

# Small bowel carcinomas in celiac or Crohn's disease: distinctive histophenotypic, molecular and histogenetic patterns

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**Non-familial small bowel carcinomas are relatively rare and have a poor prognosis. Two small bowel carcinoma subsets may arise in distinct immune-inflammatory diseases (celiac disease and Crohn's disease) and have been recently suggested to differ in prognosis, celiac disease-associated carcinoma cases showing a better outcome, possibly due to their higher DNA microsatellite instability and tumor-infiltrating T lymphocytes. In this study, we investigated the histological structure (glandular vs diffuse/poorly cohesive, mixed or solid), cell phenotype (intestinal vs gastric/pancreatobiliary duct type) and Wnt signaling activation ( $\beta$ -catenin and/or SOX-9 nuclear expression) in a series of 26 celiac disease-associated small bowel carcinoma, 25 Crohn's disease-associated small bowel carcinoma and 25 sporadic small bowel carcinoma cases, searching for new prognostic parameters. In addition, non-tumor mucosa of celiac and Crohn's disease patients was investigated for epithelial precursor changes (hyperplastic, metaplastic or dysplastic) to help clarify carcinoma histogenesis. When compared with non-glandular structure and non-intestinal phenotype, both glandular structure and intestinal phenotype were associated with a more favorable outcome at univariable or stage- and microsatellite instability/tumor-infiltrating lymphocyte-inclusive multivariable analysis. The prognostic power of histological structure was independent of the clinical groups while the non-intestinal phenotype, associated with poor outcome, was dominant among Crohn's disease-associated carcinoma. Both nuclear  $\beta$ -catenin and SOX-9 were preferably expressed among celiac disease-associated carcinomas; however, they were devoid, *per se*, of prognostic value. We obtained findings supporting an origin of celiac disease-associated carcinoma in SOX-9-positive immature hyperplastic**

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crypts, partly through flat  $\beta$ -catenin-positive dysplasia, and of Crohn's disease-associated carcinoma in a metaplastic (gastric and/or pancreatobiliary-type) mucosa, often through dysplastic polypoid growths of metaplastic phenotype. In conclusion, despite their common origin in a chronically inflamed mucosa, celiac disease-associated and Crohn's disease-associated small bowel carcinomas differ substantially in histological structure, phenotype, microsatellite instability/tumor-infiltrating lymphocyte status, Wnt pathway activation, mucosal precursor lesions and prognosis.

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Small bowel carcinomas are relatively rare neoplasms, the majority of which arise sporadically, while few cases are associated with genetic conditions such as familial adenomatous polyposis, Lynch and Peutz–Jeghers syndromes or may develop in immune-mediated inflammatory conditions, such as celiac disease or Crohn's disease.<sup>1</sup> Several small bowel carcinoma studies are available concerning their histological structure, cell phenotype, nuclear  $\beta$ -catenin or p53 protein expression and *TP53*, *KRAS*, *PIK3CA*, *HER2* or microsatellite instability-related gene mutation/methylation or copy number alteration.<sup>2–6</sup> These studies, however, deal mostly with sporadic cases or with few cases from other subgroups, with limited information on associated background disease or specific tumor histogenesis, progression and prognosis.

We recently collected a fairly large series of patients with surgically resected celiac disease-associated or Crohn's disease-associated small bowel carcinomas and identified a significantly better overall and cancer-specific survival of celiac disease-associated carcinoma patients compared with Crohn's disease-associated carcinoma patients, with no substantial difference between these two carcinoma subsets and sporadic ones, which showed intermediate survival.<sup>7</sup> Neither stage nor histopathological type and grade according to WHO 2010 classification criteria accounted for such behavior difference, which was, however, attributed to the higher prevalence of microsatellite instability status found in celiac disease-associated carcinoma, in accordance with previous observations.<sup>8,9</sup> No other molecular change, such as p53 overexpression or *TP53* gene mutation, and *KRAS* or *PIK3CA* gene mutations, had a significant prognostic impact.

Results of potential clinico-pathological relevance provided by previous studies are those suggesting a prognostic relevance of tumor cell phenotype,<sup>3,10,11</sup> a selective tumor association with mucosal metaplastic changes<sup>12</sup> or dysplastic lesions<sup>13–15</sup> and a pathogenetic role of molecular changes affecting microsatellite instability status,<sup>8,9,16,17</sup> Wnt signaling pathway<sup>18–20</sup> or p53 gene involvement.<sup>5</sup> In the present study, we reinvestigated our small bowel carcinoma series aiming to (a) look for any prognostic influence of histopathological structure, with special reference to tumor cell cohesion and stromal desmoplasia, and tumor cell phenotype, according to criteria already developed for the evaluation of

gastric cancer,<sup>21–23</sup> (b) expand our analysis to Wnt pathway activation signs such as nuclear  $\beta$ -catenin and SOX-9 transcription factor expression, including their correlation with microsatellite instability status; and (c) gain new insights into cancer histogenesis, with special reference to the carcinogenetic process at work in the inflamed non-tumor mucosa of celiac disease-associated or Crohn's disease-associated small bowel carcinoma patients.

## Materials and methods

### Study Population

This retrospective study included 76 patients with primary non-familial, non-ampullary small bowel carcinoma, including 26 celiac disease-associated small bowel carcinomas, 25 Crohn's disease-associated small bowel carcinomas and 25 small bowel carcinomas arising sporadically, ie, without a concomitant intestinal immune-mediated disorder. The patients, enrolled for a former previous clinico-pathological study, had surgical resection and complete survival data from 20 tertiary referral Italian Celiac or Inflammatory Bowel Disease Centers participating in the Small Bowel Cancer Italian Consortium.<sup>6</sup> The main exclusion criteria for all small bowel carcinoma subgroups were Lynch syndrome, Peutz–Jeghers syndrome, familial adenomatous polyposis and juvenile polyposis. Neuroendocrine neoplasms were also excluded. This study was approved by the Ethics Committee of the San Matteo Hospital Foundation of Pavia.

### Histology and Immunohistochemistry

Tissue samples were fixed in 4% formaldehyde and processed in paraffin wax. Four- $\mu$ m-thick sections were stained with hematoxylin–eosin and all cases were reinvestigated for histological findings. Histologically, small bowel carcinomas were reclassified as (a) glandular type (if >70% of the tumor exhibited glandular pattern), (b) diffuse/poorly cohesive cell type (signet ring cell cancers or cancers showing diffusely infiltrating, poorly cohesive cells, dispersed in a frequently desmoplastic stroma as single elements or as small aggregates with little to no gland formation, in >70% of the tumor), (c) mixed type (characterized by a combination of both glandular

and poorly cohesive cell/desmoplastic patterns, with at least 30% each, within the same tumor) and solid cancers (when they showed almost exclusively a solid or trabecular pattern).<sup>21,22,24</sup> Among solid cancers, (d) medullary-type cancers (characterized by tumor cells exhibiting prominent infiltration by T lymphocytes and a pushing margin) were distinguished from the (e) non-medullary solid cases lacking these features.<sup>22</sup> Four small bowel carcinomas previously classified as 'mucinous carcinomas'<sup>7</sup> were included in the glandular (three cases) or mixed (one case) type according to the predominant structure of the neoplasm. All cases were also checked for the presence of adjacent dysplastic lesions and metaplastic features of the uninvolved mucosa.

For immunohistochemistry, four- $\mu\text{m}$ -thick sections were incubated at 4 °C for 18–20 h with specific antibodies directed against: the gastric foveolar cell marker MUC5AC (monoclonal, clone CLH2, Abcam), the pancreatobiliary duct marker cytokeratin (CK) 7 (monoclonal, clone OV-TL 12/30, Dako), the pyloric and Brunner's gland marker MUC6 (monoclonal, clone CLH5, Novocastra), as well as against intestinal differentiation markers, including the transcription factor CDX2 (monoclonal, clone DAK-CDX2, Dako), the goblet cell marker MUC2 (monoclonal, clone Ccp58, Santa Cruz Biotechnology), CK20 (monoclonal, clone Ks20.8, Dako) and the small bowel brush border marker CD10 (monoclonal, clone 56C6, Dako). In addition, immunoreactions for C-terminal  $\beta$ -catenin (monoclonal, clone 14/Beta-Catenin, BD); for the transcription factor Sex-determining Region Y-Box 9 (SOX-9, polyclonal, Millipore); for the mismatch repair proteins, including MLH1 (monoclonal, clone ES05, Dako), MSH2 (monoclonal, clone FE11, Dako), MSH6 (monoclonal, clone EP49, Dako) and PMS2 (monoclonal, clone EP51, Dako); for p53 protein (monoclonal, clone DO7, Dako); for the neuroendocrine marker chromogranin-A (monoclonal, clone LK2H10, Ventana) and for CD3 (polyclonal, Dako) and CD8 (polyclonal, Dako) were also performed. Immunoreactions were developed using 0.03% 3,3'-diaminobenzidine tetrahydrochloride and sections were then counterstained with Harris' hematoxylin.

For the assessment of tumor cell phenotype, only cases with at least 10% immunoreactive cells were regarded as positive with the exception of CDX-2, for which a cutoff of 20% was applied.<sup>23</sup> Cases with nuclear accumulation of C-terminal  $\beta$ -catenin in at least 10% of dysplastic/tumor cells was recorded as positive and were also tested for the loss of nuclear expression of N-terminal  $\beta$ -catenin (monoclonal, clone E247, Abcam). Only relatively strong nuclear SOX-9 immunoreactivity, with the same intensity as the deepest intestinal crypt cells, was regarded as positive. Immunostaining of mismatch repair proteins in neoplastic cells was evaluated as positive or negative. Tumor or dysplastic growths were considered negative when there was complete absence of

nuclear staining of neoplastic cells in the presence of an internal positive control. A tumor was classified as having high density of tumor-infiltrating lymphocytes when the mean number of CD3-positive tumor-infiltrating lymphocytes per high-power field was  $> 15$ , as previously reported.<sup>7,25</sup> Tumor or dysplastic lesions were considered p53 positive when  $\geq 50\%$  of the cells showed strong nuclear p53 immunoreactivity. A central pathology review was performed by two surgical pathologists specialized in gastrointestinal pathology (AV and ES).

### Microsatellite Instability Analysis

Tumor DNA was obtained from formalin-fixed and paraffin-embedded tissues using three representative 8  $\mu\text{m}$ -thick sections of tumor samples. DNA was extracted after manual microdissection using a QIAamp DNA Formalin-Fixed, Paraffin-Embedded Tissue Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Microsatellite instability analysis was performed using a pentaplex panel of monomorphic mononucleotide repeats (BAT25, BAT26, NR-21, NR-22 and NR-24) by the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

### MLH1 Methylation Analysis

*MLH1* methylation status was examined by pyrosequencing in small bowel carcinomas exhibiting loss of *MLH1* immunohistochemical expression as previously reported.<sup>7</sup>

### Statistical Analysis

The distribution of biomarkers was reported as counts and percentage and Fisher's exact test was used to compare them across clinical groups. Cumulative survival was plotted according to the Kaplan–Meier method. The follow-up extended from the date of surgery to the date of death or last follow-up. The association candidate prognostic factors and tumor-related death was estimated by means of univariable and multivariable Cox regression. The choice of variables to be included in the multivariable model was decided *a priori* and was based on the biological knowledge of the tumor. Hazard ratios and their 95% confidence intervals were computed. The proportional hazard assumption was tested, based on Schoenfeld residuals. Model discrimination was assessed with the Harrell's *c* statistic (the closer to 1, the better) and calibration with the shrinkage coefficient (the closer to 1, the better). A two-sided *P*-value  $< 0.05$  was considered statistically significant. For *post hoc* comparisons, Bonferroni's correction applies. Stata 14 (StataCorp, College Station, TX, USA) was used for computation.

## Results

### Histological Classification

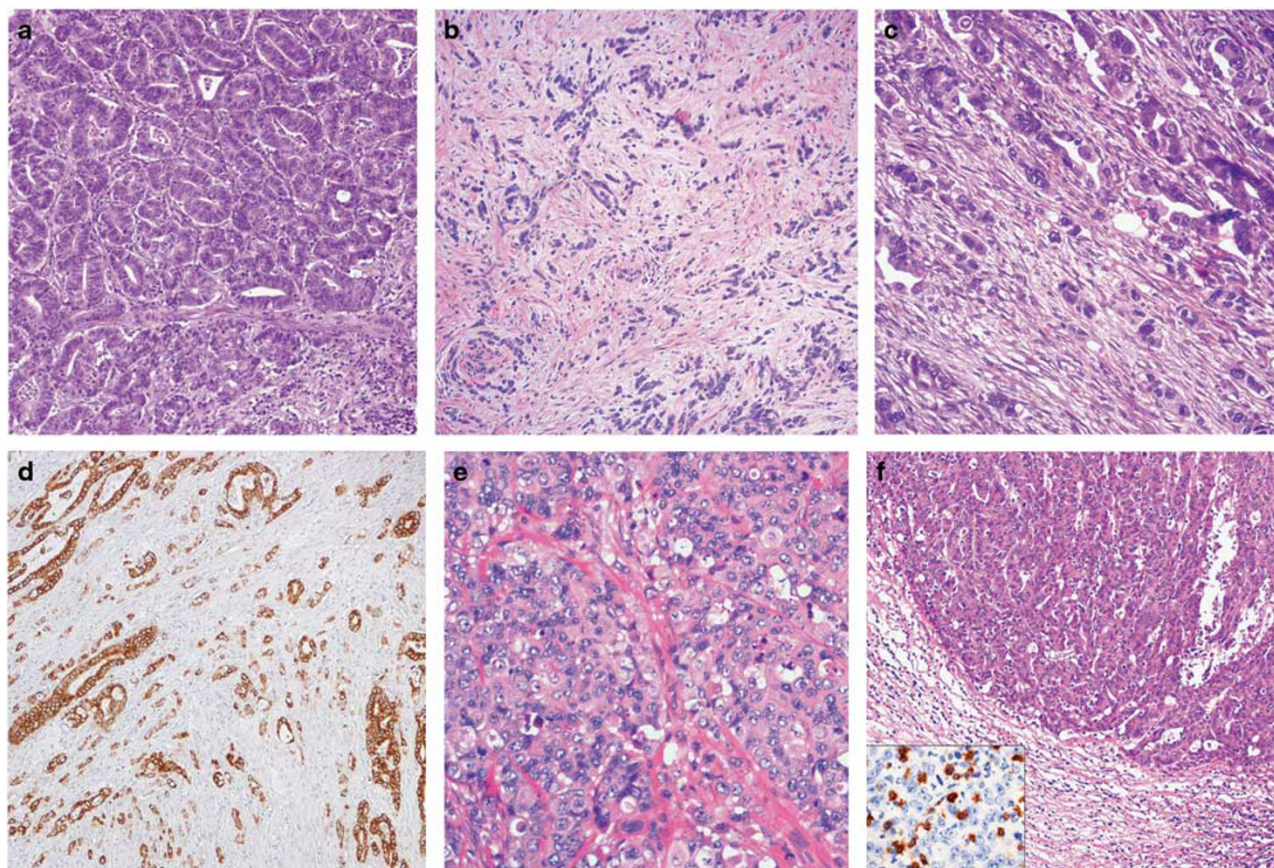
Histological analysis showed predominance of gland-forming tumors over diffuse, mixed glandular/diffuse, medullary or non-medullary solid cases (Table 1 and Figure 1). Survival analysis of these five tumor histotypes gave a trend for worse outcome of

diffuse, mixed and solid non-medullary compared with glandular or medullary cases (Figure 2a). When diffuse, mixed and solid histotypes were pooled in a single group of 28 cases (Figure 2b), a significant survival difference emerged for this group in comparison with glandular cases (hazard ratio: 4.93, 2.23–10.89,  $P < 0.001$ ). Multivariable analysis confirmed a significantly improved survival of

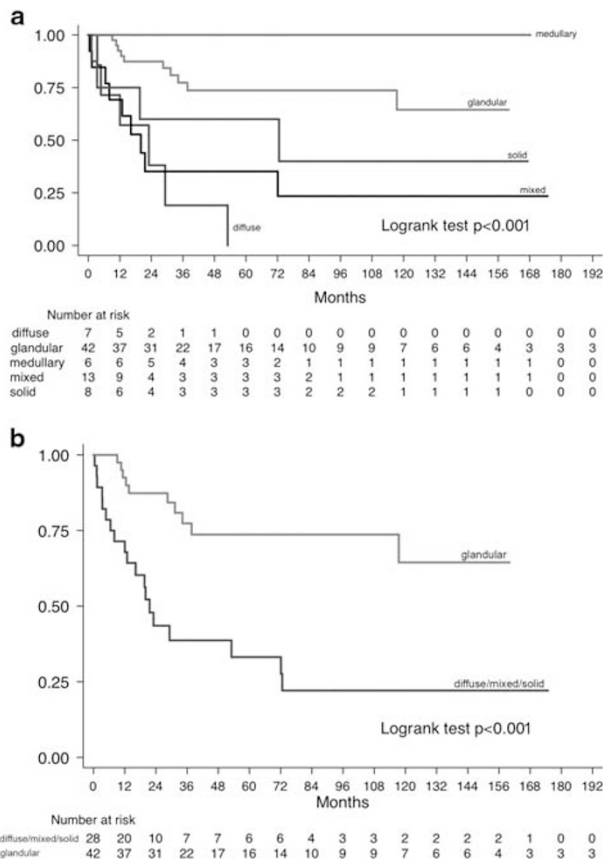
**Table 1** Histological classification of the 76 small bowel carcinomas investigated

Histotype	Distribution among clinical groups			Total
	Celiac disease-associated carcinomas (n = 26)	Crohn's disease-associated carcinomas (n = 25)	Sporadic carcinomas (n = 25)	
Glandular	14 (54%)	13 (52%)	15 (60%)	42 (55%)
Medullary	4 (15%)	1 (4%)	1 (4%)	6 (8%)
Solid	3 (11.5%)	2 (8%)	3 (12%)	8 (11%)
Diffuse	2 (8%)	4 (16%)	1 (4%)	7 (13%)
Mixed	3 (11.5%)	5 (20%)	5 (20%)	13 (23%)

No significant histotype distributive difference among the clinical groups was found.



**Figure 1** Small bowel carcinoma histotypes. (a) This glandular-type celiac disease-associated carcinoma is predominantly composed of well-formed tubular structures. (b) This diffuse type celiac disease-associated carcinoma is characterized by poorly cohesive cells, dispersed in a desmoplastic stroma as single elements or as small aggregates. (c, d) A mixed type Crohn's disease-associated ileal carcinoma characterized by a combination of both glandular and diffuse patterns within the same tumor. In panel (d), the same Crohn's disease-associated carcinoma expressing the gastric marker MUC5AC in both components. (e) A non-medullary solid-type celiac disease-associated carcinoma composed of nests of large, eosinophilic and atypical cells without intratumor T-cell infiltration. (f) This medullary-type celiac disease-associated carcinoma is characterized by solid/trabecular growth of cells, a pushing margin and prominent infiltration by numerous T lymphocytes (see CD3 immunostaining in the inset).



**Figure 2** (a) Kaplan–Meier survival estimates for all 76 small bowel carcinoma patients by histotype. *Post hoc* comparisons (significance after Bonferroni's correction set at  $P < 0.005$ ): (A) significant differences: diffuse vs glandular:  $P < 0.001$ ; diffuse vs medullary:  $P = 0.003$ ; glandular vs mixed:  $P < 0.001$ ; (B) non-significant trends: medullary vs mixed:  $P = 0.013$ ; medullary vs solid:  $P = 0.064$ ; glandular vs solid:  $P = 0.088$ ; (C) non-significant differences: diffuse vs mixed:  $P = 0.544$ ; diffuse vs solid:  $P = 0.170$ ; mixed vs solid:  $P = 0.401$ ; glandular vs medullary:  $P = 0.207$ . Notably, no difference was found between diffuse, mixed and solid histotypes, which were pooled in panel (b). (b) Kaplan–Meier survival estimates for the 70 non-medullary small bowel carcinoma patients by glandular vs diffuse/mixed/solid histotype.

patients with glandular-type small bowel carcinoma, independently of clinical group, tumor-infiltrating lymphocyte density (or microsatellite instability) and stage (Table 2).

### Tumor Cell Phenotype

The distribution of the intestinal phenotypic markers CDX2, MUC2, CK20 or CD10, the gastric marker MUC5AC and the pancreatobiliary duct marker CK7 among small bowel carcinoma subsets is outlined in Table 3, where 39 tumors with essentially intestinal phenotype (ie, positive for CDX2, MUC2, CK20 and/or CD10, whereas negative for both MUC5AC and CK7) were separated from the remaining 37 tumors expressing a metaplastic gastro-pancreatobiliary phenotype, either alone or admixed with the intestinal one (ie, positive for MUC5AC and/or

**Table 2** Overall survival by a multivariable Cox model of the 75 small bowel carcinomas patients with complete stage data

	Hazard ratio (95% CI)	P-value
<i>Clinical group</i>		
Celiac disease-associated carcinomas	1.00 (base)	0.033
Crohn's disease-associated carcinomas	4.98 (1.35–18.34)	0.016 <sup>a</sup>
Sporadic carcinomas	2.48 (0.66–9.29)	0.176 <sup>a,b</sup>
Stages III–IV vs stages I–II	9.31 (3.30–26.26)	< 0.001
CD3 <sup>+</sup> tumor-infiltrating lymphocytes > 15/high-power field vs ≤ 15	0.15 (0.03–0.69)	0.015
Glandular histotype vs non-glandular <sup>c</sup>	0.37 (0.16–0.84)	0.018

Model: LR  $\chi^2(5) = 56.72$ ,  $P$ -value < 0.001; Harrell's  $C = 0.87$ ; shrinkage coefficient = 0.91.

<sup>a</sup>For *post hoc* comparisons among the clinical groups, significance after Bonferroni's correction set at 0.017.

<sup>b</sup>Hazard ratio (95% CI): 0.50 (0.22–1.14),  $P$ -value = 0.098 vs Crohn's disease-associated small bowel carcinomas.

<sup>c</sup>Medullary histotype included among non-glandular cases.

CK7 while being positive or negative for CDX2, MUC2, CK20 and CD10). A general predominance of the intestinal phenotype among both celiac disease-associated and sporadic cases and of the non-intestinal (gastro-pancreatobiliary or mixed) phenotype among Crohn's disease cases was observed (Figure 3). Intestinal phenotype was more frequently found in glandular (32/42, 76%) compared with diffuse/mixed/solid cases (6/28, 21%,  $P < 0.001$ ). In particular, 11 of the 13 mixed, 4 of the 7 diffuse and 7 of the 8 solid small bowel carcinomas showed gastro-pancreatobiliary markers expression. Of note, all of the 10 glandular cases with the non-intestinal phenotype occurred in Crohn's disease patients.

Survival analysis showed a favorable influence of individual intestinal markers (especially CDX2 and MUC2) and a worse influence of gastro-pancreatobiliary markers. The pyloric and Brunner's gland marker MUC6 was found in a small fraction (16%) of cases, including 2 celiac disease-associated, 7 Crohn's disease-associated and 3 sporadic small bowel carcinomas, without a significant difference among clinical groups ( $P = 0.156$ ) and without prognostic value (hazard ratio: 0.80, 0.30–2.13,  $P = 0.654$ ).

Cumulative markers analysis showed better survival for patients with tumors of essentially intestinal phenotype compared with those expressing non-intestinal markers (in at least 10% of cells) (Table 3). This finding may contribute to the poor outcome of Crohn's disease-associated small bowel carcinoma patients, most of which (20/25, 80%) showed non-intestinal marker expression. In a stage-, sex- and age-inclusive survival analysis of 75 small bowel carcinomas, the favorable influence of intestinal phenotype was retained (hazard ratio: 0.30, 0.14–0.69;  $P = 0.004$ ), while it lost significance when the clinical group was also added to the model.

**Table 3** Expression of phenotypic markers in 76 small bowel carcinomas: their distribution among clinical groups and prognostic value

	Distribution among the clinical groups			Total	Survival analysis	
	Celiac disease carcinomas (n = 26)	Crohn's disease carcinomas (n = 25)	Sporadic carcinomas (n = 25)		Hazard ratio (95% confidence interval)	P-value
Cytokeratin 7	5 (19%) <sup>a</sup>	15 (60%) <sup>b</sup>	4 (16%)	24 (32%)	2.72 (1.18–6.29)	0.019
MUC5AC	5 (19%)	13 (52%)	5 (20%)	23 (30%)	2.54 (1.08–5.98)	0.032
CDX2	21 (81%) <sup>c</sup>	9 (36%)	17 (68%)	47 (62%)	0.28 (0.13–0.63)	0.002
MUC2	18 (69%)	12 (48%)	12 (48%)	42 (55%)	0.25 (0.12–0.53)	< 0.001
Cytokeratin 20	13 (50%)	9 (36%) <sup>d</sup>	20 (80%)	42 (55%)	1.19 (0.57–2.50)	0.635
CD10	8 (31%)	3 (12%)	11 (44%)	22 (29%)	0.66 (0.30–1.65)	0.302
Intestinal phenotype	17 (65%) <sup>e</sup>	5 (20%) <sup>f</sup>	17 (68%)	39 (51%)	0.39 (0.18–0.81) <sup>g</sup>	0.012

<sup>a</sup> $P=0.004$  vs Crohn's disease-associated carcinomas.

<sup>b</sup> $P=0.003$  vs sporadic carcinomas.

<sup>c</sup> $P=0.002$  vs Crohn's disease-associated carcinomas.

<sup>d</sup> $P=0.004$  vs sporadic carcinomas.

<sup>e</sup> $P=0.002$  vs Crohn's disease-associated carcinomas.

<sup>f</sup> $P=0.001$  vs sporadic carcinomas.

<sup>g</sup>Vs remaining 37 cases expressing gastro-pancreatobiliary markers.

However, in a multivariable analysis, inclusive of stage and microsatellite instability or tumor-infiltrating lymphocytes, the intestinal phenotype remained predictive of improved survival (hazard ratio: 0.35, 0.16–0.77,  $P=0.009$ ).

### Impact of Microsatellite Instability and Tumor-Infiltrating Lymphocytes on Prognostic Power of Histotype and Phenotype

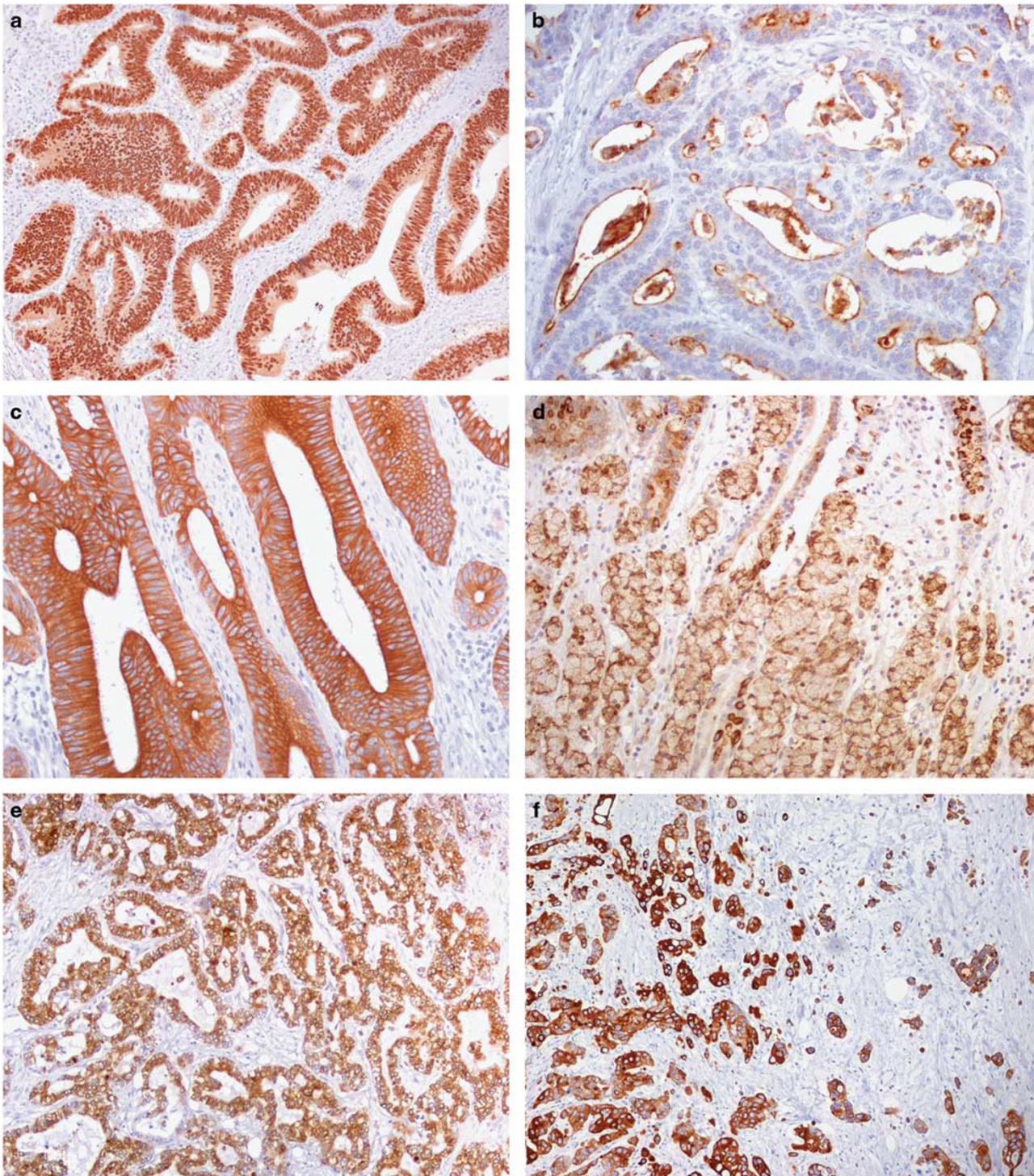
In a previous study,<sup>7</sup> the small bowel carcinoma microsatellite instability status (ascertained by concordant MLH1 nuclear loss, molecular analysis and *MLH1* promoter hypermethylation) was found to be associated with improved survival and to correlate with tumor-infiltrating lymphocytes, the likely microsatellite instability antitumor effectors.<sup>22,26</sup> Thus when we found that both microsatellite instability and high tumor-infiltrating lymphocyte density were segregating among small bowel carcinomas showing glandular or medullary histotype and intestinal phenotype, we investigated for any potential impact of microsatellite instability and/or high tumor-infiltrating lymphocytes on the prognostic power of histotype and phenotype. Separation of 25 cases showing microsatellite instability, 28 cases with high tumor-infiltrating lymphocytes or 19 cases showing both microsatellite instability and high tumor-infiltrating lymphocytes from the remaining small bowel carcinomas gave highly significant survival differences (hazard ratio: 0.22, 0.08–0.64,  $P=0.005$ ; hazard ratio: 0.09, 0.02–0.36,  $P<0.001$  and hazard ratio: 0.26, 0.12–0.57;  $P<0.001$ , respectively). Survival analysis of the 51 microsatellite stable or the 48 low tumor-infiltrating lymphocyte cases confirmed the importance of histological structure and phenotype with an improved survival of glandular vs diffuse/mixed/solid cases (hazard ratio: 0.28, 0.12–0.63,  $P=0.002$

among microsatellite stable cases and hazard ratio: 0.40, 0.18–0.87,  $P=0.022$  among low tumor-infiltrating lymphocyte cases, respectively) and of intestinal vs non-intestinal phenotype small bowel carcinoma cases (hazard ratio: 0.50, 0.23–1.08,  $P=0.080$  and hazard ratio: 0.41, 0.19–0.89,  $P=0.023$ , respectively).

### Small Bowel Carcinoma Expression of Nuclear $\beta$ -Catenin and SOX-9

As shown in Table 4 and Figures 4a and b, nuclear  $\beta$ -catenin expression was significantly concentrated within the celiac disease-associated small bowel carcinoma group, which differed significantly from the remaining small bowel carcinomas. Interestingly, N-terminally directed antibodies failed to recognize nuclear  $\beta$ -catenin in 17/40 (42%) tumors where the protein was easily detected in the nucleus by C-terminal antibodies. Both N- and C-terminal antibodies recognized membranous  $\beta$ -catenin in normal non-tumor mucosa of the same cases, thus suggesting the possibility of a tumor-selective N-terminal  $\beta$ -catenin loss. This was found to be unrelated to patient background disease and survival (Table 4). A relationship of our findings with the  $\beta$ -catenin gene deletion reported by Breuhahn *et al*<sup>27</sup> in some sporadic small bowel carcinomas seems likely. Similar to the nuclear  $\beta$ -catenin expression, the loss of MLH1 expression (which overlapped perfectly with molecularly assessed microsatellite instability status) was also highly and selectively concentrated among celiac disease-associated small bowel carcinomas ( $P=0.001$  vs remaining small bowel carcinomas). In fact, a correlation was found among the whole small bowel cancer series between the distribution of the two changes ( $P<0.001$ , Fisher's test).

The nuclear expression of SOX-9, another Wnt-related transcription factor, was significantly



**Figure 3** Phenotypic marker expression in small bowel carcinomas. (a) Uniform and intense nuclear positivity for the intestinal transcription factor CDX2 in a glandular-type celiac disease-associated carcinoma. (b) Luminal surface expression of the brush border marker CD10 in a celiac disease-associated carcinoma. (c) A sporadic small bowel carcinoma reactive for the intestinal marker CK20. (d) Signet ring cell Crohn's disease-associated carcinoma reactive for the goblet cell mucin MUC2. (e) A glandular Crohn's disease-associated carcinoma extensively expressing the gastric foveolar marker MUC5AC. (f) A mixed-type Crohn's disease-associated carcinoma reactive for the pancreatobiliary duct marker CK7.

correlated with that of  $\beta$ -catenin ( $P=0.005$ , Fisher's test) and was also highly represented among celiac disease-associated small bowel carcinomas (Figure 4c), although less selectively than  $\beta$ -catenin. Indeed, no

significant difference for SOX-9 expression was found among clinical groups. A trend for SOX-9 to be more extensively expressed in intramucosal or superficial than in deeply invasive tumor growths was noted.

Univariate survival analysis of the whole series showed a significantly better survival for patients bearing nuclear  $\beta$ -catenin-positive vs -negative small bowel carcinoma, whereas SOX-9 failed to reveal prognostic relevance. The association of nuclear  $\beta$ -catenin with more favorable outcome was retained in a stage-, age- and sex-inclusive multivariable model (hazard ratio: 0.32, 0.13–0.75;  $P=0.010$ ) or in a stage- and histotype-inclusive model (hazard ratio: 0.29, 0.13–0.65;  $P=0.003$ ), while losing significance only when clinical groups were added to the model. However, when survival of 20 small bowel carcinoma patients showing both microsatellite instability and nuclear  $\beta$ -catenin was compared with that of 20 cases showing nuclear  $\beta$ -catenin in the absence of microsatellite instability, the latter group had a worse outcome (hazard ratio: 7.14, 2.00–25.00;  $P=0.002$ ). In addition, among the 51 microsatellite stable cases, the 20  $\beta$ -catenin-positive

small bowel carcinoma cases lacked any survival difference compared with 31  $\beta$ -catenin-negative small bowel carcinoma patients (hazard ratio: 0.66, 0.31–1.43;  $P=0.296$ ).

### Small Bowel Carcinoma-Associated Dysplasia and Other Preneoplastic Changes

Evidence for residual adenomatous polypoid growth was obtained, within or adjacent to the superficial part of the neoplasm, in 10 of the 25 sporadic small bowel carcinomas, 8 of the 25 Crohn's disease-associated carcinomas and only 1 of the 26 celiac disease-associated carcinoma. Focal flat dysplasia, usually adjacent to the invasive cancer focus, was detected in 4 celiac disease-associated, 1 sporadic and 5 Crohn's disease-associated small bowel carcinomas (Figure 4d). Minute dysplastic foci were

**Table 4** Small bowel carcinoma molecular alterations: distribution among clinical groups and survival analysis

	Distribution among clinical groups			Total cases	Survival analysis	
	Celiac disease carcinomas	Crohn's disease carcinomas	Sporadic carcinomas		Hazard ratio (95% confidence interval)	P-value
Nuclear $\beta$ -catenin, C-terminal antibody	24/26 (92%) <sup>a</sup>	6/24 (25%)	10/25 (40%)	40/75 (53%)	0.30 (0.14–0.63)	0.002
Nuclear N-terminal $\beta$ -catenin loss	10/26 (38%)	2/24 (8%)	5/25 (20%)	17/75 (23%)	0.49 (0.22–1.12) <sup>b</sup>	0.091
Among C-terminal $\beta$ -catenin-positive cases	10/24 (42%)	2/6 (33%)	5/10 (50%)	17/40 (42%)	0.85 (0.24–2.97) <sup>c</sup>	0.796
SOX-9 expression	20/23 (87%)	11/22 (50%)	14/23(51%)	45/68 (66%)	0.70 (0.31–1.56)	0.380
Microsatellite instability <sup>d</sup>	17/26 (65%) <sup>e</sup>	4/25 (16%)	4/25 (16%)	25/76 (33%)	0.22 (0.08–0.64)	0.005
Among C-terminal $\beta$ -catenin-positive cases	17/24 (71%) <sup>a</sup>	1/6 (17%)	2/10 (20%)	20/40 (50%)	0.14 (0.04–0.50)	0.002

<sup>a</sup> $P < 0.001$  vs Crohn's disease-associated carcinomas or sporadic carcinomas.

<sup>b</sup>Vs remaining small bowel carcinomas.

<sup>c</sup>Vs remaining C-terminal  $\beta$ -catenin-positive cases.

<sup>d</sup>All 17 microsatellite unstable cases also had nuclear  $\beta$ -catenin among celiac disease-associated carcinomas; as against only one of the 4 among Crohn's disease-associated carcinomas and 2 of the 4 among sporadic carcinomas.

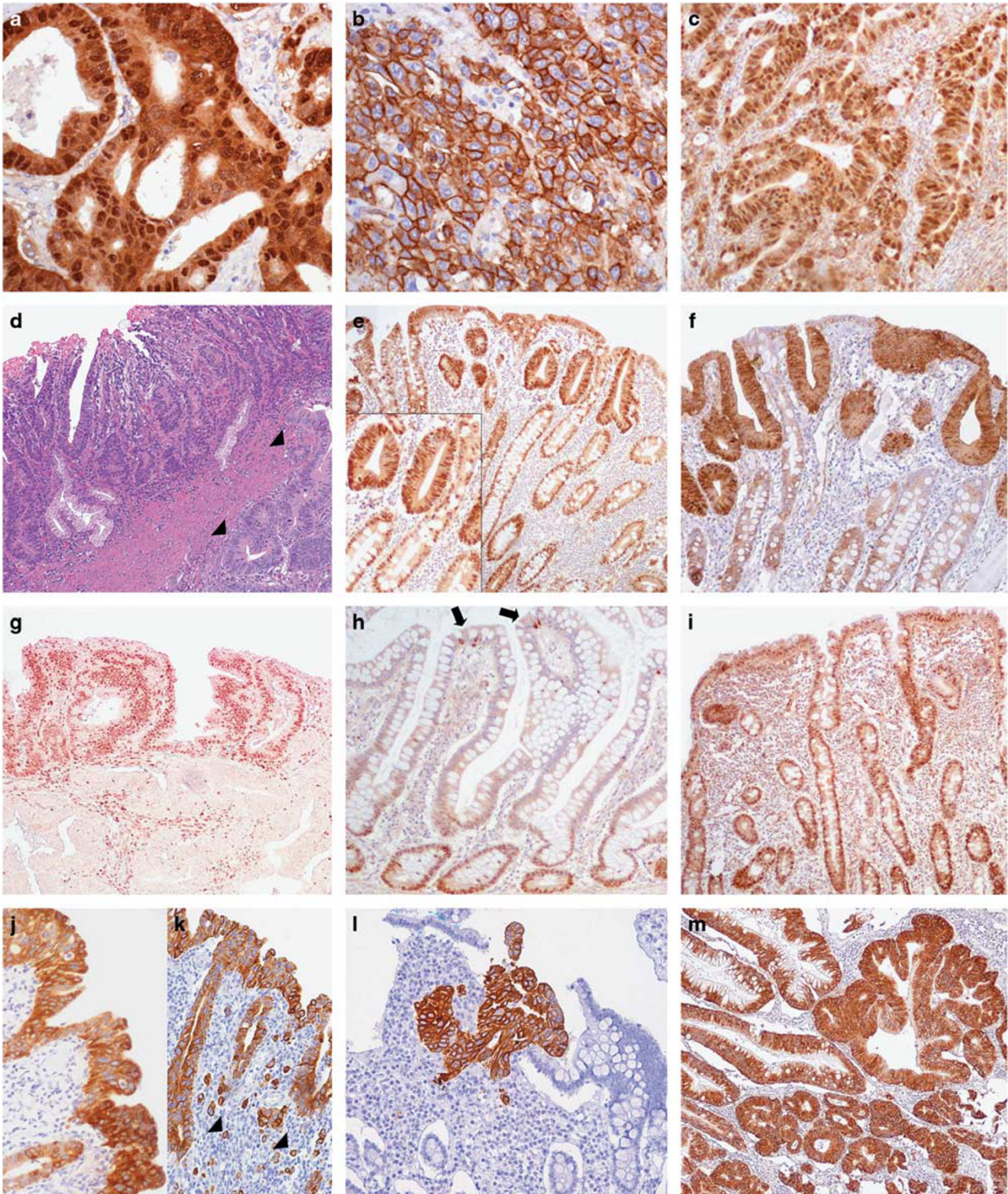
<sup>e</sup> $P = 0.001$  vs Crohn's disease-associated carcinomas or sporadic carcinomas.

**Figure 4** Molecular/cellular marker expression and preneoplastic changes. (a, b) Nuclear accumulation of  $\beta$ -catenin in a celiac disease-associated small bowel carcinoma (a), to be compared with a membranous/cytoplasmic reactivity of a Crohn's disease-associated small bowel carcinoma (b). (c) Intense SOX-9 expression in the tumor cell nuclei of a celiac disease-associated carcinoma. (d) Evidence for residual high-grade dysplasia overlying the superficial part of an invasive celiac disease-associated small bowel carcinoma (arrowheads). (e, f) Nuclear SOX-9 (e) and  $\beta$ -catenin (f) expression in a flat, low-grade, dysplastic lesion of a celiac disease-associated carcinoma case. Note the villous atrophy and, in the inset of panel (e), the extensive nuclear SOX-9 reactivity of hyperplastic crypts adjacent to dysplasia. (g) Same celiac disease-associated small bowel carcinoma case as in panel (d) showing discrepancy in MLH1 expression between the dysplastic (positive) and the underlying, invasive (negative) components. (h) A normal jejunal mucosa adjacent to a sporadic small bowel carcinoma with nuclear SOX-9 expression restricted to the deep-half of the crypts. Note a few enteroendocrine cells with SOX-9-reactive cytoplasm and unreactive nucleus (arrows). (i) SOX-9 nuclear expression in the atrophic mucosa distant from a celiac disease-associated small bowel carcinoma, showing a prominent SOX-9 expansion to involve the upper half of the crypts, the crypt/villous junction and even the surface epithelium. (j) A focus of MUC5AC-positive, high-grade dysplasia of a Crohn's disease-associated small bowel carcinoma case. (k) A dysplastic lesion and an underlying small focus of diffuse type Crohn's disease-associated small bowel carcinoma (arrowheads), both concordantly reactive for CK7. (l) An isolated, 'microscopic' CK7-positive neoplastic lesion with focal stromal invasion in the small bowel mucosa distant from a 'macroscopic' Crohn's disease-associated small bowel carcinoma. (m) Diffuse and intense MUC5AC positivity of a polypoid adenomatous dysplasia adjacent to a Crohn's disease-associated small bowel carcinoma.



found, in addition, associated with mucosal metaplastic changes in 3 Crohn's disease-associated carcinomas (see below). Total dysplastic lesions are summarized in Table 5, where it appears that nuclear SOX-9 (Figure 4e), found in 23/28 (82%) of cases investigated, is the molecular marker most widely

expressed in dysplasia, irrespective of clinical group, followed by p53 overexpression (15/29, 52%) and  $\beta$ -catenin (10/29, 34%) (Figure 4f), with only 2 of the 29 (7%) dysplastic lesions showing MLH1 loss. On the contrary, the latter change occurred in 24/76 (32%) carcinomas, including 5 of the cases retaining



**Table 5** Analysis of dysplastic and metaplastic changes associated with small bowel carcinomas

	<i>Celiac disease carcinomas</i>	<i>Crohn's disease carcinomas</i>	<i>Sporadic carcinomas</i>	<i>Total</i>
<i>Dysplasia</i>	5/26 (19%) <sup>a</sup>	16/25 (64%) <sup>b</sup>	11/25 (44%) <sup>c</sup>	32/76 (42%)
Metaplastic phenotype <sup>d</sup>	0/5 (0%)	14/15 (93%)	2/10 (20%)	16/30 (53%)
β-Catenin nuclear expression	5/5 (100%)	0/13 (0%)	5/11 (45%)	10/29 (34%)
SOX-9 expression	5/5 (100%)	10/13 (77%)	8/10 (80%)	23/28 (82%)
MLH1 loss	1/5 (20%)	1/14 (7%)	0/10 (0%)	2/29 (7%)
p53 overexpression	4/5 (80%)	7/15 (47%)	4/9 (44%)	15/29 (52%)
<i>Non-dysplastic mucosa</i>				
Metaplastic phenotype <sup>d</sup>	2/25 (9%)	19/22 (86%) <sup>e</sup>	0/24 (0%)	21/71 (30%)

<sup>a</sup>Polypoid in one and flat in four cases.

<sup>b</sup>Polypoid in eight, flat in five and minute foci in metaplasia in three cases.

<sup>c</sup>Polypoid in 10 and flat in 1 case.

<sup>d</sup>Metaplastic phenotype was defined as extensive MUC5AC and/or cytokeratin 7 expression.

<sup>e</sup>*P* < 0.001 vs either celiac disease-associated carcinomas or sporadic carcinomas.

MLH1 staining in the corresponding dysplasia (Figure 4g). This finding outlines a relatively late appearance of microsatellite instability status during carcinogenesis.

Diffuse or patchy celiac disease lesions, ie, goblet-cell poor crypt hyperplasia plus increased intraepithelial lymphocytes, either isolated (5/25) or associated with villous atrophy (17/25), were seen in the majority (22/25, 88%) of celiac disease-associated small bowel carcinomas, both in cancer-adjacent and cancer-distant small bowel mucosa. Notably, nuclear SOX-9 expression, normally restricted to the deep lower half of crypts (Figure 4h), showed a prominent expansion to involve the upper half of the crypts in non-tumor mucosa of all 19 celiac disease-associated small bowel carcinomas investigated, sometimes reaching the crypt/villous junction or even to the superficial epithelium covering the flattened mucosa resulting from villous atrophy (Figure 4i). Care was taken to discard chromogranin-A reactive cells, usually showing SOX-9-positive cytoplasm in the absence of nuclear staining and scattered as single elements along the whole crypt–villous unit (Figure 4h). Interestingly, direct continuity of SOX-9-reactive crypt hyperplasia with dysplasia and intramucosal neoplasia was consistently seen (Figure 4e). No relevant change of nuclear β-catenin expression, which remained restricted to very few cells interposed with Paneth cells within the deepest crypts, was observed in SOX-9-positive crypt hyperplasia. However, intense nuclear β-catenin appeared in adjacent dysplastic or neoplastic lesions (Figure 4f).

Although no preneoplastic change was identified in non-tumor mucosa of sporadic small bowel carcinomas, metaplastic changes showing the expression of the gastric foveolar marker MUC5AC and/or the ductal pancreatobiliary marker CK7 were extensively represented (86%) in the chronically inflamed, non-neoplastic mucosa associated with Crohn's disease-associated small bowel carcinomas, sometimes coupled with minute dysplastic or neoplastic foci

(Figure 4j). Notably, 93% of the overt dysplastic lesions associated with Crohn's disease-associated carcinomas showed the expression of CK7 and/or MUC5AC; moreover, the gastro-pancreatobiliary markers were concordantly expressed in metaplastic, dysplastic and invasive components of all but one of the 15 Crohn's disease-associated small bowel carcinomas investigated (Figures 4k–m).

## Discussion

In this study of small bowel carcinoma, retention of intestinal phenotype and gland-forming capacity by tumor cells was associated with better patient survival compared with acquisition of non-intestinal (metaplastic) phenotype or non-glandular histological structure. In addition, a small group of medullary-type microsatellite instability/high tumor-infiltrating lymphocyte cancers, found to have a good prognosis in accordance with previous studies,<sup>28–30</sup> were distinct from solid non-medullary cancers. As a previous study<sup>7</sup> showed that both high tumor-infiltrating lymphocyte density and microsatellite instability status were associated with improved survival, the prognostic power of tumor histology and phenotype was confirmed in the microsatellite stable and/or low tumor-infiltrating lymphocyte subpopulations of small bowel carcinoma patients. The prognostic power of glandular histology was also found to retain significance in a multivariable model inclusive of stage, clinical group and tumor-infiltrating lymphocyte density (or microsatellite instability status).

When substituted for glandular histology in the same model, the intestinal phenotype lost prognostic power, which, however, was retained when the clinical groups were eliminated from the model. It should be noted that the group-independent behavior of the glandular histotype fits with its relatively uniform distribution among the groups, while the group-sensitive behavior of the phenotype couples with the high, selective expression of non-intestinal

markers associated with worse prognosis among Crohn's disease-associated small bowel carcinomas. The latter finding may well contribute to the poor survival shown by Crohn's disease-associated small bowel carcinoma patients. Interestingly, the worse survival of Crohn's disease-associated carcinoma cases reached full statistical significance only with respect to celiac disease-associated carcinoma cases but not to sporadic carcinoma cases. Indeed, the microsatellite instability/high tumor-infiltrating lymphocytes pattern of celiac disease-associated carcinoma is likely to amplify its intestinal phenotype-dependent survival improvement with respect to Crohn's disease-associated small bowel carcinoma cases.

The histotype and phenotype approaches proposed here offer new tools for small bowel carcinoma prognostic evaluation, and these should be added to the clinical group characterization and microsatellite instability status suggested by our previous investigations.<sup>7</sup> Notably, while both histotype and phenotype proved to be prognostic factors independent of microsatellite instability and tumor-infiltrating lymphocyte density, only histotype was fully independent of clinical groups.

As expected, all small bowel carcinomas with medullary-type histology, ie, a solid structure with well-demarcated 'pushing' borders, had high tumor-infiltrating lymphocytes. Five of the six cases also had microsatellite instability status as a likely cause of their increased tumor-infiltrating lymphocyte density. Given the difficulty in separating, on purely histological grounds, gut medullary from lymphoepithelioma-like cancer,<sup>31</sup> often associated with Epstein-Barr virus infection, a separate investigation tested the single microsatellite stable case with Epstein-Barr virus-encoded small RNAs *in situ* hybridization, finding it to be diffusely positive.<sup>32</sup>

Similar to microsatellite instability, the nuclear accumulation of  $\beta$ -catenin was also found to be highly prevalent among celiac disease-associated small bowel carcinomas and to be apparently associated with improved survival. This seemed surprising as, unlike microsatellite instability, nuclear  $\beta$ -catenin expression has been reported by studies of other neoplasms to imply a less than favorable prognostic influence.<sup>33-35</sup> However, when among  $\beta$ -catenin-positive small bowel carcinomas, microsatellite unstable and microsatellite stable cases were compared or among microsatellite stable carcinomas,  $\beta$ -catenin-positive and -negative cases were compared, the lack of favorable prognostic influence of  $\beta$ -catenin itself, in the absence of microsatellite instability, became evident. The distributive association we found, especially among celiac disease-associated carcinomas, between nuclear  $\beta$ -catenin expression and the prognostic favorable microsatellite instability status, might account for this misleading prognostic influence of  $\beta$ -catenin. Notably, such an association is at variance with colorectal cancer findings, where nuclear

$\beta$ -catenin expression, highly prevalent among microsatellite stable cases, due to *CTNNB1* or *APC* point mutations, has been reported to be rare in microsatellite unstable cases.<sup>36</sup> Instead of point mutations, large *CTNNB1* N-terminal deletions have been reported to stabilize  $\beta$ -catenin in 20% of sporadic small bowel carcinomas,<sup>27</sup> a finding we confirmed by selective N- vs C-terminal  $\beta$ -catenin immunohistochemistry in our sporadic small bowel carcinomas and extended to about 40% of celiac disease-associated small bowel carcinomas. Whether additional stabilization mechanisms may account for the very high celiac disease-associated small bowel carcinoma rate of nuclear  $\beta$ -catenin (around 90%) and for its association with microsatellite instability status, it remains to be further investigated. On the other hand, the lack of *BRAF* mutations and the lack of correlation between *KRAS* mutations and microsatellite instability found in previous small bowel carcinoma studies<sup>5,7</sup> rule out a role of these oncogenes in inducing *MLH1* gene methylation and, consequently, microsatellite instability status, as has been shown to occur in colorectal cancers.<sup>37,38</sup> Thus more work is needed to explain the unusual association between microsatellite instability status and Wnt activation among celiac disease-associated small bowel carcinomas.

We found that another Wnt-related protein, the SOX-9 transcription factor, was also highly expressed in small bowel carcinomas, with a distribution comparable to that of nuclear  $\beta$ -catenin, at least among celiac disease-associated small bowel carcinomas. This finding seems relevant, as SOX-9 high expression has been reported in a number of cancers, including colorectal, gastric, pancreatic, hepatocellular, brain, lung and prostate cancers.<sup>39</sup> In some of these cancers, SOX-9 expression was found to be associated with tumor progression, invasion and metastasis, a pattern not seen in our small bowel carcinomas. However, an important SOX-9 role in carcinogenesis has also been proposed,<sup>39</sup> at least in part through Wnt activation via frizzled and LRP receptor overexpression.<sup>40,41</sup> In keeping with this hypothesis, SOX-9 overexpression has also been reported in some precancerous conditions, including metaplastic and dysplastic lesions of chronic *Helicobacter pylori* gastritis,<sup>42,43</sup> chronic bladder injury<sup>44</sup> and early stages of colorectal tumorigenesis,<sup>39</sup> as well as in 'acute' untreated adult celiac disease.<sup>45</sup>

This led us to investigate non-tumor mucosa of small bowel carcinoma-bearing patients for SOX-9 expression. In most celiac disease-associated small bowel carcinoma cases, we frequently observed signs of persistent mucosal damage, including atrophy of the villi, excessive intraepithelial T lymphocyte infiltration and goblet cell-poor and relatively immature crypt hyperplasia, not unlike those reported in biopsies of some non-neoplastic adult celiac disease patients, even when under gluten-free diet.<sup>46</sup> Notably, multifocal extension of nuclear SOX-9 expression, usually involving the upper half

of the hyperplastic crypts was found in most celiac disease cases investigated, even in the mucosa at a distance from the neoplasm. By itself, the topographic continuity we observed at some foci between SOX-9-positive crypt hyperplasia and intramucosal dysplastic or cancerous growths may be suggestive for a histogenetic link between such lesions.

A role for SOX-9 in the mucosa repair process of persistent celiac disease lesions seems conceivable considering its known role in intestinal mucosal repair from other types of damage.<sup>47</sup> The SOX-9-activating role of NF- $\kappa$ B transcription factor, as ascertained, for instance, in experimental *H. pylori* gastritis,<sup>43</sup> seems especially important. Indeed, NF- $\kappa$ B activity, in turn activated by pro-inflammatory cytokines, has been shown to be enhanced in many immune-inflammatory processes, including inflammatory bowel diseases and related carcinogenesis,<sup>48</sup> as well as celiac disease.<sup>49</sup> In fact, a switch from the known SOX-9 role in adult stem cell modulation to cancer stem cell activation, often chronic injury promoted and involving constitutive oncogene activation or oncosuppressor gene silencing, has been suggested by several studies.<sup>39,44,50–52</sup>

Our evidence of polypoid adenomatous remnants in the superficial part of Crohn's disease-associated and sporadic small bowel carcinomas supports the hypothesis that the adenoma-carcinoma sequence, well known from colorectal neoplasms, is also operative for these two types of small bowel carcinoma.<sup>14,15,53</sup> In celiac disease-associated carcinomas, we obtained evidence for a possible adenomatous polyp origin in only a single case, thus confirming the previous finding<sup>54</sup> of a substantial lack of such lesions in celiac disease cases, while we found four celiac disease-associated carcinomas with flat dysplasia reactive for both nuclear  $\beta$ -catenin and SOX-9.

Metaplastic changes showing the same gastric and/or pancreatobiliary phenotype as found in the associated cancer were frequent in dysplastic or non-dysplastic mucosa adjacent to Crohn's disease-associated carcinomas, though not to celiac disease-associated or sporadic carcinomas. This finding may suggest a precancerous role of such lesions in Crohn's disease. Thus two distinct histogenetic processes seem at work for celiac disease-associated and Crohn's disease-associated small bowel carcinomas, starting from immature crypt hyperplasia or epithelial metaplasia, respectively.

Although a more extensive, prospective and systematic search for hyperplastic, metaplastic and dysplastic lesions is warranted for a better understanding of small bowel carcinoma histogenesis in the two types of immune-inflammatory conditions we studied, the present findings may indicate promising lines of investigation to identify preneoplastic lesions of potential help in early cancer diagnosis.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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