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Molecular characteristics and predictors of survival in patients with malignant neuroendocrine tumors

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Abstract

To better understand the molecular pathogenesis of neuroendocrine tumors (NET), we investigated the molecular and clinical characteristics of malignant poorly differentiated colorectal NET and compared these findings with sporadic CRC and well-differentiated benign and malignant fore-/midgut NET. Tumors were analyzed and correlated for microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP). NET were scored for proliferation using Ki-67. A total of 34 malignant poorly differentiated colorectal NET, 38 well-differentiated benign and malignant fore-/midgut-NET and 150 sporadic colorectal cancers (CRC) with known MSI status were investigated. Among the sporadic CRC, CIMP was significantly correlated with MSI-high (MSI-H) ($p < 0.001$). Of the 34 colorectal NET, 0/1 of the MSI-H, 3/5 (60%) of the MSI-L and 13/19 (68%) of the MSS tumors were CIMP+ ($p = 0.17$). Of the fore-/midgut-NET, none was MSI-H. 20/34 (59%) colorectal NET *vs.* 11/38 (29%) fore-/midgut-NET were CIMP+ ($p = 0.01$). The Ki-67 index was significantly higher in poorly differentiated colorectal NET compared to the less malignant fore-/midgut-NET ($p < 0.0001$). Besides the location in the colon, Ki-67 predicted poor outcome in NET ($p < 0.0001$). CIMP status did not affect survival. In NET, *p16* methylation predicted a poor outcome ($p = 0.0004$). We conclude that molecular pathogenesis in sporadic CRC and poorly differentiated colorectal NET is different despite some similarities. Main differences between malignant well-differentiated and poorly differentiated NET are the Ki-67 proliferation rate and differential methylation in tumor-associated genes. Predictors of a poor outcome in patients with NET are poor differentiation, a high Ki-67 index and *p16* methylation.

Keywords

neuroendocrine tumors; fore-; and midgut tumors; hindgut tumors; microsatellite instability; loss of heterozygosity; CpG island methylation; promoter methylation

Gastroenteropancreatic neuroendocrine tumors (GEP-NET) represent heterogeneous tumors. The growth pattern ranges from very slow to fast growing aggressive types of tumors. Within

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these subgroups, the biological and clinical characteristics of the tumors may vary considerably.[1] The classification currently used is the revised, second edition of the "WHO classification of the endocrine tumors of the gastroenteropancreatic tract."[2]

It is well-known that tumor growth is the result of an uncontrolled cell proliferation and a defective cell death program.[3] Deregulation of this balance results in tumor progression, resistance to therapy and poor prognosis. Genomic instability is a key mechanistic component of cancer progression.[4,5] Three major mechanisms that increase the diversity of gene expression have been identified in most tumor types including colorectal cancer (CRC): microsatellite instability (MSI), chromosomal instability (CIN) and the CpG island methylator phenotype (CIMP).[4,6–11] Current data indicate that CIMP is an important mechanism of gene inactivation in human carcinogenesis, and it has been shown that a number of tumor suppressor genes, including *p16, p14, MGMT* and *hMLH1* are silenced by promoter methylation in CRC and many other tumor types.[10–12]

Several studies have been aimed to identify more precise predictors of prognosis of patients with GEP-NET. The current WHO classification attempted to define a more effective approach by introducing the concepts of cell differentiation and site-specific malignancy as well as specific criteria for carcinoma definition.[2] WHO clinicopathological correlations embed the following prognostic features: degree of cell differentiation, angioinvasion, proliferation fraction as assessed by mitotic index and Ki-67, size and functional activity. NET are categorized into low or high proliferating tumors by the Ki-67 index. Following the WHO classification, NET in this study were categorized by the Ki-67 status into the subgroups with <2%, 2–19% and ≥20% positive cells. Other prognostic variables have been identified, most of which related to specific biological features of neuroendocrine cancer cells. Nonetheless, liver or distant metastases ultimately determine patients' survival and/or response to therapy. A recent proposal of tumor grading and tumor, nodes and metastases (TNM) staging aims at a simple and practical patients' stratification.[13,14] In addition, recent studies addressed the prognostic value of immunohistochemical markers for a number of tumor-associated genes as well as clinical parameters such as age, gender and the functional activity in NET and its metastases. Only synaptophysin, cytokeratin-8 and Ki-67 had some though limited prognostic value.[15] Age above 60 was the only clinical parameter of unfavorable prognostic significance. Another study tested Ki-67, expression of p53 and bcl-2 in patients with GEP-NET.[16] Ki-67 proliferation rate and p53 were found to complement histological grading as prognostic indicators for metastatic disease, whereas bcl-2 appeared to be less useful. Recently, the role of nuclear survivin on tumor progression has been investigated in NET.[17] Expression appeared to be upregulated during progression of GEP-NET. The analysis of nuclear survivin expression identified subgroups in metastatic disease (WHO class 2) with good or less favorable prognosis. It was proposed that the determination of nuclear survivin expression may be used to individualize therapeutic strategies.

In well-differentiated GEP-NET, methylation of cancer-associated genes has recently been shown.[18,19] However, it has not been demonstrated that methylation of specific genes had a prognostic relevance. In patients with well-differentiated fore-/midgut NET, CIMP has been proposed as a prognostic marker, similar to a high Ki-67 proliferation index.[18] The role of allelic losses has not been addressed in former studies studying well-differentiated and poorly differentiated NET.

In previous studies, NET from various origins, differentiation, tumor grade and functional activity had been studied but no clear separation between these variables had been made, mostly because of low case numbers. Our aim was to compare molecular characteristics of well-differentiated and poorly differentiated NET. For this reason, we selected a group of mostly malignant and well-differentiated NET from the fore- and midgut and of malignant mainly

poorly differentiated NET from the colon and rectum. Poorly differentiated NET from the fore/midgut and well-differentiated hindgut tumors were not available at our institution. On the basis of these data, our aim was to correlate MSI, LOH, CIMP and the methylation patterns of well-differentiated NET (n = 38) with poorly differentiated NET (n = 34) in terms of tumor progression and survival. We furthermore compared mechanisms of genomic instability of poorly differentiated colonic NET with that of sporadic nonendocrine CRC.

Material and methods

Tumor tissues

Paraffin-embedded formalin-fixed tissue obtained by biopsy or surgical resection was available from 38 well-differentiated, mainly malignant fore- and midgut (WDEC, WHO class 1 and 2) and from 34 poorly differentiated colonic GEP-NET, respectively. Well-differentiated NET from the colon and rectum were not included into the study to be able to make a clear comparison between 2 cohorts of well-differentiated and poorly differentiated NET. Only sporadic GEP-NET were investigated. The MEN1-syndrome was excluded by a careful study of the personal and family history, and in cases of pancreatic NET by the chemical analysis for serum calcium and prolactin. Malignancy was scored according to WHO guidelines.[2] In detail, poorly differentiated tumors with either metastases, invasion of the muscularis propria, angioinvasion and any tumor size were scored as malignant. Well-differentiated tumors with a tumor size of >2 cm (>3 cm for pancreatic endocrine tumors) and a Ki-67 proliferation index >2% were considered to be malignant similar to those with metastases or invasion of the muscularis propria. The fore-/midgut NET were either benign or low grade malignant [well-differentiated endocrine tumors (WDET) and well-differentiated endocrine cancers (WDEC)]. Patients' characteristics of fore-/midgut NET are shown in detail in Table I. All patients with NET from the colon and rectum were malignant and mainly poorly differentiated [WDEC and poorly differentiated endocrine cancers (PDEC), WHO class 2 and 3, respectively]. Malignant NET from the colon and rectum originated from the midpart of the transverse colon to the rectum. Patients' characteristics are also shown in detail in Table II. Follow-up data were available for most NET from time of diagnosis. In addition, 150 primary colon cancers were studied which were obtained from patients with sporadic CRC. They were selected by their MSI status, which had been investigated by standard PCR methods previously.[6] Follow-up could not be obtained for these tumors.

Microdissection and DNA amplification

Serial sections from formalin-fixed paraffin-embedded matched normal and neoplastic primary tissues (5 μm) were stained with H&E, and representative normal and tumor regions were identified by microscopic examination. Normal control tissue (nontumor) was obtained from adjacent histological normal mucosa and/or normal lymph nodes.

Genomic DNA was isolated from the paraffin-embedded microdomains and removed from the slides by deparaffinization with multiple xylene washes. Subsequently, the tissues were hydrated, digested in Proteinase K and followed by DNA extraction using the QIAamp DNA mini kit (Qiagen, Valencia, CA), according to the manufacturer's instructions.

Loss of heterozygosity on chromosomes 2p16, 5q21 and 11q13

Sets of polymorphic microsatellite sequences that are tightly linked to known TSG and DNA mismatch repair (MMR) genes were used to identify significant allelic losses in the tumors.[9] DNA was amplified by PCR using ^{32}P-end-labeled primers at microsatellite loci linked to the *hMSH2* locus on 2p16 (D2S123), *APC* locus on 5q21 (D5S346) and *MEN1* locus on 11q13 (D11S913, PYGM, RH-93814) and D17S250. LOH was confirmed if a tumor allele showed at least a 50% reduction in the relative intensity of 1 allele in neoplastic tissue compared with

the matched normal DNA. LOH was defined as allelic loss in at least 1 of 6 informative markers investigated. LOH analysis was performed for all NET.

MSI analysis

Microsatellite analysis of all matched normal and tumor tissues was performed by PCR amplification using a panel of 5 NCI-workshop recommended markers that included 2 mononucleotide (BAT25 and BAT26) and 3 dinucleotide repeat sequences (D2S123, D5S346 and D17S250).[20] PCR was performed using ^{32}P-labeled primers and subsequent electrophoresis on 8% polyacrylamide gels as described previously.[18] Changes in the electrophoretic mobility of DNA amplified by PCR were used to assess MSI. Tumors showing a shift in at least 2 of the 5 markers were classified as MSI-H. MSI-L was defined as a shift in only 1 of the 5 markers. Tumors that did not show any allelic shifts were classified as microsatellite stable (MSS). MSI analysis was performed for CRC and NET.

Sodium bisulfite modification and methylation-specific PCR assays

The methylation status of DNA from human NET was determined by methylation-specific PCR (MSP). The specific primers for the methylated and unmethylated MSP for the *hMLH1* (promoter region C), *p16, APC, O^6-MGMT, PTEN, HIC1, RASSF1A, TIMP3, MEN1* and *RUNX3* genes were used as published.[9,18]

MSP was also performed on bisulfite-modified DNA templates obtained from human CRC to study the methylation status of 12 methylation targets. Among these, 9 methylation markers mapped to promoter regions of genes including *hMLH1, APC, p16^{INK4a}, p14^{ARF}, TIMP3, RUNX3, HIC1, PTEN* and *RAR*β2, and the remaining 3 markers amplified MINT (methylated in tumor) loci: MINT1, MINT2 and MINT31. All these markers were recently shown to be closely correlated with CIMP in CRC. The primer sequences, PCR conditions and product sizes for each of the methylation markers analyzed, and the specificity of the MSP assays have been described previously.[6]

Genomic DNA obtained from paraffin-embedded tissue sections was bisulfite modified to convert all unmethylated cytosine residues to uracil for subsequent detection of methylated cytosines using methylation specific primers. MSP assays were performed on the bisulfite-modified DNA using 2 sets of primers specific for amplification of methylated and unmethylated alleles as described previously.[18] Briefly, 0.5–2.0 μg of genomic DNA were denatured with NaOH, treated with sodium bisulfite, and subsequently purified using the Wizard DNA Clean-up System (Promega, Madison, WI). PCR reactions were performed in a 25-μl reaction volume containing 1× PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each PCR primer, 0.75 U of AmpliTaq polymerase, and ~25 ng of bisulfite-modified DNA. Reactions were hot-started at 95°C for 5 min. This was followed by 35–40 cycles at 95°C for 45 sec, 57–60°C for 30 sec and 72°C for 30 sec, followed by a 10-min extension at 72°C in a PTC 200 DNA Engine™ Thermocyler (MJ Research, Waltham, MA). The amplification products were separated on a 3% agarose gel and visualized by ethidium bromide staining and UV transillumination.

Human placental DNA (Sigma Chemical, St. Louis, MO) treated *in vitro* with *SssI* methylase (New England Biolabs, Beverly, MA) was used as a positive control for MSP of methylated alleles, whereas DNA from normal lymphocytes was used as a control for unmethylated alleles. Water was used as a negative PCR control to monitor for contamination.

Immunohistochemical detection of Ki-67

A standard avidin–biotin complex peroxidase method, using 3,30-diaminobenzidine as chromogen, and a monoclonal antibody (MIB-1; Dianova, Hamburg, Germany) were used for

staining nuclear Ki-67. Paraffin sections were heated for 3 5-min periods, deparaffinized, rehydrated and immersed in 10 mmol/l sodium citrate buffer (pH 6.0). The primary antibody was diluted 1:30. The number of Ki-67 positive cells was assessed as percentage of about 2,000 tumor cells in areas of highest nuclear labeling (magnification 40×). A low Ki-67 index was defined as <2% positive cells, an intermediate index as 2–19% positive cells and a high proliferation index as ≥20% positive cells according to recent guidelines from the European Neuroendocrine Tumor Society (ENET) recommendations.[13,14] Evaluation was performed independently by 2 investigators (CNA und IS). In case of disagreement, further evaluation was performed by AS-G.

Data analysis

The Fisher's exact test and the χ^2 -test were used as appropriate to test the associations between tumor subgroups. Univariate associations of baseline characteristics of the tumor subgroups and prognostic variables were assessed using the Fisher's exact test or χ^2 -test. Differences in the frequency of CIMP positive tumors and Ki-67 proliferation index between each subgroup were also analyzed with the χ^2 test. Survival analyses were performed by the Kaplan-Meyer method. The risk ratio of methylation for survival was calculated using the effect likelihood test. All reported *p*-values are 2-sided and a $p < 0.05$ was considered statistically significant.

Results

Microsatellite instability in NET

Informative results were obtained for 25/34 NET from the colon and rectum and 34 of 38 fore- and midgut NET. Clinicopathological and genetic information are shown in Tables I and II. In all noninformative cases, nonneoplastic tissue was not available for comparison. Of the fore- and midgut NET, none was MSI-H, 4/38 (11%) were MSI-L and 30/38 (89%) were MSS. Of the NET from the colon and rectum, 1/26 (4%) was MSI-H (informative markers BAT25 and D17S250), 6/26 (23%) were MSI-L (informative markers BAT25 in 3 tumors, BAT26, D5S123 and D17S250 in 1 tumor, respectively) and 19 (73%) were MSS. MSI status was not different in fore-/midgut NET *versus*. NET from the colon and rectum ($p = 0.12$).

LOH in fore-/midgut NET versus NET of the colon and rectum

LOH is a common feature in sporadic CRC and NET of various origins.[4,21,22] Of the 38 fore-/midgut NET LOH was seen in 4/24 informative tumors (17%) (tu # 3 D2S123; tu # 19 D5S346; tu # 20 D17S250; tu # 31 D17S250) (Table I). Of the 34 NET from the colon and rectum, LOH was found in 8/27 (30%) informative tumors (tu # 9 RH-93814; tu # 12 D2S123, PYGM, RH-93814; tu # 13 D17S250, PYGM; tu # 14 D11S913; tu # 18 D11S913; tu # 20 D11S913; tu # 22 D5S346, D11S913; tu # 23 D11S913) (Table II) ($p = 0.02$). The noninformative tumors were those without corresponding normal tissue. None of the LOH tumors were MSI-H.

CIMP and correlation with MSI in NET and CRC

In direct comparison, CIMP was much more frequent in poorly differentiated NET of the colon and rectum (20/34, 59%) than in well-differentiated fore-/midgut NET (11/38, 29%) ($p = 0.01$) (Table III). Comparing CRC subgrouped by MSI status, MSS NET from the colon and rectum were significantly more often CIMP positive than sporadic CRC ($p = 0.0003$) (Fig. 1a). Among all tumors, 24/94 (26%) MSS-, 10/43 (23%) MSI-L and 10/13 (77%) MSI-H sporadic CRC were CIMP positive ($p < 0.0001$), whereas 13/19 MSS informative NET from the colon and rectum (68%) and 3/6 (60%) MSI-L NET from the colon and rectum were CIMP positive ($p = 0.17$). Interestingly, the only MSI-H colon NET was CIMP negative, whereas CIMP was a predominant feature in MSI-H sporadic CRC.

Distribution of NET by Ki-67 proliferation rate and CIMP

According to their poor differentiation, NET of the colon and rectum (29/34, 85%) revealed significantly more often a high-proliferation index ≥ 20% than the well-differentiated fore-/midgut NET ($p < 0.0001$) (Table III). Fore-/midgut NET had significantly more often a proliferation index of <2% (24/38, 63%) and 2–19% (14/38, 37%) than NET from the colon and rectum (2/34, 6% and 3/34, 9%, respectively) (Table III) ($p < 0.0001$).

Upon further subgroup analysis, we categorized NET by their proliferation index and CIMP status. We found that fore-/midgut NET did not differ significantly in CIMP status (Ki-67 <2% 13/23, 57%; Ki-67 2-19% 9/14, 64%) but in NET from the colon and rectum CIMP impressively correlated with a high Ki-67 index (Ki-67 2-19% 4/4, 100%; Ki-67 ≥ 20% 16/28, 57%) ($p < 0.0001$).

Difference in promoter methylation in fore-/midgut versus NET from the colon and rectum

Among 8 analyzed loci, *HIC1*, *APC* and *RASSF1A* were more often methylated in fore-/midgut-NET, whereas *MEN1*, *MGMT*, *p16*, *hMLH1* and *TIMP3* were more frequently methylated in NET from the colon and rectum (Tables IV and V, Fig. 1b). *HIC1* was significantly more methylated in fore-/midgut NET ($p = 0.05$), whereas *MEN1* ($p = 0.02$), *p16* ($p = 0.003$) and *hMLH1* ($p = 0.03$) were significantly methylated in NET from the colon and rectum. Interestingly, promoter methylation in *p16*, *hMLH1* and *TIMP3* were only detected in NET from the colon and rectum.

The risk ratio of methylation for survival in all NET was calculated by proportional hazards analysis (Table VI). We did not find correlation with any of the investigated genes with the exception of *p16*. Methylation of *p16* clearly associated with a poor survival and was only found in poorly differentiated NET from the colon and rectum and not in any case of the fore-/midgut NET ($p = 0.01$).

Outcome by Ki-67 proliferation rate, CIMP and p16 methylation

Outcome data was available for most patients with NET. Unfortunately, follow-up was not available for patients with CRC. By this, direct outcome comparison between NET from the colon and rectum and sporadic CRC could not be performed in this study.

Our analyses demonstrated, however, that patients with poorly differentiated NET from the colon and rectum had a poorer outcome than patients with better differentiated fore-/midgut NET ($p = 0.0045$) (Fig. 2a). A three-year survival was 60% in patients with fore-/midgut NET as compared to 30% in patients with NET from the colon and rectum. The strongest predictor of survival was the Ki-67 proliferation index. Patients with NET with Ki-67 <2% had a significant longer survival than patients with a Ki-67 index of ≥ 2-19% or ≥ 20%, respectively ($p < 0.0001$) (Fig. 2b). After 2 years follow-up, 80% of the patients with Ki-67 <2% but only 30% of the patients with a Ki-67 index of ≥ 2–19% were alive ($p = 0.001$). Despite 5 patients with NET from the colon and rectum had a Ki-67 index of <5 % survival of these patients did not differ from those with a higher Ki-67 index ($p = 0.26$) (Fig. 2b). We have shown that CIMP was more frequent in poorly differentiated NET (colon and rectum) than in well-differentiated NET (fore-/midgut). However, CIMP was not a predictor for survival neither in patients with NET from the colon and rectum ($p = 0.55$) nor in patients with fore-/midgut NET ($p = 0.66$). It was further investigated if there was any influence in outcome by any of the molecular markers investigated. Interestingly, *p16* methylation was a strong predictor of survival in all NET ($p = 0.0004$). In NET from the colon and rectum, we detected a trend toward worse outcome ($p = 0.08$) (Fig. 2c). Fore-/midgut NET were not methylated in *p16*.

Discussion

In a previous study, we investigated the contribution of genetic and epigenetic alterations in molecular pathogenesis of sporadic well-differentiated fore-/midgut NET.[18] It was shown that promoter methylation was a common event and a unique methylation profile for gastrointestinal NET was described. Interestingly, there were no significant differences in the promoter methylation profiles of any tumor subgroup. We only found slight differences for less frequent hypermethylation of the *RUNX3* and the O^6-*MGMT* genes in tumor subsets. Besides genetic alterations, CIMP appeared to play an important role in tumor pathogenesis of fore-/midgut NET. These results led us to hypothesize that CIMP is involved in the molecular pathogenesis also of NET of the colon and rectum, as has been shown for sporadic CRC.

In this study, we directly compared characteristic molecular events in well and poorly differentiated NET from different anatomical sites. We could demonstrate differences in molecular events for sporadic CRC, well-differentiated NET from fore-/midgut and poorly differentiated NET of the colon, which might influence growth characteristics and proliferation rates in both cancer types. We further demonstrated the influence of several predictors on outcome in well and poorly differentiated tumors. For the first group of tumors, the Ki-67 proliferation rate was confirmed as the most important known predictor of survival. However, that was not true for undifferentiated tumors (colon and rectum) in which only methylation of *p16* appeared to influence survival. *p16* methylation in general was a predictor of worse outcome in all NET. However, the numbers of poorly differentiated hindgut tumors with a low Ki-67 index was low. CIMP was not a predictor of survival in either group.

To date, only few outcome studies have been performed for NET. Most identified the Ki-67 proliferation index as the main predictor of outcome.[23–26] Several molecular events have been described in NET including genetic or epigenetic alterations of *p53, BAX, p16INK4a/CDKN2A, BRAF, bcl-2, PTEN, DPC4/Smad4, p27*,[16–18,21–23,27–36] allelic losses[37] or differential expression of nuclear survivin and other proteins.[17,38] Most of them did not correlate with patient survival or aggressiveness of the tumors. Tumors with frequent allelic losses correlate with a higher proliferation index and a poorer survival.[22,25,37,39] Also retaining *p27* expression was a predictor of a longer survival in some patients.[40] This has been confirmed by a recent study (Grabowski 2008, submitted for publication) in which the loss of the cyclin-dependent kinase inhibitor *p27* played a critical role for the aggressiveness of GEP-NET. This was explained by the increased cell cycle progression and proliferation by lack of inhibition of cyclin E in those tumors. Patients retaining *p27* expression had a significantly longer outcome in that study. Further predictors of outcome are histological differentiation and the presence of malignancy (infiltration of adjacent structures or metastases).[2] Our study confirmed the Ki-67 proliferation rate as a predictor of survival in GEP-NET. This was not consistent for poorly differentiated NET from the colon and rectum, however, in which none of the tumors had a low proliferation rate and the differentiation into <20% or ≥ 20% positive cells had no influence on survival. The aggressiveness of NET from the colon and rectum might be explained by the poor differentiation of the tumors with frequent allelic losses and loss of *p27* expression.[25,33,37,40] In our recent study, CIMP positive tumors had a better outcome than CIMP negative tumors in WDET and WDEC from the fore- and midgut.[18] No other prognostic marker had been identified. CIMP did not influence survival in our study neither in well- and poorly differentiated NET. These discrepant results remain to be explained and might be due to the low tumor numbers investigated. Interestingly, methylation of the *p16* promoter, which has been shown to correlate with expression in different studies,[18,19] predicted a worse survival in NET and showed a trend toward a poorer outcome in poorly differentiated NET from the colon and rectum. However, the case number was too low to draw final conclusions from this finding. Methylation of *p16* was not present in fore-/midgut NET and the number of NET from the colon and rectum was too low to yield significant data. Future studies, however, should

address the role of *p16* expression in NET on survival. Despite differences in the methylation rate of *HIC1*, *MEN1*, *hMLH1* and *TIMP3*, these genes did not influence outcome in the respective tumor groups. The significance of *MEN1* methylation is still unsolved. We have shown earlier that *MEN1* expression and promoter methylation of the gene did not correlate.[18] However, *MEN1* mutations in sporadic foregut NET have been described before.[41] It remains to be elucidated how these genes influence tumor growth and/or progression in sporadic NET. Recently, a new TNM classification of GEP-NET has been proposed.[13,14] Future studies will clarify, whether this classification will represent a new outcome predictor and compliment the known predictors.

Apart from the low incidence of poorly differentiated NET of the colon and rectum compared to sporadic CRC, the molecular pathogenesis of both tumor types appears to be different although the growth characteristics of both tumors reveal some similarities. Other than in sporadic CRC, MSI is infrequent in poorly differentiated NET of the colon and rectum. MSI has also been shown to be an infrequent event in NET from different anatomic sites.[18,42,43] CIMP is common in both sporadic CRC and poorly differentiated NET from the colon and rectum, but due to the different molecular pathogenesis of the tumors the relevant methylated genes have been shown to be different.[6,9,11,18,19] Our study is the first to investigate the methylation status of tumor-associated genes in a relevant number of poorly differentiated NET from the colon and rectum.

In conclusion, our findings demonstrate that sporadic CRC and poorly differentiated NET of the colon and rectum differ in tumor pathogenesis despite the occurrence of CIMP in either. Until further studies are performed to directly compare methylation patterns in NET from the colon and rectum and sporadic CRC, we cannot know for certain whether CIMP is an acquired defect with a primary etiology or whether this abnormal pattern of promoter methylation is a random process that is selected for in tumor cells. MSI is common only in sporadic CRC and is not seen in GEP-NET from different anatomical sites and not dependent on tumor differentiation. In accordance with their poor differentiation, NET from the colon and rectum had a worse survival than well-differentiated fore-/midgut NET. Predictors of a poor outcome in this study were a high Ki-67 proliferation index and *p16* methylation. Expression of *p16* should be analyzed in future studies in a larger number of NET in order to define its value as predictor of survival that may contribute to individualize therapeutic strategies.

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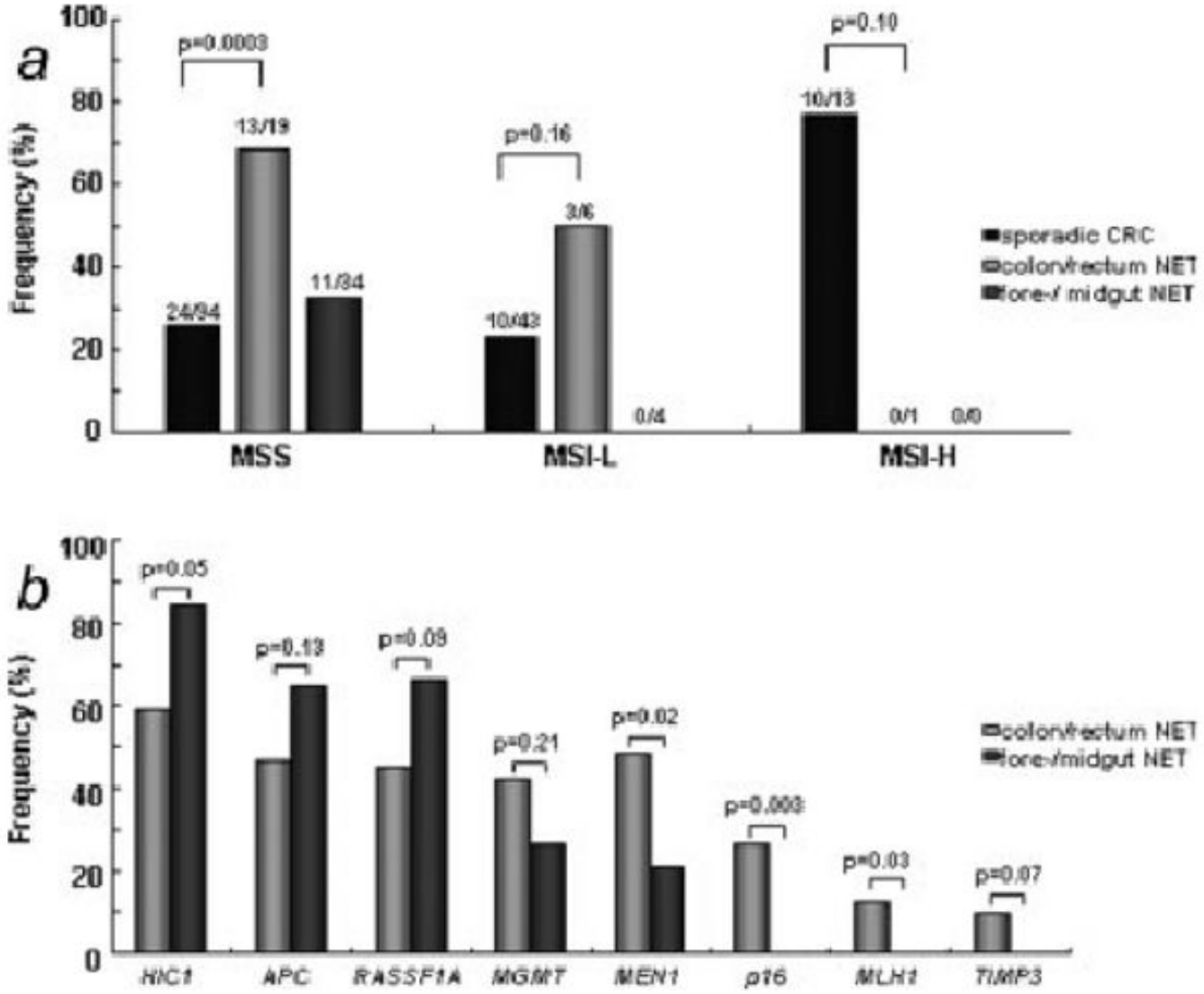

Figure 1.

(*a*) CIMP in sporadic CRC, poorly differentiated NET from the colon and rectum and well differentiated fore-/midgut NET. Tumors are sorted by their MSI status. (*b*) Methylation rates in well differentiated fore-/midgut NET and poorly differentiated NET from the colon and rectum.

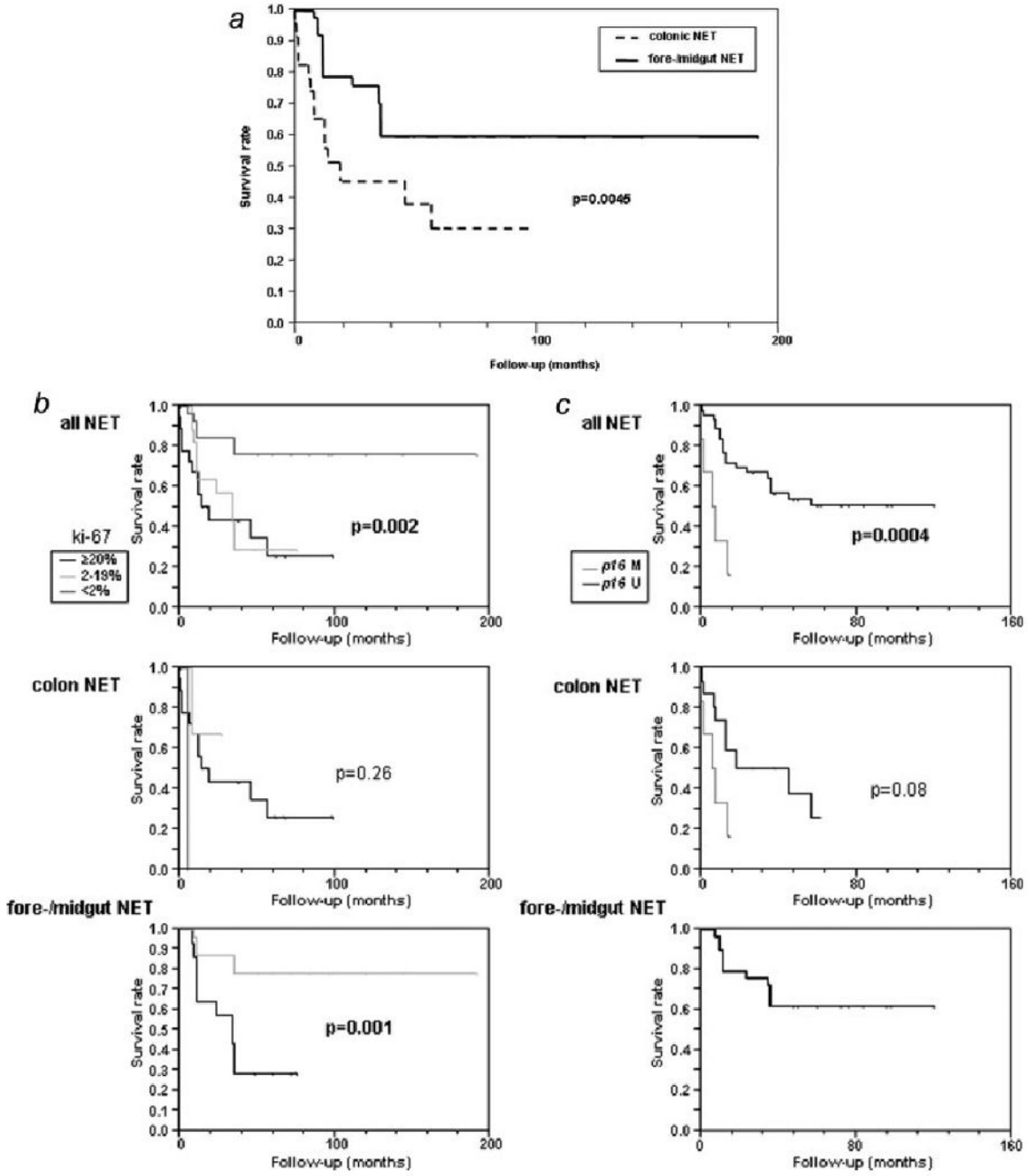

Figure 2.

(*a*) Kaplan Meyer curves for survival in well differentiated fore-/midgut NET and poorly differentiated NET from the colon and rectum. (*b*) Kaplan Meyer survival analysis by Ki-67 proliferation rate in NET. (*c*) Kaplan Meyer survival analysis by *p16* methylation in NET.

TABLE I

Characteristics of Patients with Fore-/Midgut Net According to Functional Activity, Site and KI-67 Proliferation Index

Tumor no.	Diagnosis	Origin	Behavior	Ki-67 (%)	CIMP	MSI (marker)	LOH (marker)	Follow-up time (weeks)	Survival	Gender	Age at diagnosis
8	Insulinoma	Pancreas	Malignant	<2	−	MSS	−	12	tu	m	79
14	Insulinoma	Pancreas	Benign	<2	−	MSS	−	192	Survived	f	52
16	Insulinoma	Pancreas	Benign	<2	+	MSS	−	60	Survived	f	77
19	Insulinoma	Pancreas	Malignant	2–20	−	MSS	+ (D5S346)	12	tu	f	79
12	Gastrinoma	Pancreas	Benign	2–20	−	MSS	na	36	tu	m	81
2	Vipoma	Pancreas	Malignant	2–20	−	MSS	−	60	Survived	m	56
17	Vipoma	Pancreas	Benign	2–20	+	MSS	−	76	Survived	m	56
1	Glucagonoma	Pancreas	Malignant	2–20	−	MSS	−	8	tu	f	50
5	Nonfunctional	Pancreas	Malignant	<2	+	MSS	na	84	Survived	m	61
4	Nonfunctional	Pancreas	Malignant	2–20	−	MSS	na	72	Survived	f	36
28	Nonfunctional	Duodenum	Malignant	<2	+	MSS	−	96	Survived	m	71
34	Nonfunctional	Duodenum	Malignant	<2	−	MSI-L	na	98	Survived	f	81
37	Nonfunctional	Duodenum	Malignant	<2	+	MSS	−	96	Survived	m	70
9	Nonfunctional	Duodenum	Malignant	2–20	−	MSS	na	12	tu	m	78
30	Nonfunctional	Duodenum	Malignant	2–20	−	MSS	na	35	tu	m	70
6	Gastrinoma	Duodenum	Malignant	<2	−	MSI-L	na	10	tu	f	49
15	Gastrinoma	Duodenum	Benign	<2	−	MSS	−	192	Survived	m	48
18	Gastrinoma	Duodenum	Benign	<2	−	MSS	−	50	Survived	f	49
3	Gastrinoma	Duodenum	Benign	2–20	−	MSS	+ (D2S123)	48	Survived	m	66
23	Carcinoid syndrome	Ileum	Malignant	<2	−	MSS	−	84	Survived	m	62
24	Carcinoid syndrome	Ileum	Malignant	<2	+	MSS	−	72	Survived	m	66
25	Carcinoid syndrome	Ileum	Malignant	<2	−	MSS	−	72	Survived	m	70
32	Carcinoid syndrome	Ileum	Malignant	<2	+	MSS	−	120	Survived	m	63
35	Carcinoid syndrome	Ileum	Malignant	<2	+	MSS	−	36	tu	f	56
38	Carcinoid syndrome	Ileum	Malignant	<2	−	MSS	na	144	Survived	f	60
33	Carcinoid syndrome	Jejunum	Malignant	<2	−	MSS	−	120	Survived	m	75
36	Carcinoid syndrome	Jejunum	Malignant	<2	−	MSS	na	60	Survived	m	69
13	Nonfunctional	Ileum	Malignant	<2	+	MSS	−	36	tu	f	81
21	Nonfunctional	Ileum	Malignant	<2	−	MSS	na	84	Survived	f	61

Tumor no.	Diagnosis	Origin	Behavior	Ki-67 (%)	CIMP	MSI (marker)	LOH (marker)	Follow-up time (weeks)	Survival	Gender	Age at diagnosis
22	Nonfunctional	Ileum	Malignant	<2	+	MSS	na	84	Survived	f	61
20	Nonfunctional	Ileum	Malignant	2–20	+	MSS	+ (D17S250)	36	tu	m	66
29	Nonfunctional	Ileum	Malignant	2–20	−	MSI-L	na	35	tu	f	38
31	Nonfunctional	Ileum	Malignant	2–20	−	MSS	+ (D17S250)	24	tu	m	70
10	Nonfunctional	Jejunum	Malignant	<2	−	MSS	na	12	tu	m	71
26	Nonfunctional	Jejunum	Malignant	<2	−	MSS	−	84	Survived	f	49
27	Nonfunctional	Jejunum	Malignant	<2	+	MSS	−	120	Survived	m	51
7	Nonfunctional	Jejunum	Malignant	2–20	−	MSS	−	10	tu	m	65
11	Nonfunctional	Jejunum	Malignant	2–20	−	MSI-L	na	12	tu	f	38

na, not available; tu, died from the tumor; f, female; m, male.

TABLE II

Characteristics of Patients with Net of the Colon and RECTUM According to KI-67 Proliferation Index

Tumor no.	Origin	Behavior	Ki-67	CIMP	LOH (marker)	MSI (marker)	Follow-up time (weeks)	Survival	Gender	Age at diagnosis
17	Colon	Malignant	<2	−	−	MSI-H (BAT25, D17S250)	na	na	f	70
14	Colon	Malignant	3	+	+ (D11S913)	MSI-L	8	Survived	f	62
12	Colon	Malignant	5	+	+ (D2S123, PYGM, RH-93814)	MSS	27	Survived	f	77
13	Colon	Malignant	5	+	+ (D17S250, PYGM)	MSI-L	8	tu	f	54
18	Colon	Malignant	20	+	+ D11S913	MSS	na	na	m	70
23	Colon	Malignant	30	+	+ (D11S913)	MSS	19	tu	m	32
11	Colon	Malignant	40	−	−	MSI-L	18	Survived	m	67
32	Colon	Malignant	45	−	na	MSS	62	Survived	m	54
33	Colon	Malignant	45	+	−	Na	46	tu	f	58
9	Colon	Malignant	50	+	+ (RH-93814)	Na	na	na	na	na
10	Colon	Malignant	50	+	−	Na	15	Survived	m	71
16	Colon	Malignant	50	+	−	MSS	na	na	f	57
19	Colon	Malignant	50	−	−	MSS	na	na	na	na
21	Colon	Malignant	50	+	−	MSS	13	tu	m	69
27	Colon	Malignant	50	+	−	MSS	14	tu	m	32
5	Colon	Malignant	50	+	−	Na	na	na	na	na
6	Colon	Malignant	60	−	−	Na	na	na	na	na
25	Colon	Malignant	60	+	−	MSS	0	tu	f	63
8	Colon	Malignant	60	+	−	MSS	99	Survived	m	74
1	Colon	Malignant	65	+	−	MSI-L	na	Na	na	na
34	Colon	Malignant	65	−	na	Na	38	Survived	m	56
2	Colon	Malignant	70	+	−	MSS	na	Na	na	na
29	Colon	Malignant	75	+	na	MSS	2	Tu	m	64
30	Colon	Malignant	75	−	na	MSS	1	Tu	f	92
7	Colon	Malignant	80	−	−	MSI-L	na	Na	na	na
15	Colon	Malignant	80	+	−	MSS	2	Tu	m	53
3	Colon	Malignant	85	−	na	na	na	na	na	na
31	Colon	Malignant	85	−	na	MSS	68	Survived	f	58
4	Colon	Malignant	90	−	na	na	na	na	na	na

Tumor no.	Origin	Behavior	Ki-67	CIMP	LOH (marker)	MSI (marker)	Follow-up time (weeks)	Survival	Gender	Age at diagnosis
22	Colon	Malignant	90	+	+ (D5S346, D11S913)	MSS	57	tu	m	49
28	Rectum	Malignant	<2	−	−	MSS	6	tu	m	55
24	Rectum	Malignant	50	+	−	MSS	13	tu	m	29
26	Rectum	Malignant	50	−	−	MSI-L	7	tu	m	57
20	Rectum	Malignant	90	−	+ (D11S913)	MSS	8	tu	f	81

na, not available; tu, died from the tumor; f, female; m, male.

TABLE III
Genomic Instability and Ki-67 Status in Colon/Rectum Net and Fore-/Midgut Net

Parameter		Colon and rectum NET	Fore-/midgut NET	*p*-value
Age	Mean, SD	61.2 (15.6)	62.7 (12.6)	0.69[1]
Gender	Female	11 (41%)	16 (42%)	0.91[2]
No. of samples (%)	Male	16 (59%)	22 (58%)	
CIMP status	Positive (*n* = 31)	20 (59%)	11 (29%)	0.01[2]
No. of samples (%)	Negative (*n* = 41)	14 (41%)	27 (71%)	
LOH status	Positive (*n* = 12)	8 (30%)	4 (17%)	0.28[2]
No. of samples (%)	Negative (*n* = 39)	19 (70%)	20 (83%)	
MSI status	MSI-H (*n* = 1)	1 (4%)	0 (0%)	0.17[2]
No. of samples (%)	MSI-L (*n* = 11)	6 (23%)	4 (11%)	
	MSS (*n* = 52)	19 (73%)	34 (89%)	
Ki-67 IHC score status	<2% (*n* = 16)	2 (11%)	14 (89%)	<0.0001[2]
No. of samples (%)	2–19% (*n* = 27)	3 (8%)	24 (92%)	
	>20% (*n* = 28)	28 (100%)	0 (0%)	

[1] *p*-value is based on ANOVA.

[2] *p*-values are based on X^2 test.

TABLE IV

Methylation Profile of Fore-/Midgut Net according to Site

Tumor no.	Diagnosis	*hMLH1*	*p16*	*APC*	*MGMT*	*MEN1*	*HIC1*	*RASSF1a*	*TIMP3*
3	Gastrinoma	−	−	+	−	−	na	−	−
6	Gastrinoma	−	−	−	na	−	+	+	−
12	Gastrinoma	−	na	−	−	−	na	+	−
15	Gastrinoma	−	na	na	na	na	na	na	na
18	Gastrinoma	na	−	−	na	−	na	na	na
8	Insulinoma	−	−	−	+	−	na	+	−
14	Insulinoma	−	na	+	na	na	na	−	−
16	Insulinoma	−	−	+	+	−	+	+	−
19	Insulinoma	−	na	−	+	−	−	+	−
1	Glucagonoma	−	2	+	−	−	+	+	−
2	Vipoma	−	−	+	−	−	na	na	−
17	Vipoma	−	−	+	+	+	+	+	−
23	Carcinoid syndrome	−	−	+	−	−	−	−	−
24	Carcinoid syndrome	−	−	+	+	−	+	+	−
25	Carcinoid syndrome	−	−	−	−	−	+	+	−
32	Carcinoid syndrome	−	−	+	na	+	−	−	−
33	Carcinoid syndrome	−	−	−	−	−	+	+	−
35	Carcinoid syndrome	−	−	+	+	+	+	+	−
36	Carcinoid syndrome	−	na	−	−	−	na	−	−
38	Carcinoid syndrome	na	na	+	na	na	na	na	na
4	Nonfunctional pancreas	−	−	+	−	−	+	−	−
5	Nonfunctional pancreas	−	−	+	−	−	+	+	−
7	Nonfunctional jejunum	−	−	+	−	−	na	+	na
9	Nonfunctional duodenum	−	−	−	−	−	+	+	−
28	Nonfunctional duodenum	−	−	+	−	−	+	+	−
30	Nonfunctional duodenum	−	na	+	na	na	+	na	−
34	Nonfunctional duodenum	−	−	−	−	−	na	na	−
37	Nonfunctional duodenum	−	na	+	−	+	na	−	na
10	Nonfunctional jejunum	−	−	−	−	−	+	−	−

Tumor no.	Diagnosis	*hMLH1*	*p16*	*APC*	*MGMT*	*MEN1*	*HIC1*	*RASSF1a*	*TIMP3*
11	Nonfunctional jejunum	−	na	+	na	na	+	na	−
26	Nonfunctional jejunum	−	−	+	−	−	+	na	−
27	Nonfunctional jejunum	−	−	+	−	+	na	+	−
13	Nonfunctional ileum	−	−	+	+	+	+	+	−
20	Nonfunctional ileum	−	−	+	+	−	+	+	−
21	Nonfunctional ileum	−	−	−	−	−	+	+	−
22	Nonfunctional ileum	−	−	+	−	+	−	+	−
29	Nonfunctional ileum	−	−	−	−	−	+	−	−
31	Nonfunctional ileum	−	−	+	−	−	+	−	−

Na, not available; +, methylated; −, not methylated.

TABLE V

Methylation Profile of Colon and Rectum Net According to Tumor Location

Tumor no.	Diagnosis	*p16*	*HIC 1*	*APC*	*hMLH1*	*RASSF1A*	*MEN 1*	*TIMP3*	*MGMT*
1	Colon	+	−	−	−	+	−	−	+
2	Colon	Na	+	+	−	+	−	+	−
3	Colon	−	−	−	−	−	−	−	−
4	Colon	−	na	+	−	na	−	−	na
5	Colon	−	+	−	−	−	+	−	+
6	Colon	−	−	−	−	−	−	−	−
7	Colon	na	na	−	−	−	−	−	−
8	Colon	na	+	+	+	−	−	−	−
9	Colon	−	+	+	−	+	−	−	−
10	Colon	+	−	+	−	−	−	−	+
11	Colon	−	−	+	−	+	−	−	−
12	Colon	−	+	+	−	−	+	−	+
13	Colon	+	+	+	−	−	+	−	−
14	Colon	−	+	−	−	+	+	−	−
15	Colon	−	−	−	−	+	+	−	+
16	Colon	−	+	−	−	−	+	−	+
17	Colon	−	na	−	−	na	+	−	−
18	Colon	+	+	−	−	−	+	−	+
19	Colon	−	−	na	−	−	+	−	−
21	Colon	−	+	+	−	+	+	−	−
22	Colon	−	−	+	−	+	+	−	+
23	Colon	−	−	+	−	−	+	−	+
25	Colon	+	+	−	+	+	−	−	−
27	Colon	+	+	−	−	+	+	−	−
29	Colon	+	+	+	−	−	−	−	+
30	Colon	−	na	−	−	−	na	−	−
31	Colon	na	na	−	na	na	na	−	−
32	Colon	−	na	na	na	na	−	na	na
33	Colon	−	+	+	+	+	+	+	+

Tumor no.	Diagnosis	*p16*	*HIC 1*	*APC*	*hMLH1*	*RASSF1A*	*MEN 1*	*TIMP3*	*MGMT*
34	Colon	−	−	−	+	−	−	−	+
20	Rectum	−	+	−	−	+	−	−	−
24	Rectum	−	+	+	−	+	+	+	+
26	Rectum	−	na	+	−	na	na	na	na
28	Rectum	+	−	−	−	−	−	−	−

na, not available; +, methylated; −, not methylated.

TABLE VI

Risk Ratio of Methylation for Survival

Gene	Risk ratio (confidence interval)	*p*-value
HIC1	1.46 (0.44–6.11)	0.55
MEN1	2.06 (0.72–5.99)	0.18
APC	0.40 (0.11–1.40)	0.15
RASSF1A	0.51 (0.16–1.66)	0.25
MGMT	1.32 (0.38–5.15)	0.66
p16	5.21 (1.43–18.0)	0.01
hMLH1	0.58 (0.06–5.26)	0.63
TIMP3	3.00 (0.22–23.4)	0.38

p-values were based on Effect Likelihood Tests.