Genetics and epigenetics of small bowel adenocarcinoma: the interactions of CIN, MSI, and CIMP

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Characterization of tumor genetics and epigenetics allows to stratify a tumor entity according to molecular pathways and may shed light on the interactions of different types of DNA alterations during tumorigenesis. Small intestinal adenocarcinoma is rare, and to date the interrelation of genomic instability and epigenetics has not been investigated in this tumor type. We therefore analyzed 37 primary small bowel carcinomas with known microsatellite instability and *KRAS* status for chromosomal instability using comparative genomic hybridization, for the presence of aberrant methylation (CpG island methylation phenotype) by methylation-specific polymerase chain reaction, and for *BRAF* mutations. Chromosomal instability was detected in 22 of 37 (59%) tumors (3 of 9 microsatellite instable, and 19 of 28 microsatellite stable carcinomas). Nine carcinomas (24%) were microsatellite and chromosomally stable. High-level DNA methylation was found in 16% of chromosomal instable tumors and in 44% of both microsatellite instable and microsatellite and chromosomally stable carcinomas. *KRAS* was mutated in 55, 0, and 10% of chromosomal instable, microsatellite instable, and microsatellite and chromosomally stable tumors, respectively whereas the frequencies of *BRAF* mutations were 6% for chromosomal instable and 22% for both microsatellite instable and microsatellite and chromosomally stable carcinomas. In conclusion, in this study we show that chromosomal instable carcinomas of the small intestine are distinguished from microsatellite instable and microsatellite and chromosomally stable tumors by a high frequency of *KRAS* mutations, low frequencies of CpG island methylation phenotype, and *BRAF* mutations. In microsatellite instable and microsatellite and chromosomally stable cancers, CpG island methylation phenotype and *BRAF/KRAS* mutations are similarly distributed, indicating common mechanisms of tumor initiation or progression in their molecular pathogenesis.

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Although the small intestine represents the largest segment of the gastrointestinal tract and is located between two areas of high cancer risk, the stomach and the colon, adenocarcinoma development is exceedingly rare in this location (incidence 1/100 000, SEER Cancer Statistics 2003–2007[1]). Small bowel adenocarcinoma and colorectal cancer show overlaps in morphology and hereditary and non-hereditary risk factors, including the hereditary non-polyposis colorectal cancer syndrome, familial adenomatous polyposis, and Crohn's disease.[2–4] Recent studies, however, provide evidence for a somewhat different molecular pathogenesis of the majority of sporadic carcinomas in both locations.[5–9]

Microsatellite and chromosomal instability (MSI and CIN) are two divergent types of genomic instability found in adenocarcinomas throughout the gastrointestinal tract, including the small intestine.[6,7,9–12] MSI is caused by inactivation of the DNA mismatch repair system that may either result from promoter hypermethylation of the DNA mismatch repair gene *MLH1* in sporadic carcinogenesis,[13] or following germline mutations in DNA mismatch

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repair system genes in hereditary nonpolyposis colorectal cancer.[14] CIN involves gains or losses of whole chromosomes and/or chromosomal fractions and is considered to result from defects in chromosomal segregation, telomere stability, and DNA damage repair.[15,16] In ~80% of colon cancers, CIN and/or MSI are found. A share of 20% of colorectal cancers, however, is microsatellite and chromosomally stable (MACS), and the molecular pathogenesis of these colorectal cancers is not completely understood.[17,18]

The CpG island methylation phenotype (CIMP) is an epigenetic phenomenon found in a variety of cancers.[19,20] In colorectal and gastric cancers, CIMP frequently associates with *MLH1* methylation, loss of *MLH1* transcription, and MSI-H.[19,21,22] An association of CIMP with *BRAF* mutations has been found in colorectal cancer[23] but, interestingly, mutations in *BRAF* do not have a significant role in either CIMP-positive or -negative gastric cancers.[24]

Analyses of genomic and epigenetic interrelations in human neoplasms have added largely to our understanding of tumor development and have led to a novel molecular classification of colorectal cancer.[25] As the pathogenesis of small bowel adenocarcinoma is not completely understood and interrelations of epigenetic and genetic alterations have not been investigated in this tumor type, we analyzed 37 primary small bowel adenocarcinomas stratified by MSI and *KRAS* status for chromosomal imbalances, CIMP, and the presence of *BRAF* mutations. In this study we show that the majority of small bowel adenocarcinomas display genomic instability (either CIN and/or MSI) and that a subset of 24% of tumors is microsatellite and chromosomally stable. We further demonstrate similar epigenetic alterations and relations of *BRAF/KRAS* mutations in MSI-H and MACS but not in CIN cancers, indicating MSI-H and MACS carcinogenesis to follow similar initializing or promoting epigenetic and genetic events.

Materials and methods

Tumor Samples

A total of 37 unselected primary adenocarcinomas of the small intestine, including 15 duodenal, 7 jejunal, and 7 ileal tumors, as well as 8 adenocarcinomas in small intestinal segment resection without specification of jejunal or ileal origin, were analyzed. Familial adenomatous polyposis coli, juvenile polyposis, and Peutz–Jeghers-syndrome were excluded using pathologic examination. One carcinoma (884) was associated with celiac disease and one (271) with Crohn's disease. The duodenal cancer (320) was diagnosed in a patient with hereditary nonpolyposis colorectal cancer. Hereditary nonpolyposis colorectal cancer was considered in two other patients with MSI-H carcinomas

(884, 235). In one of these patients, germline analysis of the *MLH1* gene (884) revealed no mutation. In the second patient, no germline analysis has been performed. Patient age ranged from 29 to 84 years (median age 64). The clinical data are given in Table 1. The study was approved by the institutional ethics committee (application no. 206/05) at the Medical Faculty at Heidelberg University.

DNA Isolation

For the isolation of DNA from tumor samples for methylation-specific PCR, as well as for mutational analysis of *BRAF*, tissue slides of 5-μm thickness were prepared. Tumor tissue was isolated under microscopic control of unstained slides by manual microdissection. For comparative genomic hybridization (CGH), areas of high tumor cell concentration (>70%) were marked on the slide and the corresponding area was removed from the tumor block with the help of a 0.2 mm punch needle. For CGH and PCR analyses, DNA was prepared using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

MSI Analysis

Tumor samples had previously been typed for MSI as described.[26] In brief, DNA isolated from the tumors and non-neoplastic normal mucosa was amplified. MSI analysis was performed using the standard NCI/ICG-hereditary nonpolyposis colorectal cancer marker panel[27] and CAT25.[28] PCR products were separated on an ABI3700 automated sequencer (Applied Biosystems, Warrington, UK). Tumors were classified as MSI-H if at least two of the markers displayed a shift in product length compared with the product of non-neoplastic mucosa.

CGH Analysis

CGH was performed as previously described in detail.[29] In brief, tumor DNA was biotinylated and normal control DNA was extracted from healthy human placenta tissue and labeled with digoxigenin. After hybridization of the labeled DNA on metaphase spreads (CGH target slides; Abbott Molecular, Abbott Park, IL, USA) at 37 °C for 48 h, the slides were incubated with fluorescence-labeled anti-digoxigenin and anti-biotin antibodies. Metaphase spreads from each specimen were analyzed and photodocumented using an Axiovert S100 microscope (Zeiss, Jena, Germany), a CCD camera, and hardware and software as supplied by Metasystems (Altlussheim, Germany). Fluorescence ratios were determined using both fixed cutoff values (0.8 for losses and 1.25 for gains) and cutoff values with a

Table 1 Clinical data, genetic, and epigenetic alterations of the 37 investigated cases

	loc	age	sex	1p	1q	2p	2q	3p	3q	4p	4q	5p	5q	6p	6q	7p	7q	8p	8q	9p	9q	10p	10q	11p	11q	12p	12q	13q	14q	15q	16p	16q	17p	17q	18p	18q	20p	20q	21	22	X	Mint1	Mint2	MGMT	p16	MLH1	CIMP	BRAF	KRAS
MSI carcinomas																																																
376	J/I	82	F									loss		loss	loss													gain														■	■	■	■	■	high	V600E	wt
108	D	71	M																																							■	■	■	■	■	high	wt	nd
335	J/I	82	F																																							■	■	■	■	■	high	V600E	wt
884	D	46	F																																							■	■	■	■	■	high	wt	wt
420	D	64	M										loss					gain	gain									loss										gain							■			low	wt	wt
172	J	84	M																																												low	wt	wt
410	D	74	F																																												low	wt	wt
235	D	44	F																																								■				low	wt	wt
320	D	37	M															gain	gain																												neg	wt	wt
MSS carcinomas with chromosomal imbalances (CIN)																																															
361	I	71	M			gain																				gain						loss		loss	gain	gain						■	■	■		■	high	wt	mut
656	J/I	75	F																									gain													■	■	■			high	wt	mut
323	J	63	M				gain							gain																	loss	loss		gain							■	■	■			high	wt	mut
263	I	54	F	loss	loss				gain							gain	gain	loss	gain	gain										loss	loss	loss												■			low	nd	mut
401	J/I	59	M							loss	loss																				loss	loss												■			low	wt	wt
591	J	37	M								loss		loss					loss													loss	gain													■		low	wt	mut
303	I	66	M					gain	loss	gain	loss	gain	gain	gain		gain	gain	gain							gain		gain	gain			gain	gain				gain	gain					■		■		low	V600E	wt
249	I	80	F													gain	gain								gain	gain																■				low	wt	mut
970	D	74	M																													loss									■					low	wt	wt
469	J/I	79	M									gain																																			neg	wt	mut
109	J	69	M			gain	gain			loss	loss					gain	gain	loss										gain			loss	loss	loss	loss				gain									neg	wt	wt
195	D	76	M				gain		gain									loss				gain								loss	loss	loss														neg	wt	mut
208	D	54	F			loss	loss		loss		loss				loss					loss						gain					loss	loss														neg	wt	wt
240	D	62	M				gain					gain	gain	gain	loss	gain	gain								gain						loss	gain													neg	wt	wt
2358	J/I	67	F									gain													loss		loss	loss	gain	gain				gain										neg	wt	wt
180	D	64	M																				gain										gain												neg	wt	wt
246	J	29	M									loss													loss																					neg	wt	mut
271	I/J	32	m											gain	gain	loss																gain						gain							neg	wt	wt
502	J	30	M									loss																																		neg	wt	mut
MACS carcinomas																																															
174	D	60	M																																							■	■	■	■	■	high	wt	wt
215	D	69	F																																							■	■	■		■	high	V600E	wt
256	D	34	M																																							■	■	■			high	wt	wt
311	J/I	45	F																																									■	■		high	wt	wt
343	I	83	M																																									■			low	wt	mut
398	J	55	F																																									■			low	Δ604	wt
820	I	72	M																																								■				low	wt	wt
716	D	54	M																																												neg	wt	wt
486	I	69	F																																												neg	wt	wt

Age, age at diagnosis; loc, location; D, duodenal; J, jejunal; I, ileal; J/I, segment small bowel resection without specification of ileum or jejunum; wt, wild type; n.d., not determined.

twofold s.d. Chromosomal abnormalities detected with both approaches were scored. Centromeric and telomeric chromosomal areas as well as chromosome 19 were excluded from scoring. CGH data of 21 of the 37 carcinomas have previously been reported.[7,8]

Methylation-Specific PCR

Methylation-specific PCR was performed for five 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*;[30] *MGMT*;[31] *p16*;[32] *MLH1*[33]). DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. One-fifth of a conversion reaction was used as template.

PCR products were visualized on 2% agarose TAE gel or 6% nondenaturing polyacrylamide gels (PAA). Methylation status was assessed based on the presence or absence of amplified product in the methylation-specific PCR. CIMP-high (CIMP-H), CIMP-low (CIMP-L), and CIMP-negative (CIMP-neg) were scored when >50, 20–50%, and 0% of the investigated loci were positive for methylation-specific PCR, respectively (Figure 1). Methylation-specific PCR data of 21 carcinomas were the subject of a previous study on the pathogenesis of celiac disease-associated small bowel adenocarcinomas.[34]

BRAF Mutations

For analysis of *BRAF* mutations, the following primers were used: *BRAF* forward 5′-CCTAAACTCT TCATAATGCTTGCTC-3′, *BRAF* reverse 5′-CCACA AAATGGATCCAGACA-3′. PCR conditions were as follows: denaturing 92 °C (45 s), annealing at 60 °C (1 min), extension 72 °C (1 min); 35 cycles. PCR products were analyzed by single-strand conformation polymorphism (SSCP) method. Tumors with aberrantly shifted bands were subjected to sequencing. The PCR fragments of these tumors were purified using the high Pure PCR Purification kit (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer, subjected to direct cycle sequencing using a bigDye termination kit (Applied Biosystems), and automated sequencing by ABIPrism 377 (Applied Biosystems).

Results

Stratification of Small Bowel Adenocarcinomas According to Chromosomal and Microsatellite Instability Identifies Four Genomic Subgroups

Of 37 carcinomas, 22 (19 microsatellite stable, 3 MSI-H) displayed chromosomal imbalances. According to the microsatellite status and the presence of chromosomal imbalances, four groups of tumors could be distinguished.

Group 1, MSS/CIN
In all, 19 microsatellite stable (MSS) carcinomas displayed chromosomal imbalances. The most consistent finding was a deletion at chromosome 18q found in 11 of 19 carcinomas in this group. This alteration was commonly found in combination with additional chromosomal imbalances. Deletions at a lower frequency were detected for chromosomal arms 4q (5/19), 6q (4/19), 8p (4/19), and 17p (4/19) whereas gains were common at chromosomes 20q (8/19) and chromosome 7 (5/19).

Group 2, MSI-H/CIN
In all, three MSI-H carcinomas displayed chromosomal alterations. Two tumors showed gains at chromosome 8. No other recurrent imbalances were found. All tumors lacked deletions at 18q.
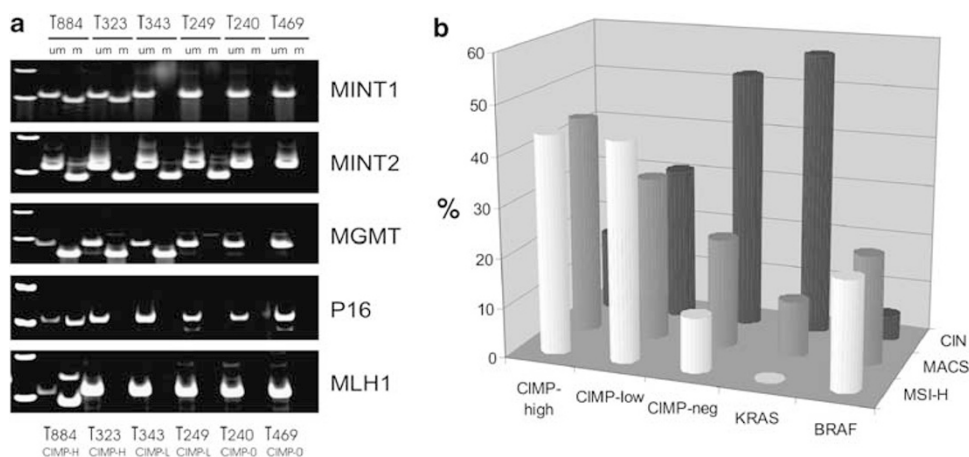

Figure 1 (**a**) Representative results of methylation-specific PCR in six tumors showing two examples each of CIMP-H, CIMP-L, and CIMP-neg. m, methylated; um, unmethylated. (**b**) Graphical summary of genetic and epigenetic findings in small bowel cancer stratified by the presence of MSI-H, CIN, and MACS.

Group 3, MSI-H/CIN negative
Of nine MSI-H tumors, six did not show chromosomal alterations by CGH analysis.

Group 4, MSS/CIN negative
Of 28 MSS tumors, 9 did not show chromosomal alterations by CGH analysis. The results of CGH are summarized in Table 1.

Stratification of Small Bowel Adenocarcinomas According to Methylation Status: CIMP-H Is Associated with MSI-H and MACS

Among the 37 investigated carcinomas, 24 tumors were found to be methylated for at least one investigated locus. A total of 11 carcinomas were scored CIMP-H (3 CIN/MSS, 4 MSI-H, and 4 MACS), 13 carcinomas CIMP-L (4 MSI-H, 6 CIN/MSS, and 3 MACS), and 13 CIMP-neg (10 CIN/MSS, 1 MSI-H, and 2 MACS). CIMP-H was found in 15% (3/19) of CIN/MSS carcinomas, in 44% (4/9) of MSI-H, and in 44% (4/9) of MACS tumors. *MINT2* and *MINT1* represented the most commonly methylated loci (17 and 16 cases, respectively), followed by methylation at *MGMT* (14 cases), *p16* (8 cases), and *MLH1* (7 MSI-H and 1 MSS case). Whereas methylations at single loci were found for *MGMT*, *MINT1*, and *MINT2*, methylation at *MLH1* was only seen in combination with at least one additionally methylated locus, and methylation of *p16* was only observed in CIMP-H carcinomas.

BRAF Mutations Are Associated with MSI-H, MACS, and CIMP

BRAF mutations were observed in five carcinomas. In four cases, a codon 600 mutation (GTG to GAG; V600E) was found, and one additional case displayed a previously published mutation consisting of a 3-bp deletion (c1869–1871[35]) predicted to result in a loss of W604 on the protein level. All tumors with *BRAF* mutations were CIMP positive, including three CIMP-H and two CIMP-L carcinomas. *BRAF* mutations were observed in 2 of 9 MSI-H carcinomas, in 2 of 9 MACS carcinomas, and in 1 of 19 CIN carcinomas.

Discussion

In this study, we aimed at elucidating the association of genetics, epigenetics, and mutations in *KRAS/BRAF* in small bowel adenocarcinomas. As a first step, we therefore stratified 37 carcinomas by the presence of genomic instability. Among 9 MSI-H and 28 MSS carcinomas investigated, we identified 3 of the MSI-H (33%) and 19 of the MSS carcinomas (68%) to display CIN. We thus found MSI-H to be inversely associated with CIN. Moreover, compared with MSS cancers with CIN, the extents and chromosomal locations of CIN in MSI-H cancers were different (Table 1), indicating a selective growth advantage of divergent chromosomal alterations in MSI-H and MSS cancers. These data are comparable with the situation in colorectal cancer, in which generally lower numbers and different types of chromosomal alterations are detected in MSI-H compared with MSS carcinomas.[36,37]

MSS small bowel carcinomas with CIN presented the largest genetic subgroup (51%). Among the alterations seen in this group, deletions at chromosome 18q were the most common shared feature (58% in the CIN group, 30% of all investigated carcinomas) and were commonly associated with gains at chromosomes 20q and 7 as well as with losses of chromosomes 4q, 8p, and 17p (Table 1). Our finding of 30% 18q deletions among all investigated small bowel adenocarcinomas is in contrast with a previous matrix CGH study by Diosdado *et al*[38] reporting <10% of 18q deletions in 48 small intestinal adenocarcinoma, whereas the frequencies of deletions at 4q, 8p, 17p, and gains at chromosomes 7 and 20 were similar to those of our study. The reason for this discrepancy is not clear. It may be explained by sampling variation, and in part result from the high number of celiac disease-associated carcinomas (15/48) included in the study by Diosdado *et al*[38] (1/37 in our study), which have been shown to follow a somewhat different genetic pathway than nonceliac disease-related sporadic carcinomas.[38,39]

Chromosome 18q harbors the *SMAD4* gene, a mediator of TGFβ signaling, which was previously described as a mutational target for a variety of cancers, including small bowel adenocarcinoma.[7,40] The importance of *SMAD4* inactivation for small bowel carcinogenesis is underlined by the fact that deletions at the *SMAD4* locus were found in two carcinomas with only minimal numbers of other chromosomal imbalances (T401, T970), and that somatic intragenic mutations of *SMAD4* were identified in two tumors lacking 18q loss (T246, T502; data not shown).

Although the majority of the carcinomas investigated in this study could be assigned to one type of genomic instability, a significant share of 24% of tumors were found microsatellite and chromosomally stable. This finding is similar to the situation in colorectal cancer, where MACS is identified in 15–30% of tumors.[17,41–43] The pathogenesis of MACS tumors is not understood. It is noteworthy, however, that we have previously identified[44] large-scale N-terminal deletion mutations of β-catenin in three small bowel adenocarcinomas characterized as MACS in the present study, indicating that inactivation of DNA repair mechanisms other than those implicated in mismatch or chromosomal repair could be involved in MACS tumorigenesis.

To investigate the interaction of genomic instability types with epigenetics, we analyzed the tumors for CIMP. We observed 11 carcinomas displaying high-level CIMP (CIMP-H). These 11 carcinomas were not evenly distributed among CIN, MSI-H, and MACS cancers. Similar to the situation in colorectal

cancer, CIMP-H with *MLH1* methylation was particularly frequent in MSI-H carcinomas, providing evidence for a link of CIMP-H and MSI-H in small bowel adenocarcinomas also. Interestingly, we found that CIMP-H is similarly frequent in MACS and is less frequently present in MSS tumors with CIN. These data are in line with a previous observation in colorectal cancer reporting an inverse correlation of aberrant methylation and CIN.[45,46] *BRAF* mutations have been reported to associate with CIMP-H in colorectal cancer. Our data show that the situation is similar in small bowel cancer. We found *BRAF* mutations in three CIMP-H and two CIMP-L carcinomas. The *BRAF* mutation frequency was identical in MSI-H and MACS carcinomas (2/9 cases each), whereas only 1 of 19 CIN carcinomas was *BRAF* mutated, indicating that not only CIMP-H but also *BRAF* mutations preferentially occur in the absence of CIN. In contrast, mutations in *KRAS* were commonly found in MSS/CIN carcinomas (58%) and were infrequent in MSI-H and MACS carcinomas.

Our comprehensive analyses of genetic and epigenetic alterations show that small bowel adenocarcinomas can be stratified into different molecular subgroups. Our findings reveal similar principles of molecular tumorigenesis than recently reported in two comparable studies on colorectal cancer[45,46] (Figure 2), and indicate that CIMP is related to MSI- and MACS-associated intestinal carcinogenesis, whereas it is inversely correlated with CIN. According to our analysis, 50% of small bowel adenocarcinomas are CIN, frequently *KRAS* mutated, and usually show no or a low level of methylation. Among the remaining 50% of chromosomally stable

tumors, MSI-H and MACS cancers had an equal share. MSI-H and MACS small bowel carcinomas usually lack *KRAS* mutations and display similar frequencies of high-level methylation and *BRAF* mutations, indicating common mechanisms to trigger MSI-H and MACS. Future analysis may show if the molecular subtypes of small bowel adenocarcinoma correlate with tumor biology and prognosis.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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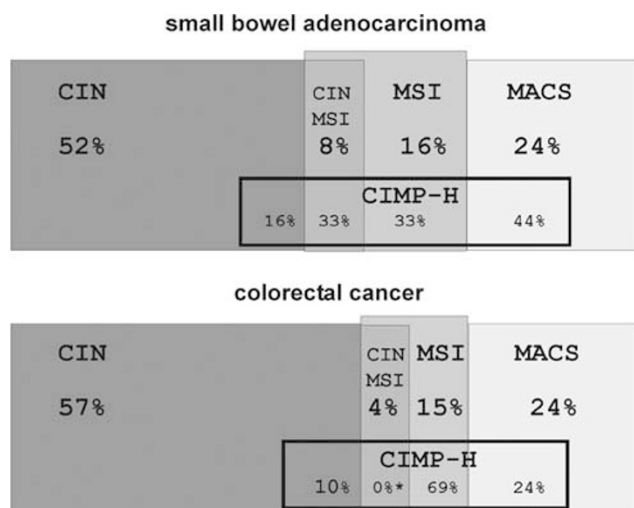
Figure 2 Comparison of the distribution of CIN, MSI, and MACS in small intestinal adenocarcinoma and colorectal cancer. The results of this study and the average values of two similar studies on colorectal cancer[45,46] are presented. The frequency of CIMP-H in the individual genetic subtypes highlights the predominant occurrence of CIMP-H in MSI and MACS carcinomas. *The frequency of CIMP-H in MSI/CIN colorectal carcinomas could only be calculated for one study[45] (0/3 cases).

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