Small bowel adenocarcinomas in celiac disease follow the CIM-MSI pathway

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Abstract. Celiac disease (CD) is an inflammatory disorder associated with an increased risk of small bowel adenocarcinoma. Recent studies have demonstrated aberrant CpG island methylation (CIM) in chronic inflammation, aging and cancer. We hypothesized that CIM may link CD to small bowel carcinogenesis. We determined microsatellite instability (MSI), CIM, and expression of MLH1 and MGMT in 3 CD-associated small bowel carcinomas and corresponding non-neoplastic mucosa. The results were compared to those of small bowel mucosa from CD patients without carcinoma and 20 small bowel carcinomas from a non-CD origin. A high level CIM/MSI phenotype was found in all of the 3 CD-associated carcinomas and was associated with loss of MLH1 expression due to hypermethylation of the *MLH1* promoter. This phenotype was noted in only 2 of the 20 investigated non-CD-associated carcinomas. Low-level CIM was already detectable in 9 of the 12 non-neoplastic mucosa samples of CD patients and in non-CD-associated carcinomas of elderly patients. In conclusion, our data reveal that the high-level CIM/MSI pathway is typical of CD-associated small bowel carcinomas and indicate that aberrant CpG island methylation links CD and carcinogenesis. The data further suggest that CD should be considered in patients with small bowel adenocarcinoma, particularly when the tumors display MSI.

Introduction

Celiac disease (CD) is an inflammatory disorder caused by intolerance to gluten. Previously considered a disease of early infancy, late age of clinical onset and diagnosis is currently well recognized (1,2). In CD patients, exposure to gluten results in chronic inflammation of the small bowel mucosa characterized by intraepithelial lymphocytosis, crypt hyperplasia and villous atrophy (3,4). CD is a well-established risk factor for small bowel lymphoma (5-7). In several recent studies on CD patients, the association with small bowel adenocarcinomas (SBAs) was also investigated, and a relative risk of 10- to 80-fold for this tumor type was reported for CD (6,8,9). Given the comparatively high, yet not precisely determined, prevalence of CD in various populations (0.15-2.6%) (10), one would expect a significant share of CD-associated SBAs in a larger collection of these tumors. The results of two studies concerning this issue, however, are conflicting. While Howdle *et al* (9) reported 13% of SBAs collected in a nation-wide survey in the UK to be CD-associated (9), no signs of CD were reported among 69 SBAs collected in Northern Ireland, an area of high CD prevalence (11).

The association of gluten-induced chronic inflammation with small bowel tumorigenesis is reminiscent of colon cancer in inflammatory bowel disease. Aberrant methylation at CpG islands (CIM) is an epigenetic phenomenon originally described in cancer (12,13), but CIM has also previously been reported in association with non-neoplastic conditions including aging and chronic inflammation (14-16). Since CIM can contribute to carcinogenesis through transcriptional silencing of tumor-suppressor genes, it may provide an explanation for the well-known association of aging and chronic inflammation with tumor risk.

We investigated a large cohort of consecutively collected SBAs for the presence of CD and analyzed the molecular pathogenesis of CD-associated carcinomas in comparison to non-CD-associated carcinomas. Herein, we showed that in contrast to non-CD-associated SBAs, all CD-associated carcinomas followed a molecular pathway characterized by high-level CIM, *MLH1* inactivation and microsatellite instability (MSI). Our data indicate CD-associated carcinogenesis to be a sequential process preceded by CIM, initiated by methylation-induced *MLH1* silencing and promoted by MSI.

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Materials and methods

A consecutive cohort of 70 primary SBAs collected over a time-span of 20 years were retrieved from our Institute of Pathology. Histologic sections were reviewed for the presence of CD. Diagnosis of CD was based on CD typical morphology of non-neoplastic mucosa and on clinical information. Three patients with CD-associated SBAs were identified. In one patient CD was diagnosed in early childhood; in the other two cases, CD was diagnosed at the time of tumor resection. In one case a small bowel resection for anaplastic T-cell lymphoma was performed 4 years before small bowel carcinoma resection; in the other 2 cases, no synchronous or metachronous tumors occurred.

Twenty non-CD-associated carcinomas previously analysed for MSI (17) [4 MSI-H, 16 microsatellite stable (MSS)], non-neoplastic mucosa of 5 non-CD carcinoma cases as well as 9 biopsy specimens of CD patients without carcinoma were chosen for the comparative study of methylation. Clinical data for the individual cases is documented in Table I together with the results of methylation and MSI analysis. The study was approved by the Institutional Ethics Committee (application no. 206/05) of the Medical Faculty of Heidelberg University.

DNA isolation. For isolation of DNA from tumor and non-neoplastic mucosa, tissue slides (5-μm) were prepared. Tumor tissue and normal mucosa were isolated by manual microdissection of unstained slides. DNA from non-neoplastic mucosa was taken from the resection margins whenever possible (2 of the 3 CD-associated SBAs and in 4 of 5 non-CD-associated SBAs) or was taken at least a distance of 0.5 cm from the tumor. DNA was prepared using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

MSI analysis. Tumor samples were typed for MSI using the standard NCI/ICG-HNPCC marker panel (18) and CAT25 as described previously (19).

Methylation-specific PCR. Methylation-specific (MSP) PCR was performed for 5 CpG islands known to be methylated in colorectal cancer: *MINT1*, *MINT2*, *MGMT*, *MLH1* and *p16* (*CDKN2A*). Primers and PCR conditions were used as described previously [MINT1 and MINT2 (20); MGMT (21); p16 (22); MLH1 (23)]. DNA was bisulfite-converted using the EZ DNA Methylation-Gold kit (Zymo Research, USA) according to the manufacturer's instructions. One-fifth of a conversion reaction was used as template.

PCR products were visualized on a 2% agarose TAE gel. Methylation status was assessed based on the presence or absence of amplified product in the MSP PCR. CIM-high, CIM-low and CIM-negative were scored when more than 50%, 20-50% and 0% of the investigated loci were positive for MSP PCR, respectively.

Immunohistochemistry. To compare the methylation status of *MLH1* and *MGMT* with protein expression, immunohistochemistry was performed on 5-μm paraffin sections. For the mismatch repair protein MLH1 (clone G168-15, BD Pharmingen, Heidelberg, Germany; dilution 1:100) and MGMT (clone MT3.1, Thermo Scientific, Fremont, CA, USA; dilution 1:30) slides were pre-treated with 10-min microwave boiling in Dako target retrieval buffer, pH 9.0 (Dako, Hamburg, Germany). The antibody was incubated for 30 min at room temperature. For visualization purposes, the labeled immunoperoxidase method with AEC as a chromogen was applied (Dako). In non-neoplastic mucosa, loss of MGMT expression was scored when all cells within a crypt or groups of crypts were negative for nuclear staining while stromal cells were positive. In tumors, loss of MGMT and MLH1 expression was scored when all tumor cells were negative while the stromal cells and adjacent non-neoplastic mucosa were positive.

Results

Microsatellite instability in CD- and non-CD-associated SBAs. MSI was detected in all of the 3 CD-associated adenocarcinomas. Among the 20 non-CD-associated adenocarcinomas used for the comparative study of methylation, MSI-H was noted in 4 tumors. All three CD-associated adenocarcinomas displayed loss of MLH1 expression (Fig. 1), and all of the 4 MSI-H carcinomas in the non-CD carcinoma cohort were MLH1-negative.

CpG island methylation in SBAs and non-neoplastic mucosa of patients with and without CD. All 3 CD-associated SBAs and 5 of the 20 non-CD-associated SBAs were CIM-high. Eight of the 20 non-CD-associated SBA were CIM-low, and 7 of 20 were CIM-negative. CIM was related to patient age. While the average age of the patients with CIM-negative carcinomas was 55 years, it was 73 years for patients showing CIM. CIM-high with *MLH1* methylation and loss of MLH1 expression was found in all 3 of the CD-associated SBAs and in 2 of the 4 MSI-H non-CD-associated SBA. CIM-high without *MLH1* methylation was found in 3 MSS SBAs. CIM-low with *MLH1* methylation was noted in 2 of the MSI-H non-CD-associated carcinomas. The results of the methylation analyses in individual cases and at the tested loci are summarized in Table I.

Non-neoplastic mucosa. CIM-low was identified in the non-neoplastic mucosa of all of the CD-associated SBA cases (Table I, Fig. 1). CIM-low was also identified in 6 of the 9 CD mucosa samples not associated with SBAs as well as in 4 of the 5 non-neoplastic mucosa samples of the non-CD-associated carcinomas. No methylation of *MLH1* or *CDKN2A* was noted in the non-neoplastic mucosa specimens. *MINT1* was methylated in 5 of the 9 CD mucosa samples and in 3 of the 5 non-CD mucosa samples. *MINT2* methylation was found in 2 CD and in 2 non-CD mucosa samples. *MGMT* methylation was observed in 1 non-CD mucosa sample.

Except for case 401, where *MGMT* methylation was exclusively found in non-neoplastic mucosa, methylation of a specific locus in non-neoplastic mucosa was also found in the corresponding tumor tissue (Table I).

Expression of MLH1 and MGMT. Loss of MLH1 expression was found in all of the 3 CD-associated carcinomas and it

Table I. Clinical data, tumor location, methylation and microsatellite status of the investigated carcinomas.

Case	Location	Age/gender	MGMT	MINT1	MINT2	P16	MLH1	MSI/MSS
Non-neoplastic mucosa and tumors in celiac disease								
N884				■				
T884	Duo	47/female	■	■	■	■	■	MSI-H
N418			■	■				
T418	Jej/Ile	57/male	■	■	■	■	■	MSI-H
N332				■	■			
T332	Jej/Ile	59/male		■	■	■	■	MSI-H
Non-neoplastic mucosa and tumors without celiac disease								
N401			■	■				
T401	Jej/Ile	59/male		■				MSS
N174				■	■			
T174	Duo	61/male	■	■	■	■		MSS
N180								
T180	Duo	64/male						MSS
N343				■				
T343	Ile	83/male	■	▒				MSS
N376					■			
T376	Jej/Ile	81/female	■	■	■	■	■	MSI-H
Tumors without celiac disease								
T108	Duo	71/female	■	■	■	■	■	MSI-H
T410	Duo	74/female		■			■	MSI-H
T172	Ile	84/male		■			■	MSI-H
T361	Ile	71/male	■	■	■	■		MSS
T656	Jej/Ile	75/male	■	■	■	■	▒	MSS
T820	Ile	72/female			■	■		MSS
T970	Duo	74/male		■				MSS
T249	Ile	80/female			■			MSS
T303	Duo	76/male	■					MSS
T246	Ile	29/male						MSS
T502	Jej/Ile	30/male					▒	MSS
T208	Duo	54/female					▒	MSS
T235	Jej/Ile	68/female						MSS
T486	Ile	69/female						MSS
T195	Duo	76/male						MSS
Marsh			Non-neoplastic mucosa in celiac disease					
CD142	II-III	62/male		■				
CD743	0	62/female		■				
CD359	III	53/female		■	■			
CD505	III	45/female		■				
CD507	III	46/female			■			
CD418	III	72/male		■				
CD271	III	46/female						
CD417	0	40/female						
CD413	I	45/male						

Black squares, methylation; blank squares, no methylation; grey squares, not determined.

correlated well with a strong PCR signal for the methylated DNA at the *MLH1* promoter while the corresponding PCR signal for non-methylated DNA was faint or absent (Fig. 1). MLH1 expression was retained in the surrounding non-neoplastic mucosa of the CD cases where no *MLH1* methylation was found. Loss of MLH1 expression together with *MLH1* methylation was present in all 4 MSI-H non-CD carcinomas (17). *MGMT* methylation correlated with loss of MGMT expression in the CD-associated SBAs and in the non-neoplastic mucosa of CD case 418 (Fig. 1). Loss of MGMT expression was noted in 2 non-CD carcinomas and methylation of *MGMT* was found in both (T361, T343). Five non-CD carcinomas with *MGMT* methylation showed retained expression of MGMT indicating incomplete *MGMT*-silencing by promoter methylation.

Discussion

Among a cohort of 70 consecutively collected small bowel adenocarcinomas, we identified 3 cases associated with CD (4.3%). Given a CD prevalence of 0.5% in the German population (10) our data are in line with a nation-wide survey in the UK (9), where CD is approximately twice as common as in Germany (24). In this study, Howdle *et al* (9) reported

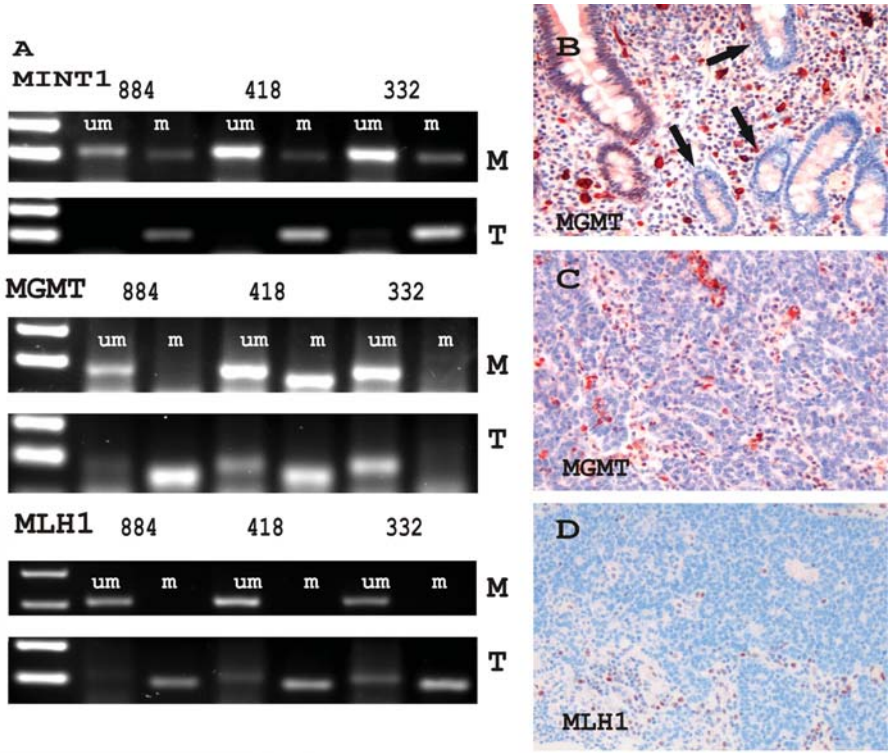

Figure 1. Methylation analysis of CD-associated carcinomas (T) and corresponding non-neoplastic mucosa (M) for MINT1, MGMT and MLH1 (A). Methylation (m) of MINT1 is noted in all of the cases of non-neopolastic mucosa and methylation of MGMT in the non-neoplastic mucosa of 418. No MLH1 methylation is noted in the non-neoplastic mucosa but in all of the tumors with a very faint amplification of the unmethylated allele indicating biallelic methylation. Methylation of *MGMT* correlates with loss of protein expression in singular crypts of the non-neoplastic mucosa (N418; B, arrows). Methylation of MGMT and MLH1 correlate with complete loss of MGMT (C) and MLH1 (D) expression in the tumor cells while stromal cells and lymphocytes retain protein expression (T418; C and D).

13% CD-associated tumors among 169 primary small bowel adenocarcinomas. However, in a similar study in Northern Ireland, in which pathology reports were reviewed for villous atrophy, CD was identified in 18% of small bowel lymphomas but in none of the 69 adenocarcinomas. The reason for this disparity is not clear, but some explanations should be considered. On the one hand, the infiltrative and hyperplastic types of CD (MARSH types 1 and 2) lack significant changes in villous architecture. Therefore, absence of villous atrophy does not rule out CD. On the other hand, CD's association with SBA is less known to pathologists and clinicians than its association with lymphoma, the enteropathy-type lymphoma in particular. There is no tumor entity defined as enteropathy type-associated adenocarcinoma. When assessing a resection specimen of small intestinal adenocarcinoma, subtile inflammatory alterations of CD may remain unrecognized by the pathologist.

Diagnosis of CD in SBA is of clinical importance in order to avoid misinterpretation of postoperative malabsorption and to prevent CD-associated complications by a gluten-free diet (25). The small bowel lymphoma risk in CD significantly decreases with successful treatment (26). This may not be the case for the risk of SBA in CD. Kingham *et al* (27) reported the occurrence of CD-associated SBA despite the remission of CD under a gluten-free diet. Corroborating this finding, one of the patients presented herein developed SBA although CD diagnosed in early childhood was successfully treated.

SBA is rare (incidence rate 2, 3/100,000, SEER), and compared to colorectal cancer its occurrence is more likely to be related to a cancer-prone condition. Apart from CD and Crohns's disease, FAP and HNPCC are well-established risk factors (28-30). Young age of onset and detection of MSI are typical for the development of SBA in the setting of HNPCC. Our data and the findings of two other studies (31,32) identifying MSI in 67 and 73% of CD-associated carcinomas, respectively, show that these features also occur in CD-associated SBAs. Therefore, CD should be included in the differential diagnosis of SBAs exhibiting MSI.

Aberrant methylation at CpG islands (CIM) is an epigenetic phenomenon found in cancer and non-neoplastic conditions including chronic inflammation and aging (14-16). By evaluating the methylation status in CD-associated carcinomas and non-neoplastic mucosa samples, we aimed to identify a molecular link between CD-associated inflammation and carcinogenesis. In line with a causative role of CD for aberrant methylation, CIM was identified in the majority of the non-neoplastic mucosa samples of the CD patients and in all of the CD-associated carcinomas investigated herein. While methylation at the *MINT* and *MGMT* loci was noted in both non-neoplastic and neoplastic tissues, *MLH1* methylation and loss of MLH1 expression were exclusively found in carcinomas of the CD patients indicating *MLH1* methylation to be the critical step in CD-associated carcinogenesis. CIM, however, was not found to be specific for CD. We detected low-level CIM in the majority of non-CD tissues investigated,

indicating alternative causes of CIM. The average age of patients with CIM-negative and CIM-positive non-CD carcinomas was 55 and 73 years, respectively, supporting the hypothesis of aberrant methylation occurring in the course of aging (14). High-level CIM, methylation-induced silencing of *MLH1* and MSI were found in 2 non-CD carcinomas and were not specific for CD-associated SBAs. Detection of this pathway in all of the CD-associated SBAs in the present study, however, provide evidence for a link of this epigenetically initiated pathway to chronic inflammation.

In conclusion, we confirmed CD as a risk factor for SBA and further demonstrated the high-level CIM/MSI pathway to be typical of CD-associated small bowel carcinomas. Our findings suggest that CD should be considered in patients with small bowel adenocarcinoma, particularly when the tumors display MSI.

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