cell tumors (30%) than in nonmucinous carcinoma (64%) ($P<0.005$) (Table 1).

BRAF and *KRAS* Mutations, and TP53 Immunohistochemistry

The most common *BRAF* mutation is the p.Val600Glu mutation (V600E, previously called 'V599E' mutation). All other mutations comprised only appro-

Table 1 MSI, MLH1 loss, and 18q LOH in colorectal carcinoma with signet ring cell and mucinous components and nonmucinous adenocarcinoma

Type of colorectal carcinoma	*MSI-H (%)*	*MLH1 loss (%)*	*18q LOH present (%)*
Carcinoma with signet ring cell component (the signet group)			
≤19%	7/25 (28%)[b1]	7/25 (28%)[b3]	6/20 (30%)[d6]
20–49%	2/3 (67%)[a1]	2/5 (40%)	1/2 (50%)
≤49%	9/28 (32%)[d1]	9/30 (30%)[d4]	7/22 (32%)[b5]
≥50%	2/8 (25%)	2/7 (29%)	4/7 (57%)
Any	11/36 (31%)[d2]	11/37 (30%)[d5]	11/29 (38%)[b6]
Carcinoma with mucinous component (the mucinous group)			
≤19%	11/70 (16%)[b2,c]	8/61 (13%)	30/54 (56%)
20–49%	15/44 (34%)[b2,f1]	11/37 (30%)[e1]	17/30 (57%)
≤49%	26/114 (23%)[a2,d3]	19/98 (19%)[b4]	47/84 (56%)
≥50%	20/53 (38%)[c,a2,f2]	14/50 (28%)[e2]	20/38 (53%)
Any	46/167 (28%)[f3]	33/148 (22%)[e3]	67/122 (55%)
Nonmucinous adenocarcinoma	38/351 (11%)[b1,a1,d1,d2,f1,d3,f2,f3]	36/352 (10%)[b3,d4,d5,e1,b4,e2,e3]	194/304 (64%)[d6,b5,b6]

Superscripts for statistical significance: [a1,a2]$P<0.05$; [b1–b6]$P<0.025$; [c1]$P<0.01$; [d1–d6]$P<0.005$; [e1–e3]$P<0.001$; [f1–f3]$P<0.0001$.
LOH, loss of heterozygosity; MSI-H, microsatellite instability-high.

Table 2 *BRAF* and *KRAS* mutations and TP53 immunohistochemistry in colorectal carcinoma with signet ring cell and mucinous components and nonmucinous adenocarcinoma

Type of colorectal carcinoma	*BRAF mutants (%)*	*KRAS mutants (%)*	*TP53 positive (%)*
Carcinoma with signet ring cell component (the signet group)			
≤19%	7/21 (33%)[d1]	9/27 (33%)	4/12 (33%)
20–49%	0/2 (0%)	1/3 (33%)	4/6 (67%)
≤49%	7/23 (30%)[d2]	10/30 (33%)	8/18 (44%)
≥50%	2/9 (22%)	0/8 (0%)	3/4 (75%)
Any	9/32 (28%)[d3]	10/38 (26%)	11/22 (50%)
Carcinoma with mucinous component (the mucinous group)			
≤19%	9/61 (15%)	32/66 (48%)[e3]	22/54 (41%)[a2]
20–49%	10/41 (24%)[d4]	17/39 (44%)[a1]	11/27 (41%)
≤49%	19/102 (19%)[b1]	49/105 (47%)[d5]	33/81 (41%)[b2]
≥50%	14/51 (27%)[e1]	15/49 (31%)	13/42 (31%)[d7]
Any	33/153 (22%)[e2]	64/154 (42%)[d6]	46/123 (37%)[d8]
Nonmucinous adenocarcinoma	30/348 (8.6%)[d1,d2,d3,d4,b1,e1,e2]	102/376 (27%)[e3,a1,d5,d6]	181/322 (56%)[a2,b2,d7,d8]

Superscripts for statistical significance: [a1,a2]$P<0.05$; [b1,b2]$P<0.025$; [d1–d8]$P<0.005$; [e1–e3]$P<0.001$.

Table 3 Loss of MGMT and p16 (CDKN2A), and COX2 expression in colorectal carcinoma with signet ring cell and mucinous components and nonmucinous adenocarcinoma

Type of colorectal carcinoma	*MGMT loss (%)*	*p16 loss (%)*	*COX2 positive (%)*
Carcinoma with signet ring cell component (the signet group)			
≤19%	5/19 (26%)	4/16 (25%)	9/14 (64%)
20–49%	0/2 (0%)	1/1 (100%)	0/2 (0%)
≤49%	5/21 (24%)	5/17 (29%)	9/16 (56%)
≥50%	0/4 (0%)	1/4 (25%)	1/4 (25%)
Any	5/25 (20%)	6/21 (29%)	10/20 (50%)[c]
Carcinoma with mucinous component (the mucinous group)			
≤19%	16/41 (39%)	6/45 (13%)[a2,e1]	35/44 (80%)
20–49%	10/19 (53%)[b]	4/19 (21%)	13/20 (65%)
≤49%	26/60 (43%)[a1]	10/64 (16%)[a3,e2]	48/64 (75%)
≥50%	5/28 (18%)[b,a1]	11/31 (35%)[a2,a3]	23/35 (66%)
Any	31/88 (35%)	21/95 (22%)[e3]	71/99 (72%)
Nonmucinous adenocarcinoma	71/208 (34%)	101/240 (42%)[e1,e2,e3]	346/457 (76%)[c]

Superscripts for statistical significance: [a1–a3]$P<0.05$; [b]$P<0.025$; [c]$P<0.01$; [e1–e3]$P<0.001$.

Table 4 FASN expression in colorectal carcinoma with signet ring cell and mucinous components and nonmucinous adenocarcinoma

Type of colorectal carcinoma	*3+ FASN (%)*	*2+ FASN (%)*	*1+ FASN (%)*	*Negative*	*Total cases analyzed*
Carcinoma with signet ring cell component (the signet group)					
≤19%	1 (7.1%)	3 (21%)[a2]	5 (36%)	5 (36%)[b4]	14
20–49%	0	0	0	2 (100%)	2
≤49%	1 (6.3%)	3 (18%)[b1]	5 (31%)	7 (44%)[d]	16
≥50%	0	0	1 (100%)	0	1
Any	1 (5.9%)	3 (18%)[c2]	6 (35%)	7 (41%)[c5]	17
Carcinoma with mucinous component (the mucinous group)					
≤19%	8 (19%)[c1]	25 (58%)[a2,b2]	7 (16%)[a3]	3 (7.0%)[b4,b5]	43
20–49%	0	11 (58%)	5 (26%)	3 (16%)	19
≤49%	8 (13%)	36 (58%)[b1,c3]	12 (19%)	6 (10%)[d,b6]	62
≥50%	4 (12%)	16 (50%)	6 (19%)	6 (19%)	32
Any	12 (13%)[a1]	52 (55%)[c2,c4]	18 (19%)[b3]	12 (13%)[c5,a4]	94
Nonmucinous adenocarcinoma	19 (5.9%)[c1,a1]	126 (39%)[b2,c3,c4]	101 (31%)[a3,b3]	75 (23%)[b5,b6,a4]	321

Superscripts for statistical significance: [a1–a4]$P<0.05$; [b1–b6]$P<0.025$; [c1–c5]$P<0.01$; [d]$P<0.005$.

ximately 4% of *BRAF* mutations in carcinomas. *BRAF* mutations were more frequent in the signet group (22–33%) and in the mucinous group (15–27%) than nonmucinous carcinoma (8.6%) (Table 2). *KRAS* mutation distributions (ie, prevalence of each codon 12 or codon 13 mutation among all *KRAS* mutations) in the signet group, the mucinous group, and nonmucinous carcinoma did not significantly differ (data not shown). The *KRAS* mutation frequency in ≤49% mucinous tumors (47%), but not that in ≥50% mucinous tumors (31%), was significantly higher than nonmucinous carcinoma (27%) (Table 2). TP53 positivity was less frequently observed in the mucinous group (31–41%) than in nonmucinous carcinoma (56%) (Table 2).

Expression of MGMT, p16 (CDKN2A), COX2, and FASN

MGMT and p16 immunohistochemistry is shown in Figure 1. MGMT loss was less frequent in the signet group (0–26%) than in the 20–49% mucinous tumors (53%) and in nonmucinous carcinoma (34%), though statistical significance was not reached (Table 3). Interestingly, MGMT loss was more common in 20–49% mucinous tumors (53%) than in ≥50% mucinous tumors (18%) ($P<0.025$). Loss of p16 was less common in ≤49% mucinous tumors (16%) than in nonmucinous tumors (42%; $P<0.001$) (Table 3).

COX2 and FASN immunohistochemistry is shown in Figures 2 and 3, respectively. There was no significant difference in distributions of COX2 staining intensities among the mucinous group and nonmucinous carcinoma (~5% showing 3+ expression, 30–50% 2+ expression, 20–30% 1+ expression) (data not shown). COX2 expression was less common in tumors with any signet ring cell component (50%) in nonmucinous carcinoma (76%; $P<0.01$) (Table 3).

FASN expression was remarkable in that the mucinous group showed the highest levels of expression (58–77% with 3+ or 2+ positivity), followed by nonmucinous carcinoma (70% with 2+ or 1+ positivity), and the signet group most often showed low levels or no expression (~75% showing 1+ or negative staining) (Table 4).

Discussion

Signet ring cell colorectal carcinoma and mucinous colorectal adenocarcinoma are pathologically related, specific subtypes of colorectal adenocarcinoma. By convention, at least 50% of signet ring cell or mucinous component is required for the designation of signet ring cell carcinoma or mucinous carcinoma, respectively. We hypothesized that tumors with even less than 50% signet ring cell or mucinous component in colorectal adenocarcinoma imply molecular features similar to carcinoma with 50% or more signet ring cell or mucinous component, respectively. Our results support this hypothesis. There were some molecular differences, specifically frequencies of MSI and MGMT loss, between ≥50% mucinous carcinoma and ≤49% mucinous carcinoma. Statistically significant results by some of these pairwise comparisons might represent a result of multiple hypothesis testing, or a true biological difference. Nonetheless, in general, ≥50% mucinous carcinoma and ≤49% mucinous carcinoma appear similar, if not exactly the same. With regard to ≥50% signet ring cell carcinoma and ≤49% signet ring cell carcinoma, it seems that there is no significant difference. However, the number of tumors with a signet ring cell component, especially those with ≥50% signet ring cell component, is small and more cases are necessary to draw definitive conclusions. In light of our observations, we recommend that pathologists try to identify and report any minor component of signet ring cell

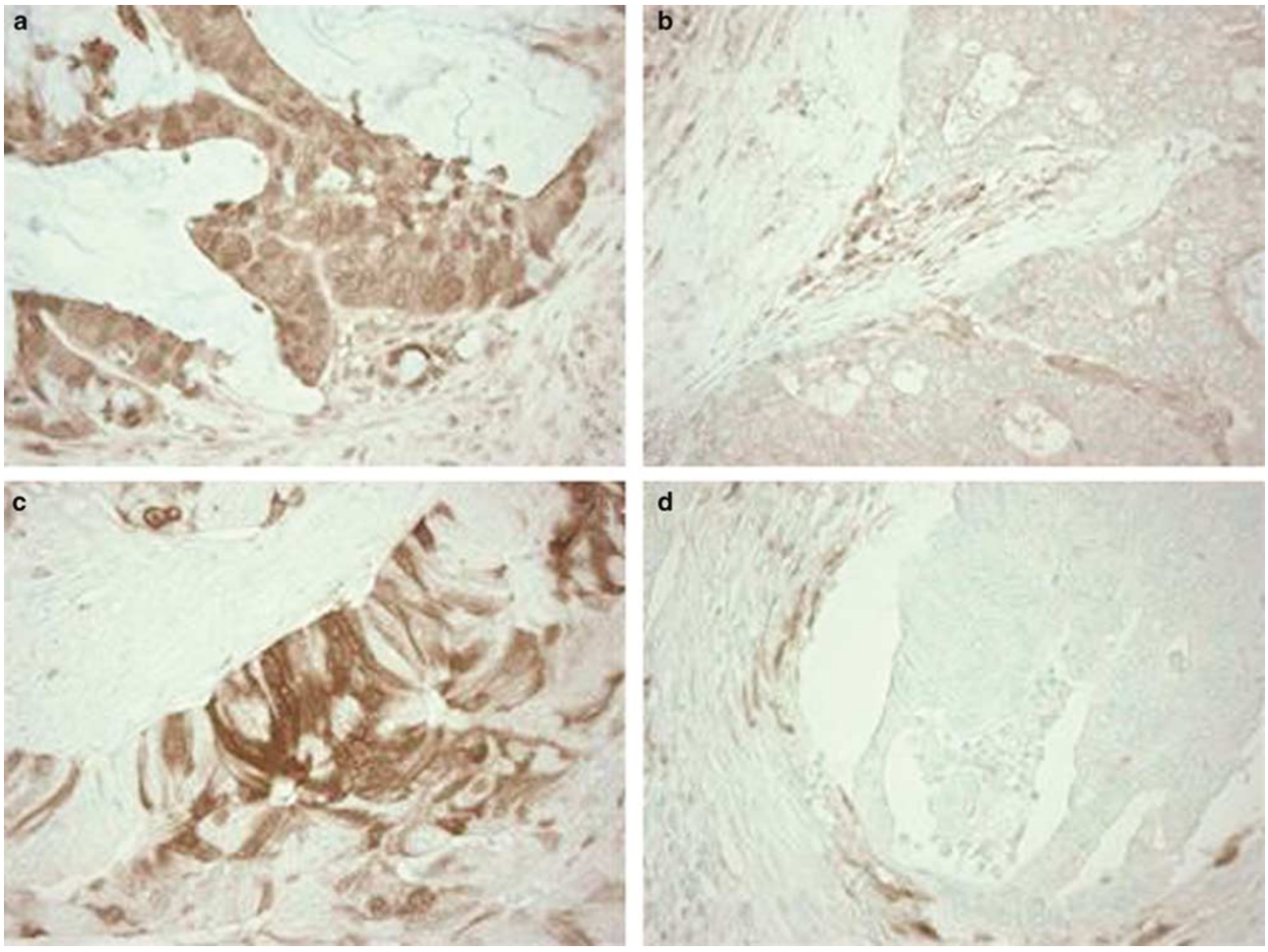

Figure 1 MGMT and p16 (CDKN2A) immunohistochemistry in colorectal carcinoma. (**a**) MGMT expression in mucinous carcinoma. (**b**) Loss of MGMT expression in nonmucinous carcinoma. Note that positive staining in mesenchymal and inflammatory cells serves as internal positive controls. (**c**) p16 expression in nonmucinous carcinoma. Note that p16 staining is focal. (**d**) Loss of p16 expression in nonmucinous carcinoma. Note that positive staining in mesenchymal cells serves as internal positive controls (original magnifications all $\times 400$).

or mucinous areas in colorectal carcinoma, with efforts to quantify the amount of such a component. Since targeted therapy against specific deranged oncoproteins or signal transduction pathways may be available in the future, any findings that imply distinct molecular features should be reported.

We compared various molecular features between carcinoma with signet ring cell component (the signet group) and carcinoma with mucinous component, but no signet ring cell component (the mucinous group). There are molecular similarities among these groups, including higher frequencies of *BRAF* mutation, MSI, and MLH1 loss. However, there are a number of molecular differences between the signet and mucinous groups. The mucinous group showed more frequent *KRAS* mutations, higher levels of COX2, and FASN expression than the signet group. Our results suggest that the signet and mucinous groups have overlapping, but distinct, pathogenetic mechanisms from each other. Since chemotherapeutic agents that inhibit the activity of FASN have potential activity against many different cancers with FASN overexpression,[24,28] our results of FASN expression (the highest expression in the mucinous group, followed by nonmucinous carcinoma, and the lowest expression in the signet group) may have some implications in treatment for colorectal cancer by FASN inhibitors in the future.

There are a number of differences between the signet group and nonmucinous non-signet ring cell carcinoma (simply referred to as 'nonmucinous carcinoma'). Compared to nonmucinous carcinoma, the signet group has more frequent *BRAF* mutations, MSI and MLH1 loss, less frequent 18q LOH, and lower COX2 level. There are also a number of differences between the mucinous group and nonmucinous carcinoma. Compared to nonmucinous carcinoma, the mucinous group has more frequent *BRAF* mutations, MSI, and MLH1 loss, less frequent TP53 mutation, and higher level of FASN expres-

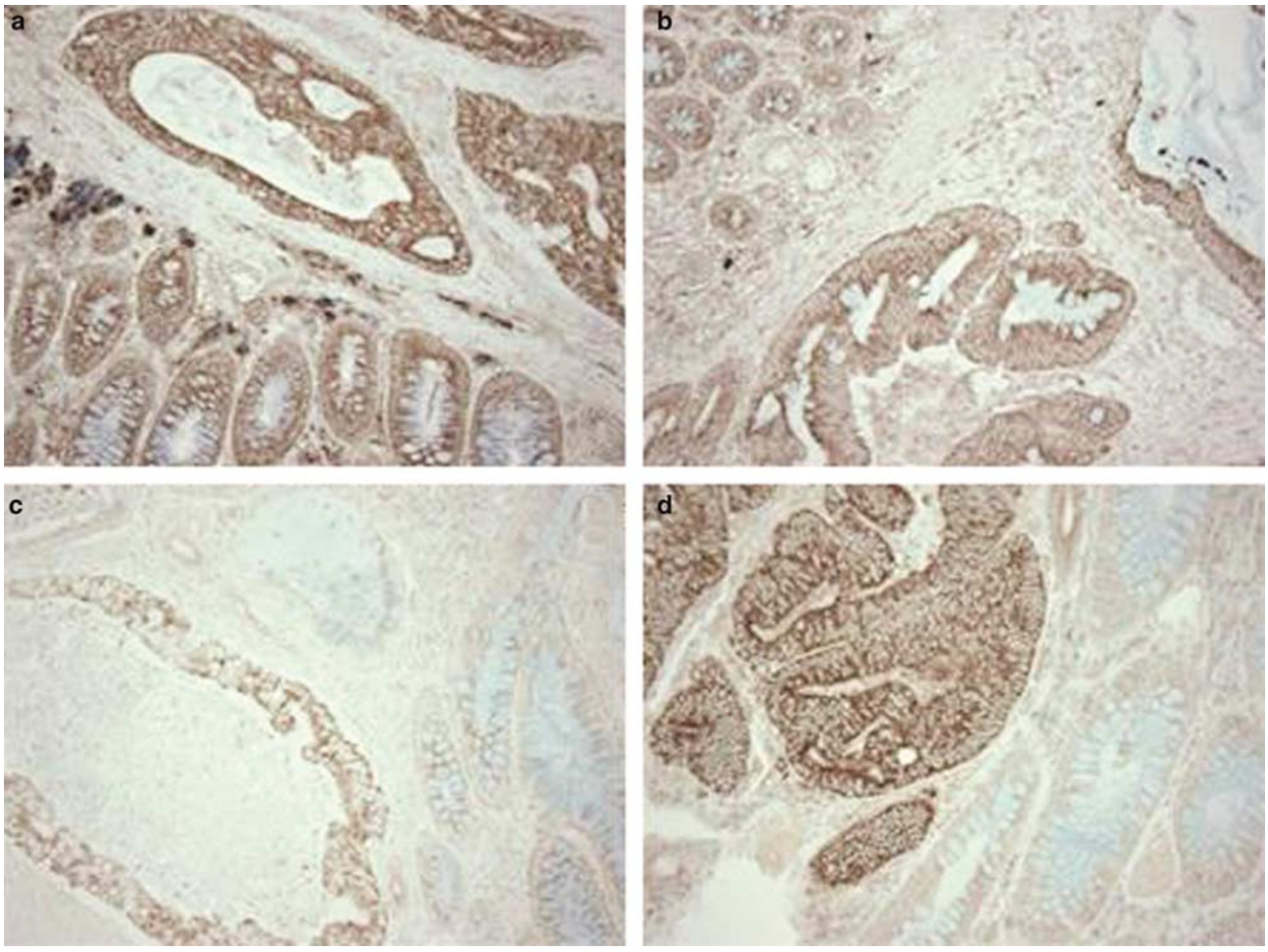

Figure 2 COX2 immunohistochemistry in colorectal carcinoma. (**a**) No or little COX2 overexpression in colorectal carcinoma (top) relative to normal mucosa (bottom). (**b**) Weak (1+) COX2 overexpression in carcinoma with mucinous component (bottom and right) relative to normal mucosa (top left). (**c**) Moderate (2+) COX2 overexpression in colorectal carcinoma (left) relative to normal mucosa (right). (**d**) Strong (3+) COX2 overexpression in colorectal carcinoma (left) relative to normal mucosa (right) (original magnifications all $\times 200$).

sion. Both signet ring cell carcinoma and mucinous colorectal carcinoma have been associated with MSI.[3–5] *KRAS* mutations were less common in signet ring cell carcinoma (4/11 = 36%) and mucinous carcinoma (11/29 = 38%) than in nonmucinous carcinoma (18/30 = 60%) in a Japanese study.[1] Other unique molecular abnormalities described in mucinous tumors include less frequent APC inactivation and *KRAS* mutation (28%).[4] Another study showed *KRAS* mutation frequency of 29% in mucinous tumors.[41] Our results of *KRAS* mutation in 33% of mucinous tumors are also consistent with these findings.

Cyclooxygenase 2 (COX2) overexpression in colorectal carcinoma has been associated with poor prognosis.[14] Expression of phospholipase A2, a key enzyme for prostaglandin synthesis together with COX2, has been associated with TNF-alpha-induced apoptosis in colon cancer cells.[13] As lower COX2 levels have been reported in colorectal carcinoma with MSI,[42] we examined whether COX2 was expressed any differently among the signet group, the mucinous group, and nonmucinous carcinoma, depending on different MSI status. However, we did not observe any significant modifications of COX2 levels due to MSI status among these tumor groups (data not shown). Since COX2 is an attractive target of chemoprevention as well as targeted therapy for colorectal carcinoma,[16,43] further investigations on various aspects of COX2 in colorectal carcinogenesis are awaited.

In conclusion, a minor signet ring cell or mucinous component in colorectal carcinoma implies molecular features similar to carcinoma with $\geq 50\%$ signet ring cell component (signet ring cell carcinoma) or carcinoma with $\geq 50\%$ mucinous component (mucinous carcinoma), respectively. Colorectal carcinoma with a signet ring cell component and carcinoma with a mucinous component are related subtypes of colorectal adenocarcinoma, but have distinct molecular features from each other.

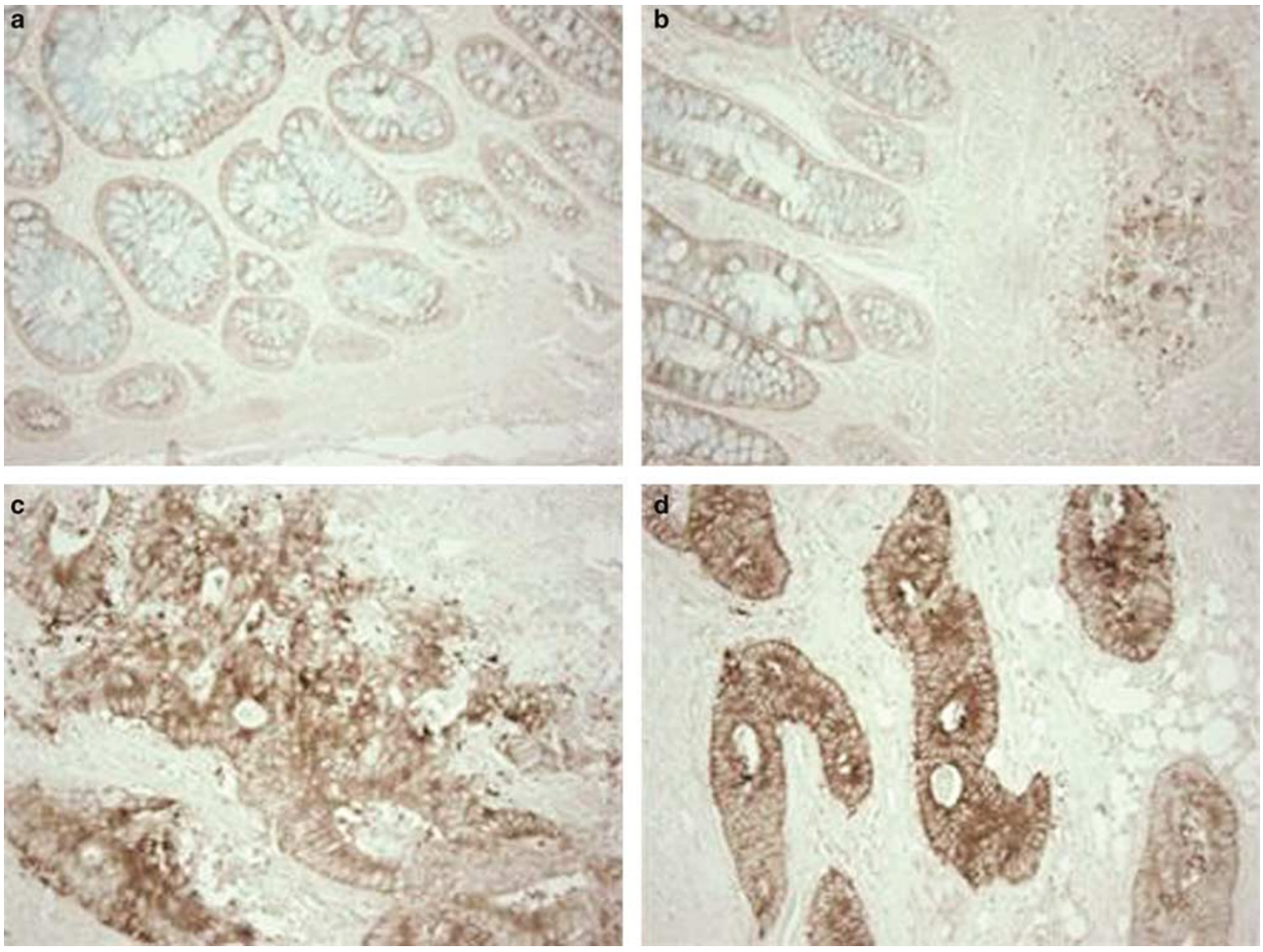

Figure 3 FASN immunohistochemistry in colorectal carcinoma. (**a**) Little FASN expression in normal colonic mucosa. (**b**) Weak (1+) FASN overexpression in colorectal carcinoma (right). Little FASN expression in normal mucosa (left). (**c**) Moderates (2+) FASN overexpression in colorectal carcinoma. (**d**) Strong (3+) FASN overexpression in colorectal carcinoma (original magnifications all $\times 200$).

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Notes added in proofs

Detailed immunohistochemical methods for MLH1, CDKN2A (p16), MGMT and COX2 were as follows:

For MLH1 immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in 10 mM citrate buffer (BioGenex, San Ramon, CA, USA) in a pressure cooker by a microwave for 30 min. Tissue sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase for 15 min, and then incubated with 10% normal goat serum in phosphate-buffered saline (10 min). Tissue sections were further incubated with avidin block (Vector Laboratories, Burlingame, CA, USA) for 20 min and then with biotin block (Vector Laboratories) for 20 min. Primary antibody against MLH1 (BD Pharmingen, San Jose, CA, USA) (dilution 1:100) was applied for 30 min at room temperature. Multilink secondary antibody (BioGenex) (20 min), and then streptavidin horse radish peroxidase (BioGenex) were applied (20 min). Sections were visualized by diaminobenzidine (DAB) (5 min) and methyl-green counterstain. Normal colonic epithelial cells and inflammatory cells served as internal positive controls.

For CDKN2A (p16) immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in 10 mM citrate buffer (BioGenex) by a microwave for 30 min. Tissue

sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase for 20 min, and then incubated with 10% horse serum (Vector Laboratories) in phosphate-buffered saline (20 min). Primary antibody Ab-7 clone against CDKN2A (LabVision, Fremont, CA, USA) (dilution 1:200) was applied overnight at 4°C. Secondary antibody (Vector Laboratories) (30 min) and then avidin–biotin complex conjugate (Vector Laboratories) were applied (30 min). Sections were visualized by DAB (5 min) and methyl-green counterstain. Some mesenchymal cells and inflammatory cells served as internal positive controls.

For MGMT immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in 10 mM citrate buffer (BioGenex) by a microwave for 15 min. Tissue sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase for 20 min, and then incubated with 10% horse serum (Vector Laboratories) in phosphate-buffered saline (20 min). Tissue sections were further incubated with avidin block (BioGenex) for 15 min and then with biotin block (BioGenex) for 15 min. Primary antibody against MGMT (Chemicon, Temecula, CA, USA) (dilution 1:50) was applied overnight at 4°C. Secondary antibody (Vector Laboratories) (30 min) and then avidin–biotin complex conjugate (Vector Laboratories) were applied (30 min). Sections were visualized by DAB (5 min) and methyl-green counterstain. Normal colonic epithelial cells and inflammatory cells served as internal positive controls.

For COX2 immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in citrate buffer (BioGenex) by a microwave for 15 min, and letting the sections cool for at least 40 min. Tissue sections were incubated with 3% H_2O_2 (20 min) to block endogenous peroxidase, and then incubated with Avidin Block (Vector Laboratories) (15 min), then with Biotin Block (Vector Laboratories) (15 min). Primary anti-COX2 antibody (Cayman Chemical, Ann Arbor, MI, USA) (dilution 1:300) was applied overnight at 4°C. Then, secondary anti-mouse antibody (Vector Laboratories) was applied (20 min), avidin–biotin complex conjugate (Vector Laboratories) was added and sections were visualized by DAB (5 min) and methyl-green counterstain. COX2 expression was interpreted as negative, weak (1+), positive (2+) and strongly positive (3+), using normal epithelial and inflammatory cells as reference.

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