Colorectal Poorly Differentiated Neuroendocrine Carcinomas and Mixed Adenoneuroendocrine Carcinomas: Insights Into the Diagnostic Immunophenotype, Assessment of Methylation Profile, and Search for Prognostic Markers

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Abstract: Colorectal poorly differentiated neuroendocrine carcinomas (NECs) and mixed adenoneuroendocrine carcinomas (MANECs) are well-recognized entities generally known to be associated with biological aggressiveness and poor patient survival. However, a few published papers have highlighted the existence of a subgroup of tumors with a better survival than expected; however, to date, there are no established parameters that usefully identify this category. In the present study we have investigated the morphologic features, the CpG methylator phenotype (CIMP), microsatellite instability (MSI), and the immunohistochemical profile, including the expression of transcription factors (TTF1, ASH1, CDX2, and PAX5), stem cell markers (CD117 and CD34), and cytokeratins 7 and 20, in a series of 39 carcinomas (27 NECs and 12 MANECs) to better characterize such neoplasms and to search for prognostic indicators. No different patient survival was observed between NECs and MANECs. Neoplasms showed a heterogenous spectrum of morphologic and immunohistochemical features; however, only large-cell subtype, significant peritumoral lymphoid reaction, CD117 immunoreactivity, vascular invasion, and MSI/CIMP+ status were significantly correlated with prognosis on univariable analysis. Furthermore, vascular invasion and CD117 immunoreactivity were independent prognostic markers on multivariable analysis. In addition to these prognostic features, neoplasms showed different expression of transcription factors, stem cell markers, and cytokeratins that should be considered for diagnostic purposes and, especially, for discriminating among possible differential diagnoses.

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Colorectal poorly differentiated neuroendocrine carcinomas are defined as neuroendocrine carcinomas (NECs) according to the 2010 WHO classification and are rare neoplasms accounting for about 0.6% of all colorectal cancers.[37] Although there are typical histologic and immunohistochemical features that strongly suggest the diagnosis of such carcinomas, assessment of proliferative activity, including both mitotic and Ki67 index count, is now mandatory in the diagnostic guidelines.[38] Indeed, NECs are by definition carcinomas with >20 mitoses X 10 HPF and/or $>20\%$ Ki67 proliferative index.[37,39] Cytologically, NECs have been subdivided into 3 main types: small-cell, large-cell, and intermediate-cell categories.[42] Tumor cells grow in large sheets with abundant areas of necrosis and express general neuroendocrine markers such as synaptophysin, chromogranin A, neuron-specific enolase, and CD56. They may be associated with a non-neuroendocrine component, mainly represented by foci of adenocarcinoma or, more rarely, squamous cell carcinoma. When both neuroendocrine and non-neuroendocrine components are conspicuous, representing at least 30% of the neoplastic tissue, tumors are classified as mixed exocrine-neuroendocrine carcinomas or, as suggested by the 2010 WHO classification, as mixed adenoneuroendocrine carcinomas (MANECs).[37]

The pathogenesis of colorectal NECs is poorly understood, and little is known about the role of gene methylation in their development and/or progression. Indeed, CpG island methylation, which is a common epigenetic event in colorectal adenocarcinomas, has been poorly investigated in colorectal NECs.[3] The CpG island methylator phenotype (CIMP) indicates the simultaneous methylation of several genes. CIMP has been found to closely correlate with microsatellite instability (MSI) and characteristic clinicopathologic features in colorectal

adenocarcinomas.[1,16] However, whether CIMP and MSI can be associated with specific clinical, morphologic, or immunophenotypical features or whether they can play a role in tumor progression with diagnostic and prognostic implications in colorectal NECs is still unknown.

The expression of transcription factors (TFs) and stem cell markers in colorectal NECs and their possible role as diagnostic and/or prognostic indicators remain to be clarified. Some TFs are known to be specifically expressed in neuroendocrine cells and related tumors of different sites and have therefore been investigated as diagnostic/prognostic markers. Examples are TTF1 expression in lung neuroendocrine cells and related tumors,[26] CDX2 in intestinal EC cells and EC cell tumors,[5,29] and Isl-1 and PDX1 in islet cells and neuroendocrine tumors of the pancreas.[40] In addition, it has been recently reported that the expression of hematopoietic stem cell markers such as CD117 correlates with prognosis in neuroendocrine neoplasms.[48]

From a clinical point of view, colorectal NECs are high-grade cancers associated with poor prognosis.[37] However, survival data regarding patients with colorectal NECs mainly refer to small series, and analyses of a large number of cases are rare in the literature.[42] In addition, data from a few published studies[36,42] and from anecdotal experience seem to suggest that there is a fraction of patients with colorectal NECs who have a better survival rate than expected, but there are no available parameters that can identify this small group of patients.

In the present study we have systematically investigated the clinicopathologic features, the immunohistochemical profile including the expression of several TFs and stem cell markers, MSI status, and the methylation phenotype of 39 colorectal NECs to better characterize this tumor category and identify possible diagnostic and/or prognostic markers.

MATERIALS AND METHODS

Cases

The pathology database at the Department of Pathology of the Ospedale di Circolo in Varese was searched for primary colorectal neoplasms with one of the following diagnoses during the years 1980–2010: "poorly differentiated neuroendocrine carcinoma," "small cell carcinoma," "adenocarcinoma with neuroendocrine features," and "mixed exocrine-(neuro)endocrine carcinoma." Cases included specimens from the surgical or endoscopic departments of the hospital in Varese and neoplasms sent for consultation from other hospitals. All cases were reviewed, and only neoplasms with pure morphologic and immunohistochemical neuroendocrine features or mixed exocrine-neuroendocrine tumors with a neuroendocrine component $> 30\%$ were considered for the study. Moreover, patients with a history of lung primary neuroendocrine neoplasms or with well-differentiated (grade 1 or 2, according to 2010 WHO classification) colonic neuroendocrine tumors (carcinoids) were excluded. Following these criteria, 39 cases were enrolled in the investigation. The series included 38 primary cancers and 1 liver metastasis. Surgical material was available for 36 cases, whereas for 3 cases only endoscopically removed biopsies were available. This study was conducted according to the clinical standards of the 1975 and 1983 Declaration of Helsinki.

Morphologic Investigation

All tissues were fixed in buffered formalin (formaldehyde 4% wt/vol and acetate buffer 0.05 M) and routinely processed in paraffin wax. Five-micrometer-thick sections were stained with hematoxylin-eosin and alcian-blue/periodic acid-Schiff. The 39 colorectal NECs were thoroughly investigated for the following parameters: cytologic subtype (small-cell, large-cell, intermediate-cell types), number of mitoses per 10 HPF, presence of necrosis (absent vs. focal or extensive with typical "geographic chart" appearance), lymphovascular invasion evaluated in both H&E-stained and CD34-stained sections, perineural invasion, infiltrative or expanding type of growth, size, level of colonic wall invasion, peritumoral lymphoid infiltration, staging according to UICC (2010), and ENETS.[39]

Immunohistochemistry

For immunohistochemistry, 3-μm-thick sections were mounted on poly-L-lysine–coated slides, deparaffinized, and hydrated through graded alcohols to water. After endogenous peroxidase activity inhibition, performed by dipping sections in 3% hydrogen peroxide for 10 minutes, incubation with primary antibodies (Table 1) was carried out at 4°C for 18–20 hours, followed by the avidin-biotin complex procedure. Immunoreactions were developed using 0.03% 3,3′diaminobenzidine tetrahydrochloride, and then sections were counterstained with Harris hematoxylin. A tumor was considered positive when at least 5% of cells were immunoreactive for a specific antibody.

MSI Analysis and Methylation-specific Multiplex Ligation Probe Amplification (MS-MLPA)

Tumor DNA of each patient was obtained from formalin-fixed and paraffin-embedded tissues using representative 8-μm-thick sections of tumor samples. Three sections of every specimen were treated twice with xylene and then washed twice with ethanol. DNA was extracted using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Neuroendocrine neoplastic areas were manually microdissected for DNA extraction and contained at least 70% of tumor cells to minimize contamination by normal cells.[13]

MSI analysis was carried out using 5 mononucleotide repeat microsatellite targets (BAT-25, BAT-26, NR-21, NR-24, and NR-22) in a pentaplex PCR system according to the protocol previously reported.[44] The PCR fragments were separated using an ABI Prism 310 Genetic Analyser and further analyzed using GeneMapper 4.0 (Applied Biosystems, Foster City, CA). MSI at high

TABLE 1. Antibodies and Antisera Used

Antibodies/Antisera	P/M (Clone)	Dilution	Source
Synaptophysin	M (snp88)	1:100	BioGenex Laboratories, San Ramon, CA
Chromogranin A	M (LK2H10)	1:1	Ventana, Tucson, AZ
NSE	M (MIG-N3)	1:200	Monosan, Uden, The Netherlands
CD56	M (123C3)	1:100	Monosan
TTF1	M (SPT24)	1:100	Novocastra, Newcastle, UK
ASH1	M (24B72D11.1)	1:100	BD Biosciences, San Jose, CA
CD117	P	1:100	Dako, Glostrup, Denmark
CD34	M (QBE nd/10)	1:1	Novocastra
PAX5	M (C-20)	1:2000	Santa Cruz, Biotechnology, Santa Cruz, CA
Cytokeratin 20	M (Ks 20.8)	1:100	Dako
Cytokeratin 7	M (OV-TL 12/30)	1:200	Dako
CDX2	M (CDX2-88)	1:100	BioGenex Laboratories
hMLH1	M (G-168.15)	1:100	Pharmingen, San Diego, CA
hMSH2	M (Fe11)	1:100	Oncogene, Cambridge, MA
hMSH6	M (44)	1:100	BD Transduction Laboratories, Lexington, KY
hPMS2	M (A16-4)	1:100	BD Biosciences
p53	M (D07)	1:500	Dako
Ki67	M (MIB1)	1:100	Dako

NSE indicates neuron-specific enolase; P/M, polyclonal/monoclonal.

frequency was scored when at least 40% of the microsatellites analyzed were unstable.

The SALSA MS-MLPA ME001 tumor suppressor-1 kit and SALSA MS-MLPA ME002 tumor suppressor-2 kit (MRC-Holland, Amsterdam, The Netherlands) were used to perform promoter methylation analysis on all tumors included in the study. A total of 34 different tumor suppressor genes (Table 2) were examined by MS-MLPA according to the manufacturer's instructions after checking each DNA sample for integrity and amplifiability by BIOMED-2 multiplex PCR.[45] The MS-MLPA products were separated by capillary electrophoresis on an ABI 310 Automatic DNA Sequencer (Applied Biosystems) and analyzed by GeneMapper 4.0 genotyping software (Applied Biosystems). Two replicates were performed for each sample. Values corresponding to peak size in base pairs and peak areas were used for further data processing using Coffalyser V7 software (MRC-Holland), and the methylation ratio (MR) was calculated by dividing each normalized peak value of the digested sample by that of the corresponding undigested sample. Sensitivity and specificity of the MS-MLPA assay were determined by a titration experiment, mixing fully methylated DNA (CpGenome Universal Methylated DNA, Millipore) with unmethylated DNA (CpGenome Universal UnMethylated DNA, Millipore) in dilutions of 0%, 10%, 30%, 50%, 80%, and 100%. MRs obtained in the titration experiments were evaluated by receiver operating characteristic (ROC) curve analysis.[49] For each gene we defined the best cutoff points of MR to score the presence of aberrant methylation as a categorical variable (Table 2).

Statistical Analysis

The cutoff value of the MR of each gene with corresponding sensitivity, specificity, and accuracy was estimated by ROC curve analysis.[49] The best statistical cutoff was calculated by minimizing the distance between the point with specificity = 1 and sensitivity = 1 and the points on the ROC curve.

Correlation analyses were performed using the Fisher exact test, analysis of variance, and the independent sample *t* test. The relationship between morphologic and immunohistochemical variables and patient survival was evaluated using the Kaplan-Meier method and statistically checked with the log-rank test. All predictors statistically correlated with survival on univariable study were included in a multivariable analysis using the Cox proportional regression model. A *P*-value of < 0.05 was considered statistically significant. All analyses were performed using MedCalc Statistical Software (version 11.0.1.0).

RESULTS

Clinicopathologic Findings

Of the 39 cases included in the study, 12 showed a non-neuroendocrine component representing at least 30% of the tumor tissue, and, for this reason, they were reclassified as MANECs, in line with the 2010 WHO classification.[37] In 2 MANECs the non-neuroendocrine component was represented by squamous cell carcinoma, in 4 by adenocarcinoma, in 4 by adenoma, and in 2 by adenoma combined with squamous cell carcinoma. All the cases showing the squamous cell carcinoma component were localized in the right colon. In addition to MANECs, a focal non-neuroendocrine component (< 30% of the tumor tissue) was also observed in 11 other NECs. The patients' main clinicopathologic features are summarized in Table 3. Both NECs and MANECs were more frequently localized in the right colon and were more frequently observed in men (M:F = 1.4:1) with an average age of 67 years (range, 34 to 87 y). However, patients with MANECs showed a slightly higher average age (72 y) at diagnosis than did patients with NECs (65 y). Most tumors (77%) showed lymph node metastases at the

TABLE 2. Genes Examined by MS-MLPA Analysis

No.	Gene	Position	ID Number	Amplicon Size	MR (Cutoff Used)
1	TP73	1p36.3	NM_005427.2	400*; 238†	0.32
2	CASP8	2q33.2	NM_001228	265*	0.41
3	VHL	3p25.3	NM_000551	355*; 265†	0.17
4	RARB	3p24.2	NM_000965	193*; 454†	0.27
5	MLH1	3p22.3	NM_000249	166*; 463*	0.2
6	RASSF1	3p21.3	NM_007182	328*; 382*	0.57
7	FHIT	3p14.2	NM_002012	409*	0.24
8	APC	5q22	NM_000038	148*	0.44
9	ESR1	6q25.1	NM_000125	373*; 301†	0.42
10	PAX5A	9p13	NM_016734	211†	0.3
11	CDKN2A (p14)	9p21.3	NM_058195	160*	0.24
12	CDKN2A (p16)	9p21.3	NM_058195	427†	0.36
13	DAPK1	9q22	NM_004938	346*	0.22
14	PTEN	10q23.3	NM_000314	292*; 184†	0.16
15	MGMT	10q26.3	NM_002412	193†; 382†	0.38
16	CD44	11p12	NM_000610	319*; 463†	0.66
17	GSTP1	11q13	NM_000852	454*; 274†	0.28
18	PAX6	11p13	NM_001604	409†	0.23
19	WT1	11p13	NM_000378	247†	0.31
20	ATM	11q23	NM_000051	184*; 160†	0.26
21	IGSF4	11q23	NM_014333	427*; 355†	0.35
22	CDKN1B	12p13.2	NM_004064	274*	0.25
23	CHFR	12q24.3	NM_00116134	238*; 292†	0.34
24	BRCA2	13q13.1	NM_000059	301*; 148†	0.36
25	RB1	13q14.2	NM_000321	319†; 472†	0.2
26	THBS1	15q15	NM_003246	346†	0.25
27	PYCARD	16p11.2	NM_013258	400†	0.21
28	CDH13	16q23.3	NM_001257	436*; 220†	0.58
29	TP53	17p13.1	NM_000546	166	0.21
30	HIC1	17p13.3	NM_006497	220*	0.22
31	BRCA1	17q21.3	NM_007294	247*; 142†	0.31
32	STK11	19p13.3	NM_000455	373†	0.23
33	GATA5	20q13.3	NM_080473	436†	0.24
34	TIMP3	22q12.3	NM_000362	142*	0.28

*ME001-C1 MS-MLPA 1.
†ME002-A1 MS-MLPA kit (MRC-Holland).
Probe sequences and the locations of the target *Hha*I sites are given in the MRC-Holland web site.

time of diagnosis, whereas distant metastases at the time of diagnosis were observed in 10 cases (8 NECs and 2 MANECs). Most tumors (89%), both NECs and MANECs, were diagnosed at stage III or IV, independently of the scheme used (UICC or ENETS).

The morphologic features are summarized in Table 4 and shown in Figure 1. The majority (79%) of tumors were composed of small-to-intermediate cells, whereas only 8 (21%) carcinomas (6 NECs and 2 MANECs) were composed purely of large cells. Lymphatic vessel infiltration was observed in all but one case; perineural invasion was observed in the majority of carcinomas, as was vascular invasion. Necrosis, either focal or diffuse, was observed in 33/39 (92%) cases. In 8/39 (21%) cases (7 NECs and 1 MANEC) a significant lymphoid infiltrate was observed at the periphery of the neoplasms.

Immunohistochemical Profile

Immunohistochemical findings are summarized in Table 5. All tumors were first tested for chromogranin A and synaptophysin expression, and both were generally well expressed. For 5 cancers in which these markers were negative, CD56 and neuron-specific enolase immunostainings were performed to confirm the neuroendocrine nature of the tumors. Immunoreactivity for TTF1 and ASH1, 2 TFs known to be expressed in lung NECs, was observed in 18% and 26% of cases, respectively, whereas the expression of CDX2, a TF expressed in intestinal neoplasms, was found in 29/39 (74%) carcinomas (19 NECS and 10 MANECs). We decided to use the expression of TTF1 and/or ASH1 in the absence of CDX2 to define a "lung-type" NEC or MANEC (5 cases), whereas the expression of CDX2 in the absence of both TTF1 and ASH1 identified an "intestinal-type" NEC or MANEC (21 cases). In addition to these 2 subgroups, 6 cancers expressing both CDX2 and TTF1 and/or ASH1 (mixed type) and 7 cases negative for all of these TFs (null type) were found. These subgroups of tumors did not show a prognostic significance in this specific tumor series but were correlated with morphologic cell features. In this respect it is worth noting that 7/8 (87.5%) large-cell carcinomas showed an "intestinal-type" phenotype versus 14/31 (45%) non–large-cell type. The expression of hematopoietic stem cell markers including CD117, CD34, and PAX5 was observed in 54%, 8%, and 18% of cases,

TABLE 3. Clinicopathologic Characteristics

	NECs 27 Cases	MANECs 12 Cases	Total 39 Cases
Sex			
Male	15 (60%)	8 (67%)	23 (59%)
Female	12 (40%)	4 (33%)	16 (41%)
Age (y)			
Average	65	72	67
Range	34-85	51-87	34-87
Location*			
Right colon	17 (65%)	8 (67%)	25 (64%)
Left colon	9 (35%)	4 (33%)	13 (33%)
Lymph node metastasis	19 (70%)	11 (92%)	30 (77%)
Distant metastasis	8 (30%)	2 (17%)	10 (26%)
Tumor stage (UICC)†			
Stage I	0	0	0
Stage II	4 (17%)	0	4 (11%)
Stage III	12 (50%)	10 (83%)	22 (61%)
Stage IV	8 (33%)	2 (17%)	10 (28%)
Tumor stage (ENETS)†			
Stage I	0	0	0
Stage II	3 (13%)	0	3 (8%)
Stage III	13 (54%)	10 (83%)	23 (64%)
Stage IV	8 (33%)	2 (17%)	10 (28%)
Follow-up (mo)			
Mean time	43.5	16.6	35.8
Range time	1-257	1-72	1-257
DOC	2 (7%)	0	2 (5%)
DOD	19 (70%)	7 (59%)	26 (67%)
AFD	4 (15%)	3 (25%)	7 (18%)
POD	1 (4%)	1 (8%)	2 (5%)
Lost	1 (4%)	1 (8%)	2 (5%)

*For 1 case information regarding tumor site was not available.
†For 3 endoscopic NECs staging was not performed.
AFD indicates alive and free of disease; DOC, died of other causes; DOD, died of disease; POD, postoperative death.

respectively. p53 nuclear immunoreactivity was found in all MANECs and in 70% of NECs. Both CK7 and CK20 were found in a small group of tumors (Table 5).

TABLE 4. Morphologic and Proliferative Features

	NECs 27 Cases	MANECs 12 Cases	Total 39 Cases
Cell types			
SC	4 (15%)	1 (8%)	5 (13%)
SC/IC	8 (30%)	5 (42%)	13 (33%)
IC	4 (15%)	3 (25%)	7 (18%)
LC/IC	5 (18%)	1 (8%)	6 (15%)
LC	6 (22%)	2 (17%)	8 (21%)
Mean mitotic count	61.4	34.5	53.2
Mitotic range	15-140	20-60	15-140
Mean Ki67 index	66%	60%	64%
Ki67 index range	40-90%	40-90%	40-90%
Lymphatic invasion*	24 (100%)	11 (92%)	35 (97%)
Angioinvasion*	18 (75%)	11 (92%)	29 (80%)
Perineural invasion*	23 (96%)	11 (92%)	34 (94%)
Necrosis*	23 (96%)	10 (83%)	33 (92%)
Peritumoral lymphocytes*	7 (29%)	1 (8%)	8 (21%)

*For 3 NECs only endoscopic biopsies were available; hence, the parameter was not evaluable.
IC indicates intermediate cells; LC, large cells; SC, small cells.

No significant morphologic and immunohistochemical differences were observed between tumors located on the right and left, although carcinomas of the right colon were more frequently of "intestinal-type" with extensive CDX2 expression.

Methylation Profiles and MSI Status

Methylation analysis of all 34 genes was possible in 30 of the 39 tumor samples included in the study. Three genes, namely *ESR1*, *WT1*, and *GATA5,* were methylated in $>70\%$ of cases. Intermediate levels of methylation between 10% and 50% were observed for the following genes: *CDH13*, *CHFR*, *DAPK1*, *CASP8*, *RARB*, *TIMP3*, *PTEN*, *APC*, *PAX5A*, *IGSF4*, *RASSF1*, *MGMT*, *HIC1,* *PYCARD*, *THBS1*, and *GSTP1*. The remaining genes were rarely methylated, showing methylation in $<10\%$ of cases (Fig. 2). A subgroup of 11 tumors (37% of cases) showed extensive gene methylation involving $>25\%$ of the promoters examined, suggesting the presence of a CIMP+ phenotype. The methylation of the following 10 genes was significantly correlated with CIMP+: *HIC1* ($P<0.0002$), *DAPK1* ($P=0.0007$), *THBS1* ($P=0.001$), *PTEN* ($P=0.005$), *TIMP3* ($P=0.006$), *IGSF4* ($P=0.007$), *RARB* ($P=0.007$), *GSTP1* ($P=0.02$), *PAX5A* ($P=0.03$), and *MGMT* ($P=0.03$).

The presence of MSI was observed in 5 of 34 tumors (15% of cases), and it was positively associated with a CIMP+ phenotype (100% of MSI tumors vs. 63% of MSS tumors; $P=0.02$). MSI/CIMP+ cancers were all negative for hMHL1 and hPMS2 protein expression, did not show an exocrine component, were more frequently of large-cell subtype, and showed an abundant peritumoral lymphoid infiltrate. In contrast, they showed neither vascular invasion nor CD117 immunoreactivity, and none of them was diagnosed at stage IV (Fig. 3).

Survival Analysis

Follow-up information was available for all but 2 patients. The mean follow-up time was 35.8 months: 43.5 months for NECs and 16.6 months for MANECs. Twenty-six of 39 (67%) patients died of disease: specifically, 19/27 (70%) patients with NECs and 7/12 (59%) with MANECs. Interestingly, 7 patients (4 with NECs and 3 with MANECs) were alive and free of disease after a mean follow-up time of 104 months (range, 11 to 257 mo), and 2 other patients who died of unrelated causes after a mean follow-up time of 190 months (range, 150 to 230 mo) should be added to this group. No statistically significant ($P=0.82$) different survival rate was observed between patients with NECs and MANECs (Fig. 4). For this reason, the prognostic impact of all parameters considered has been evaluated on the entire series of 39 cancers, independently of the NEC or MANEC diagnosis.

Factors statistically influencing survival on the univariable analysis (Fig. 4) were large-cell type versus non–large-cell type ($P=0.009$), presence of vascular invasion ($P=0.0002$) and of peritumoral lymphoid infiltration ($P=0.005$), CD117 immunoreactivity

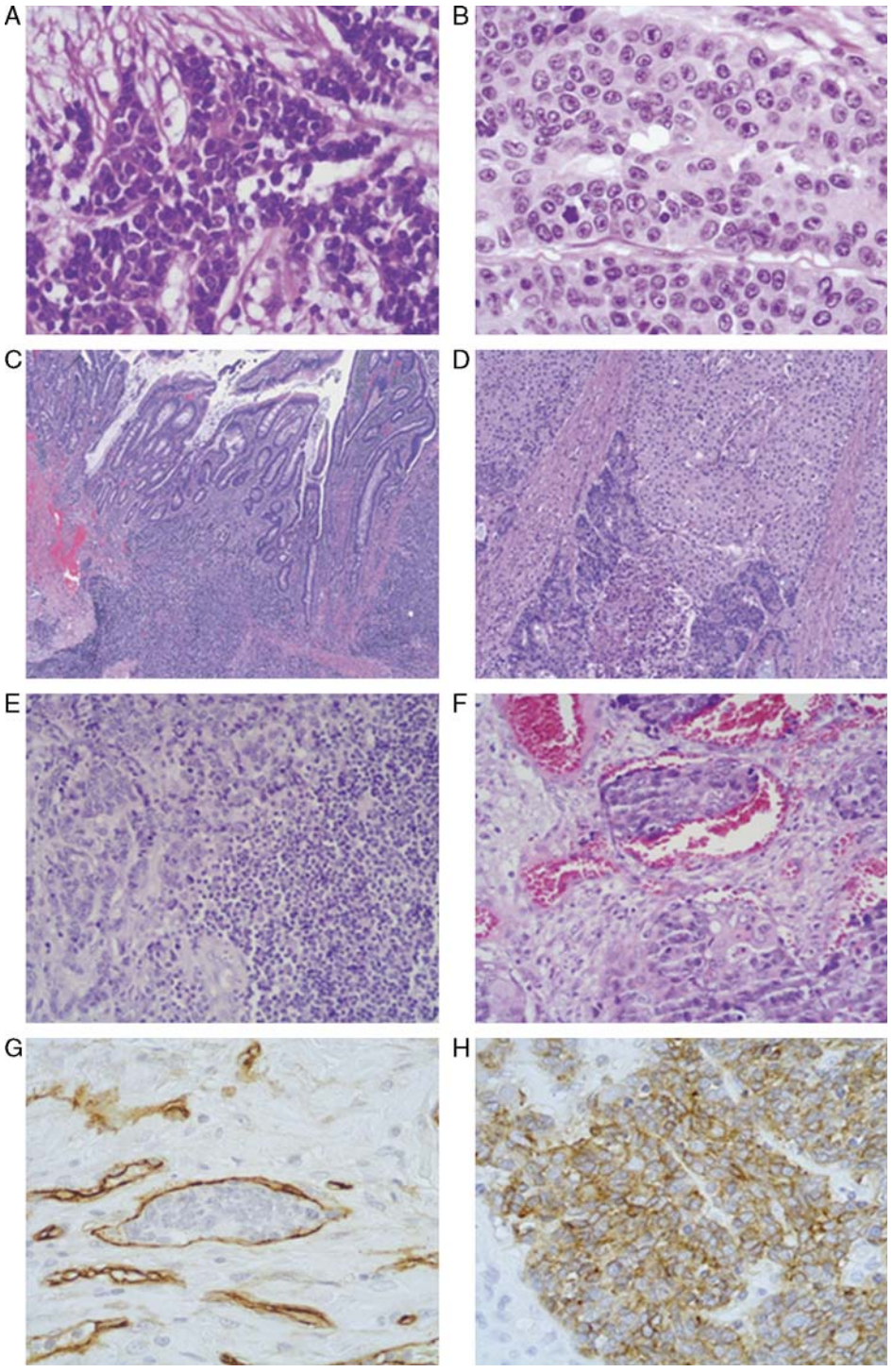

FIGURE 1. Small-cell NEC is composed of atypical small cells with hyperchromic nuclei without nucleoli and scarce cytoplasm (A), whereas the large-cell subtype is composed of atypical large cells with nuclei showing dispersed chromatin, well-evident nucleoli, and abundant eosinophilic cytoplasm (B). C, An example of a MANEC composed of a tubulovillous adenoma (top) and a small-cell NEC infiltrating the bowel wall (bottom). D, An example of a MANEC composed of a moderately differentiated adenocarcinoma and a large-cell NEC invading the muscular layer. An abundant lymphoid infiltration (E) at the tumor periphery was observed in some cases and it was found to be a good prognostic marker on univariable statistical analysis. In contrast, vascular invasion, detected in both H&E-stained (F) and CD34 (G)-stained sections, was an independent worse prognostic factor, as well as CD117 expression (H).

TABLE 5. Immunohistochemical Findings

	NECs 27 Cases	MANECs 12 Cases	Total 39 Cases
Chromogranin A	8 (30%)	6 (50%)	14 (36%)
Synaptophysin	21 (77%)	12 (100%)	33 (85%)
TTF1	4 (15%)	3 (25%)	7 (18%)
ASH1	8 (30%)	2 (17%)	10 (26%)
CD117	12 (44%)	9 (75%)	21 (54%)
PAX5	5 (18%)	2 (17%)	7 (18%)
CD34	2 (7%)	1 (8%)	3 (8%)
CK20	1 (4%)	5 (42%)	6 (15%)
CK7	2 (7%)	3 (25%)	5 (13%)
CDX2	19 (70%)	10 (83%)	29 (74%)
p53	19 (70%)	12 (100%)	31 (79%)

($P = 0.004$), and MSI/CIMP+ status versus stable (MSS) carcinomas with or without CIMP ($P = 0.04$). These parameters were included in a multivariable analysis, and,

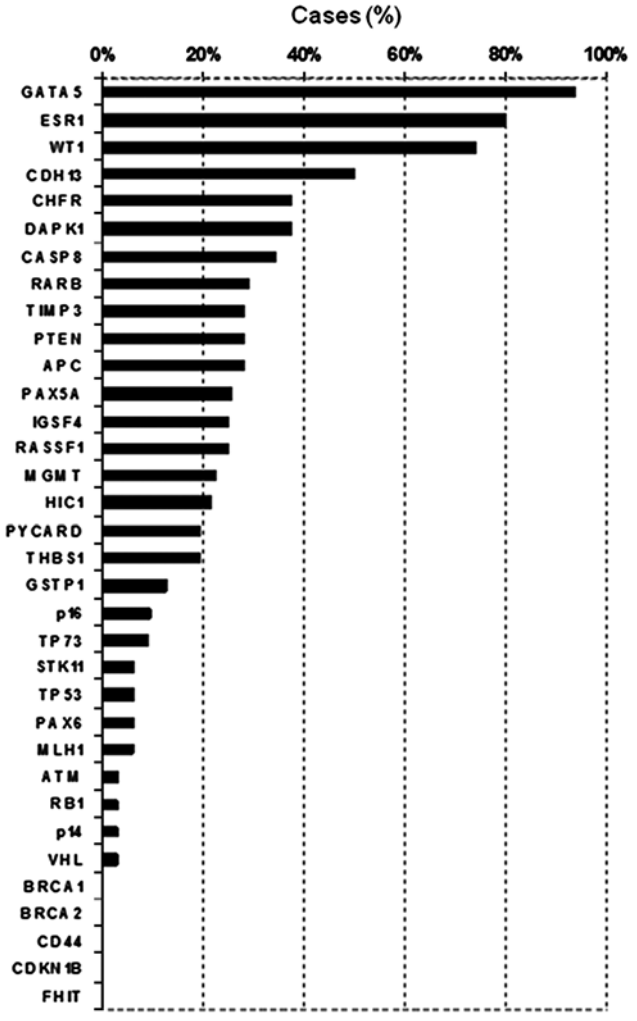

FIGURE 2. Gene methylation rates in the 34 tumors analyzed by MS-MLPA.

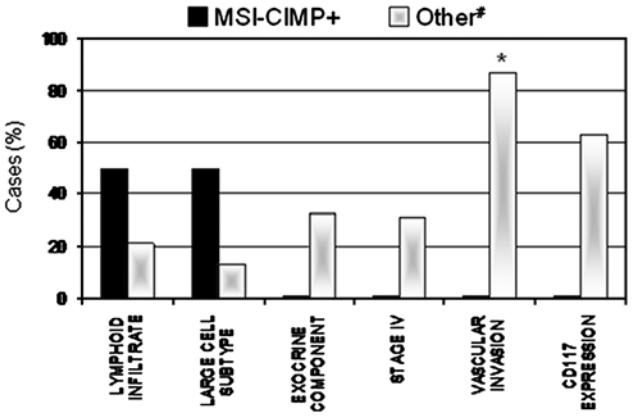

FIGURE 3. Main clinicopathologic features differentiating MSI-CIMP+ NECs/MANECs (black column) from the other tumors (#) that included stable NECs/MANECs with or without CIMP profile (gray column). *$P = 0.003$.

among them, vascular invasion ($P = 0.006$; HR: 9.7; 95% CI, 1.893-50.353) and CD117 expression ($P = 0.02$, HR: 3.995; 95% CI,1.195-13.078) were independent prognostic parameters correlating with shorter patient survival.

DISCUSSION

Colorectal NECs are high-grade malignant neoplasms composed of small-to-intermediate or large cells and showing an organoid structure, multifocal necrosis, and high proliferation. Although the presence of an exocrine component in gastrointestinal NECs has often been reported, MANECs are rare and are, by definition, neoplasms in which either component represents at least 30% of the lesion.[37] In the present series we subdivided the 39 enrolled cases into 27 NECs and 12 MANECs, which showed similar clinical features including prognosis. This is not surprising considering that the neuroendocrine component observed in MANECs was represented by high-grade NECs that negatively influenced the prognosis even when the exocrine component was an adenocarcinoma or a squamous cell carcinoma.[30] It has to be underlined that this result is not in line with findings reported by Shia et al,[42] who observed that patients with NECs had a survival rate worse than that of patients with MANECs. However, the series they investigated included both gastric and colonic neoplasms, and the result may have been influenced by the presence of gastric neoplasms for which a different survival rate between NECs and MANECs has been previously demonstrated,[27] suggesting that some clinical differences between NECs and MANECs may be site related.[25]

In the present series, patients with NECs or MANECs exclusively composed of large neuroendocrine cells showed a better survival rate compared with patients with small-to-intermediate-cell or mixed large-to-intermediate-cell carcinomas. An association with a better prognosis has also been registered in a series of gastric large-cell NECs[27] but was not found in cases of gastrointestinal

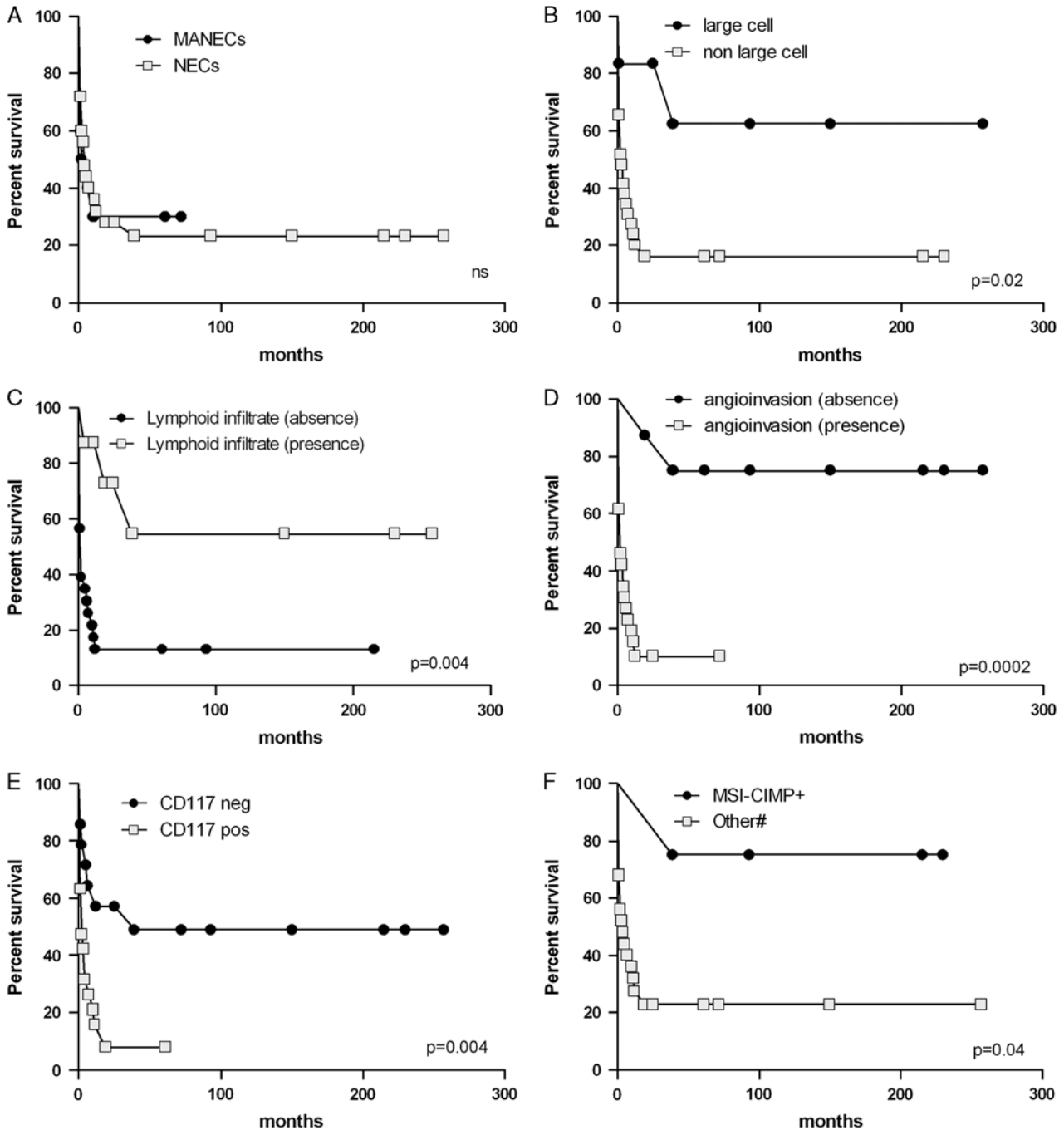

FIGURE 4. Kaplan-Meier curves demonstrating that survival of patients with NECs is not statistically different from that of patients with MANECs (A). Large-cell subtype (B), the presence of peritumoral lymphoid infiltrate (C), and a MSI/CIMP+ profile (F) correlated with a better patient survival, whereas vascular invasion (D) and CD117 immunoreactivity (E) were associated with a worse patient outcome on univariable analysis.

tumors examined by Shia et al.[42] The difference in survival rates we observed between large-cell and non–large-cell colorectal NECs/MANECs is interesting because it underlines the importance of an easily detectable prognostic parameter and suggests a different biology of these 2 tumor subgroups. Another interesting and not previously reported finding for colonic NECs is the favorable prognostic meaning of the peritumoral lymphoid infiltration, which indicates a host lymphoid response to tumor and which has already been positively correlated with better survival of patients with colorectal adenocarcinomas.[9,10,35] Interestingly, Dahlin and colleagues have recently demonstrated that patients with colorectal cancers showing abundant T-cell lymphoid infiltration had a longer survival compared

with patients with colorectal carcinomas showing scarce lymphoid response. A high total T-cell score maintained its statistical prognostic power in multivariable analysis also, in which other parameters including MSI and CIMP status lost their prognostic meaning.[10] However, in the present study the prognostic power of peritumoral lymphoid infiltrate and that of the large-cell subtype was lost in the multivariable analysis; hence, their usefulness needs to be checked in future studies based on a larger tumor series. Vascular invasion was an independent prognostic marker on multivariable analysis, and for this reason it appears to be a significant morphologic parameter that must be included in the pathology report. The prognostic impact of vascular invasion is not surprising considering that it has been previously demonstrated in gastroenteropancreatic neuroendocrine neoplasms.[27,28]

The extensive immunohistochemical investigation we performed included the assessment of the neuroendocrine profile, p53, and, for the first time, the expression of several TFs, stem cell markers, and cytokeratins 7 and 20. Synaptophysin was expressed in the majority of tumors with a higher percentage than chromogranin A, and this result is in line with previously published data.[42] However, we did not observe any relationship between synaptophysin expression and survival as described by Shia et al.[42] The expression of p53 in the majority of tumors completely confirmed previous findings demonstrating p53 nuclear immunoreactivity and p53 gene mutation in NECs of different sites.[12,27,28]

The TTF1 and ASH1 expression that we observed in a subgroup of tumors is not surprising, considering that these TFs have been identified previously in gastrointestinal NECs.[26,43] Interestingly, we found CDX2 nuclear immunoreactivity in 29/39 (74%) neoplasms, suggesting its possible role as a diagnostic marker indicating the intestinal origin. However, CDX2 expression has already been reported in a small number of lung NECs[5,29]; therefore CDX2, although more frequently expressed in intestinal NECs, cannot be considered as a univocal marker of intestinal origin. On the basis of the expression of TTF1, ASH1, and CDX2 we tentatively subdivided tumors into 4 groups (see Materials and methods) with the aim of identifying different phenotypic/diagnostic/prognostic categories: lung-type, intestinal-type, mixed-type, and null-type colorectal NECs/MANECs. Although these subgroups of tumors did not show a prognostic significance, they should be considered for diagnostic purposes because they indicate the phenotypical heterogeneity of colorectal NECs. In this respect it is worth noting that 7/8 (87.5%) large-cell carcinomas showed an "intestinal-type" phenotype versus 14/31 (45%) non–large-cell type. We also investigated 2 hematopoietic stem cell markers: CD117 and CD34. Immunoreactivity for CD117 has already been described in neuroendocrine neoplasms, and it has also been suggested as a marker of malignancy for pancreatic neuroendocrine tumors and NECs.[11,47,48] Interestingly, in the present series, CD117 immunoreactivity was found in 21/39 (54%) neoplasms and was correlated with a worse prognosis on univariable and multivariable analyses. Previous investigations did not demonstrate c-kit gene mutation in CD117 immunoreactive colorectal NECs.[2,20] This suggests that in this cancer subset CD117 overexpression may not be mediated through activating mutations, indicating that imatinib mesylate (Glivec) therapy may not be useful in these patients.[15]

The usefulness of cytokeratins 7 and 20 in the diagnostic pathway of colorectal NECs has not been widely investigated yet. A few papers have reported that gastrointestinal NECs did not express CK7 and CK20,[8,22,34] although Kato et al[21] described a large-cell NEC of the colon that was CK20 positive. We have systematically investigated the expression of these 2 cytokeratins in all 39 neoplasms and found that they were expressed in only a small fraction of tumors and did not show any correlation with prognosis. However, this finding may be important for diagnostic purposes. The knowledge that a fraction of colorectal NECs/MANECs can be CK20 and CK7 positive should be taken into account for the differential diagnosis with poorly differentiated adenocarcinomas, especially in small bioptic specimens.

In addition to these morphologic and immunohistochemical features, new insights have been provided by the methylation profiling and MSI analysis. To the best of our knowledge, our study is the second one investigating gene methylation and MSI status in a relevant number of colorectal NECs. In agreement with our results, Arnold et al[3] found that aberrant DNA methylation is a common event in neuroendocrine tumors of the colon and rectum, showing comparable frequencies to those usually observed in exocrine colorectal carcinomas.[23] Moreover, our data support the findings reported by previous studies that NECs/MANECs are characterized by high frequencies of methylation of specific genes known to be rarely methylated in exocrine colorectal carcinomas. Specifically, we observed high frequencies of *APC*, *CASP8*, and *RASSF1* methylation, which has been shown to be a common event in neuroendocrine tumors from different anatomic sites.[4,19,31,32] These findings suggest that there are significant differences in DNA methylation profiles between NECs/MANECs and exocrine colorectal carcinomas, emphasizing that epigenetic mechanisms target different genes in these 2 tumor classes.

In contrast, a novel result of our work was the observation that some genes, known to exhibit a cancer-specific hypermethylation in exocrine colorectal carcinomas, are also methylated in NECs/MANECs. Among these genes, the extensive methylation of GATA5 in over 90% of the tumors examined (Fig. 2) is of particular interest. Indeed, methylation of GATA4/5 is a common and specific event in exocrine colorectal carcinomas, and recent studies have highlighted its potential diagnostic value for early detection and risk assessment of colonic cancer.[17,33] Our analysis has also highlighted the potential utility of the *WT*1 gene as an early diagnostic marker of NECs/MANECs because of its extensive methylation in the present series. To date, a widespread methylation of this

gene has been reported in colorectal cancer, although very few studies have been published on this issue.[18,46] Taken together, these data have important clinical and scientific implications because they support the hypothesis that specific epigenetic changes may be the driving force in the earliest steps of colorectal tumorigenesis[24,41] and may characterize tumors destined to progress differently, such as exocrine colorectal carcinomas and NECs/MANECs. This idea is supported by a lot of evidence demonstrating that silencing of genes by methylation plays a role in the initiation of colorectal tumors and tumors of other sites.[6,7]

Another aim of the present work was to analyze the MSI status to evaluate the frequency of this marker in NECs/MANECs and the potential association between MSI and CIMP phenotype. A few previous studies reported that MSI is infrequent in NECs,[3,4,14] but the series examined were too small to draw final conclusions, and no information was available about the association between MSI and CIMP phenotype. In this study, we demonstrated that MSI was present in 15% of NECs/MANECs (5 of 34 tumors), and all the 4 MSI tumors in which the methylation analysis was possible showed a CIMP phenotype. For the first time, these results suggest that MSI is present in NEC/MANECs and in exocrine colorectal cancers with very similar frequencies and confirm that a close association between MSI and the CIMP phenotype is evident in NECs in the same way as in exocrine colorectal cancers. The important clinical implication of this finding is that CIMP+ tumors with MSI represent a distinct entity associated with a better prognosis than the remaining cancers (Fig. 4F). In general, our molecular investigation demonstrated that aberrant gene methylation is a common abnormality in NEC/MANECs and that a specific epigenetic characterization of these tumors may provide clues for early diagnostic markers and for tumor-specific methylation profiles. The simultaneous presence of MSI and a widespread gene methylation seem to be a predictor of better outcome in patients with NEC/MANECs. For the future we are planning a study of a cohort of grade-matched and stage-matched colorectal adenocarcinomas without any neuroendocrine differentiation to verify whether they share some of the new molecular findings that we have detected in NEC/MANECs.

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