Microsatellite unstable gastrointestinal neuroendocrine carcinomas: a new clinicopathologic entity

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Abstract

Gastroenteropancreatic (GEP) neuroendocrine carcinomas (NECs) and mixed adenoneuroendocrine carcinomas (MANECs) are heterogeneous neoplasms characterized by poor outcome. Microsatellite instability (MSI) has recently been found in colorectal NECs showing a better prognosis than expected. However, the frequency of MSI in a large series of GEP-NEC/MANECs is still unknown. In this work, we investigated the incidence of MSI in GEP-NEC/MANECs and characterized their clinicopathologic and molecular features. MSI analysis and immunohistochemistry for mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) were performed in 89 GEP-NEC/MANECs (six esophageal, 77 gastrointestinal, three pancreatic, and three of the gallbladder). Methylation of 34 genes was studied by methylation-specific multiplex ligation probe amplification. Mutation analysis of *BRAF* and *KRAS* was assessed by PCR-pyrosequencing analysis. MSI was observed in 11 NEC/MANECs (12.4%): seven intestinal and four gastric. All but two MSI-cases showed *MLH1* methylation and loss of MLH1 protein. The remaining two MSI-cancers showed lack of MSH2 or PMS2 immunohistochemical expression. MSI-NEC/MANECs showed higher methylation levels than microsatellite stable NEC/MANECs (40.6% vs 20.2% methylated genes respectively, $P<0.001$). *BRAF* mutation was detected in six out of 88 cases (7%) and *KRAS* mutation was identified in 15 cases (17%). *BRAF* mutation was associated with MSI ($P<0.0008$), while *KRAS* status did not correlate with any clinicopathologic or molecular feature. Vascular invasion ($P=0.0003$) and MSI ($P=0.0084$) were identified as the only independent prognostic factors in multivariate analysis. We conclude that MSI identifies a subset of gastric and intestinal NEC/MANECs with distinct biology and better prognosis. MSI-NEC/MANECs resemble MSI-gastrointestinal adenocarcinomas for frequency, molecular profile and pathogenetic mechanisms.

Key Words
- microsatellite instability
- mismatch repair
- gene methylation
- neuroendocrine carcinomas
- NEC
- MANEC

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Introduction

High-grade (or poorly differentiated) gastroenteropancreatic (GEP) neuroendocrine carcinomas (NECs) are aggressive cancers with a high propensity for distant metastases. Like the more frequent pulmonary counterparts, they have traditionally been divided into the small- and large-cell subtypes, depending on the morphological features of neoplastic cells (La Rosa & Sessa 2014). Small-cell carcinomas are composed of small to medium-sized (two to four times the size of a lymphocyte), round to oval cells with scant cytoplasm and hyperchromatic nuclei with indistinct nucleoli. Large-cell subtypes are composed of large cells with vesicular nuclei showing prominent nucleoli and abundant eosinophilic cytoplasm. In both cases, tumor cells grow forming sheets or nests, although in the large-cell subtype a more structured trabecular or organoid architecture is frequently observed. Infiltration of the gut wall or peripancreatic tissue, extensive necrosis, high mitotic count, and perineural and vascular invasion is the rule.

Cancers with these morphological features have been named in the past few years using different names including poorly differentiated NECs and high-grade NECs. Accordingly to the 2010 WHO classification of tumors of the digestive system, they are currently named NECs and, by definition, they are grade 3 (G3) neoplasms characterized by more than 20 mitoses per ten high power fields (HPF) and/or a Ki67 proliferative index >20%, independently of the morphological features (Rindi *et al.* 2010). NECs express general neuroendocrine markers such as synaptophysin and chromogranin A and may be associated with a non-neuroendocrine component. When both the neuroendocrine and non-neuroendocrine components are conspicuous, representing at least 30% of the neoplastic tissue, tumors are classified as mixed adenoneuroendocrine carcinomas (MANECs; Rindi *et al.* 2010). Both the exocrine and neuroendocrine components can have different morphological features, ranging from adenomas to adenocarcinomas or squamous cell carcinomas with different degrees of differentiation in exocrine components and from well-differentiated to poorly differentiated neuroendocrine tumors in neuroendocrine components (La Rosa *et al.* 2012*a*,*b*).

The pathogenesis of GEP-NECs and MANECs is still largely unknown (Smith & Reidy-Lagunes 2013). Regardless of anatomic site, *TP53* alterations have been found to be frequent in NECs (Dacic *et al.* 2002, Furlan *et al.* 2005), together with the impairment of the CDKN2A/p16-Rb pathway (Parwani *et al.* 2003, Nassar *et al.* 2005, Yachida *et al.* 2012). Most GEP-NECs have high chromosomal instability (Furlan *et al.* 2005, Lubensky & Zhuang 2007), while microsatellite instability (MSI) has rarely been observed (Ghimenti *et al.* 1999, Nassar *et al.* 2005, Stelow *et al.* 2006, La Rosa *et al.* 2012*a*,*b*) and the role of other epigenetic mechanisms is still poorly understood.

Although GEP-NECs are generally aggressive cancers, showing ominous prognosis with a median survival (MS) ranging from 6 to 12 months (La Rosa & Sessa 2014), recent findings from a few published studies (Shia *et al.* 2008, Power *et al.* 2010, La Rosa *et al.* 2012*a*,*b*) and from anecdotal experience seem to suggest that there is a fraction of patients with GEP-NECs showing a better than expected survival rate. We have recently demonstrated that a subset of colorectal NECs exhibiting MSI and extensive gene hypermethylation showed a better prognosis than NECs without these features (La Rosa *et al.* 2012*a*,*b*). Apparent similarities between MSI-NECs and MSI-adenocarcinomas of the colon–rectum has led to the hypothesis that similar pathogenetic mechanisms may be involved in the development of these two tumor subsets (Furlan *et al.* 2013). However, there are no studies on large series of GEP-NECs demonstrating that MSI and gene hypermethylation can occur in other sites of the GEP system and that this is related to patients' prognosis.

In this study, we investigated the incidence of the MSI phenotype in a large and well-characterized cohort of GEP-NEC/MANECs in order to characterize the clinicopathologic features and the outcome of such carcinomas, evaluating the type of mismatch repair (MMR) defect and its correlation with high levels of gene hypermethylation and *KRAS* and *BRAF* mutations.

Materials and methods

Cases

Tissue samples from 89 surgically resected GEP-NECs and MANECs cohort were collected from the files of the Departments of Pathology of the Ospedale di Circolo – Univeristy of Insubria, Varese and from the archives of the Institute of Pathology of the IRCCS Policlinico San Matteo-University of Pavia, Pavia, Italy. All the cases were reviewed to confirm the diagnoses before starting the investigation. The cases included 53 NECs and 36 MANECs located as follow: six in the esophagus, 36 in the stomach, four in the duodenum, 37 in the colon–rectum, three in the gallbladder, and three in the pancreas. The presence of Lynch syndrome or other inherited tumor

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syndromes was carefully explored clinically and was not found in any patient. Germline studies were not performed because several patients were died at the time of this retrospective investigation. None of the patients had a history of primary pulmonary neuroendocrine neoplasms. Clinical information including sex, age, type of surgery, clinical history, the presence of distant metastases, and follow-up findings was collected by consulting clinical charts.

This study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethical Committee of the Ospedale di Circolo of Varese (no. 0008465).

Morphological and immunohistochemical study

All tissue samples were fixed in buffered formalin (formaldehyde 4% w/v and acetate buffer 0.05 M) and routinely processed in paraffin wax. Five micrometre-thick sections were stained with hematoxylin–eosin (H&E) and alcian-blue/periodic acid-Schiff for the morphological evaluations. All cases were thoroughly investigated for the following histological features: neuroendocrine cytologic subtype (small and large cell), type of the exocrine component (adenomas, adenocarcinomas, squamous cell carcinomas), vascular and perineural invasion, presence of necrosis, mitotic count per ten HPF, level of gut wall invasion or infiltration of peripancreatic tissues, and presence of metastases in local lymph nodes. Intra and peritumoral lymphoid infiltration was evaluated using H&E stained sections and CD3 immunostaining and following the criteria proposed by Walsh *et al.* (2013): peritumoral lymphocytes infiltration was defined as a mantle or cap of lymphoid cells at the deepest point of direct spread. We also counted the number of lymphocytes in three different areas at ×40 magnification at this level. Crohn's-like lymphocytic reaction was considered when at least three nodular lymphoid aggregates were observed deep into the invasive margin in a ×4 field magnification. Intratumoral-infiltrating lymphocytes were considered when at least four intraepithelial lymphocytes per ×40 field magnification were observed. The Ki67 proliferative index was calculated in all NECs and in the neuroendocrine components of all MANECs. It was expressed as a percentage value corresponding to the count of Ki67-positive cells in 2000 tumor cells performed in areas of the highest immunostaining as previously reported (La Rosa *et al.* 2009, 2011). Finally, a stage was assigned according to the ENETS criteria.

For immunohistochemical analyses, 3 μm-thick sections were mounted on poly-L-lysine coated slides, deparaffinized, and hydrated through graded alcohols to water. Endogenous peroxidase activity inhibition was carried out by dipping the sections in 3% hydrogen peroxide for 10 min, followed by incubation with the primary antibodies (Supplementary Table 1, see section on supplementary data given at the end of this article) at 4 °C for 18–20 h and subsequently the avidin–biotin complex procedure. Immunoreactions were developed using 0.03% 3,3′diaminobenzidine tetrahydrochloride and then sections were counterstained with Harris' hematoxylin.

MSI analysis

Tumor DNA from each patient was obtained from formalin-fixed and paraffin-embedded tissues using three representative 8 μm-thick sections of tumor samples. DNA was extracted after manual microdissection, using a QIAamp DNA FFPE tissue kit according to the manufacturer's protocol (Qiagen). Integrity and amplifiability of each DNA sample were evaluated using BIOMED-2 multiplex PCR (van Dongen *et al.* 2003). MSI analysis was carried out using a pentaplex panel of monomorphic mononucleotide repeats (BAT25, BAT26, NR-21, NR-22, and NR-24) as previously reported (Suraweera *et al.* 2002).

Methylation-specific multiplex ligation probe amplification

Methylation analysis of 34 gene promoters was performed in two replicates for each sample using methylation-specific multiplex ligation probe amplification (MS-MLPA) with the SALSA MS-MLPA ME001 Tumor Suppressor-1 Kit and SALSA MS-MLPA ME002 Tumor Suppressor-2 Kit (MRC-Holland, Amsterdam, The Netherlands), which were previously validated with other techniques (Furlan *et al.* 2013). Methylation ratio was calculated using the Coffalyser V7 software (MRC-Holland) and the presence or absence of promoter methylation was scored as discrete variables using the cut-off values previously reported (La Rosa *et al.* 2012*a*,*b*). We also analyzed the promoter methylation of *MMR* genes using the SALSA MS-MLPA ME011 MMR Kit (MRC-Holland) in MSI-cancers. All the genes examined by MS-MLPA are listed in the Supplementary Table 2, see section on supplementary data given at the end of this article.

MLH1 methylation analysis by bisulphite pyrosequencing

MLH1 methylation status was confirmed by pyrosequencing analysis in all the cases that were methylated with MS-MLPA. Bisulfite modification of genomic DNA

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(300 ng) was performed with an EpiTect bisulfite kit (Qiagen) according to the manufacturer's recommendations. A region of 84 nucleotides inside the Deng C-region (Gausachs *et al*. 2012) was amplified in two independent reactions using the following primers: forward: 5′-BIOTIN-GAGTTTTTAAAAAAGAATTAATAG-GAAGAG-3′ and reverse 5′-ATACTACCCCCTACC-TAAAAAAATAT-3′. To improve the PCR efficiency using bisulfite-treated DNA, PCR was performed with EpiTaq HS (Takara, Shiga, Japan) using 5 µl of the bisulfite-converted DNA (30–60 ng assuming 100% yield) in a 50 µl reaction containing 2.5 mM $MgCl_2$, 0.3 µM primer pairs, and 200 µM dNTPs. Thermal cycling conditions were as follows: 2 min at 95 °C, 35 cycles of 95 °C/25 s, 57 °C/30 s, 72 °C/30 s and 72 °C/2 min. Pyrosequencing was carried out on the DNA strand purified by streptavidin-coated beads, addressing five consecutive cytosines starting from the sequencing primer: 5′-CTACCCCCTACCTAAAAAAATATAC-3′. Analytical sensitivity and linearity of the assay was assessed using a serial dilution of fully methylated DNA and unmethylated DNA (Chemicon International, Inc., Billerica, MA, USA). Coefficient of variation inter-/intra-assay was calculated using 20 DNA samples from healthy donors and was 3.5%. A sample was classified as methylated when the mean of all the five cytosines was >10%.

BRAF and *KRAS* mutation analyses

Mutations in codon 600 of *BRAF* and codons 12 and 13 of *KRAS* gene were analyzed in duplicate by PCR-pyrosequencing using the anti EGFR MoAb response KRAS Status Kit and anti EGFR MoAb response BRAF status Kit (Diatech Pharmacogenomics, Jesi, Italy) according to the manufacturer's instructions.

Statistical analyses

Association analyses were performed using the Fisher exact test, ANOVA analysis, and the independent sample *t*-test. We used a model-based cluster algorithm (Fraley & Raftery 2002) to appropriately define a threshold for the Ki67 percentage and for gene hypermethylation levels. The method is based on the estimation of different models (mixed Gaussian distribution), characterized by different number of groups; thus, the best model is selected according to the Bayesian Information Criterion or BIC (Schwarz 1978). This analysis was performed with R software (http://www.r-project.com) with the mclust package (Schwarz 1978, Fraley & Raftery 2002). The patient survival was evaluated using the Kaplan–Meier method and statistically tested with the log-rank test. Patients who died within 1 month of surgery were excluded from the survival analyses. Multivariate analysis was performed with the Cox proportional hazard model using the backward method. A P value <0.05 was considered to be statistically significant. These analyses were performed using MedCalc Statistical (version 11.0.1.0) and GraphPad Prism V5.0 software (San Diego, CA, USA).

Results

Morphological features of the 89 NEC/MANECs investigated

All NECs and the neuroendocrine components of all MANECs were composed of poorly differentiated cells of either small- (49 cases) or large-cell (40 cases) subtype showing, by definition, >20% of Ki67 proliferative index. Small-cell NECs were found in all sites examined, including the esophagus where the large-cell subtype was not observed. Barrett metaplasia was found in only one esophageal MANEC. No cases presented as morphologically well/moderately differentiated neoplasms, corresponding to the recently described, but not finally accepted, group of the so-called 'NET G3' (Vélayoudom-Céphise *et al*. 2013, Hijioka *et al*. 2014, La Rosa *et al*. 2014).

The exocrine component of MANECs was represented by squamous cell carcinoma in the two esophageal MANECs, while in MANECs of other sites it was represented by an adenocarcinoma in eight cases, by a tubular or tubulo-villous adenoma in six cases, by an adenoma with adenocarcinoma in 17 cases, by a squamous cell carcinoma in two cases, and by a combination of adenoma with squamous cell carcinoma in one case.

For 16 MANECs, we were able to evaluate both the primary neoplasm and the relative metastasis (Supplementary Table 3 and Supplementary Fig. 1, see section on supplementary data given at the end of this article). Interestingly and as expected, in the metastatic sites the NEC component was more extensively represented than the exocrine one (P=0.0002).

MSI GEP NEC/MANECs

MMR defect and clinicopathologic profile Microsatellite analysis allowed us to identify 11 unstable carcinomas (seven MSI-NECs and four MSI-MANECs) representing 12.4% of our series of 89 cases (Table 1).

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Table 1 Clinicopathologic characteristics of unstable (MSI) and stable (MSS) neuroendocrine carcinomas

	MSI 11 cases	MSS 78 cases	Total 89 cases
Gender			
Male	4 (36%)	53 (71%)	57 (66%)
Female	7 (64%)	22 (29%)	29 (34%)
Age (years)			
Average	74	65.1	66.3
Range	61–91	34–93	34–93
Location			
Esophagus	0	6 (7%)	6 (6%)
Stomach	4 (36%)	32 (41%)	36 (41%)
Duodenum	1 (10%)	3 (4%)	4 (5%)
Colon–rectum	6 (54%)	31 (40%)	37 (42%)
Pancreas	0	3 (4%)	3 (3%)
Gallbladder	0	3 (4%)	3 (3%)
Classification			
NEC	7 (64%)	46 (59%)	53 (60%)
MANEC	4 (36%)	32 (41%)	36 (40%)
Lymph node metastasis	10 (91%)	67 (89%)	77 (89%)
Distant metastasis	0	30 (38%)	30 (34%)
Tumor stage (ENETS)			
Stage I	0	0	0
Stage II	4 (36%)	6 (9%)	10 (13%)
Stage III	7 (64%)	42 (63%)	49 (63%)
Stage IV	0	19 (28%)	19 (24%)
Follow-up (months)			
Mean time	85.7	19.5	27.7
Range time	14–230	1–257	1–257
DOC	1 (9%)	2 (3%)	3 (3%)
DOD	7 (64%)	59 (75%)	66 (74%)
AFD	2 (18%)	10 (13%)	12 (14%)
POD	0	3 (4%)	3 (3%)
Lost	1 (9%)	4 (5%)	5 (6%)

NEC, neuroendocrine carcinoma; MANEC, mixed adenoneuroendocrine carcinoma; DOC, died of other cause; DOD, died of disease; AFD, alive free disease; POD, post-operative death.

In all of them, we evaluated the size of allelic shifts at each marker and we observed that all five loci were affected by deletions ranging from 5.4 to 9.7 bp. BAT-26 showed the longest allelic shifts with a mean size of 9.7 bp $\pm$3.4. The MSI group included 1/6 (16.6%) proximal gastric (cardial), 3/30 (10%) antral/body gastric, 1/4 (25%) duodenal, 3/21 (14.3%) right colonic, and 1/12 (8.3%) left colonic neoplasms. For four colonic cases, including two MSI-carcinomas, the exact site was not specified. The remaining 78 cancers (87.6%), including all esophageal, pancreatic, and gallbladder NEC/MANECs, were classified as stable neoplasms (MSS-NEC/MANECs), as they did not show instability at any microsatellite locus.

All MSI-NEC/MANECs showed lack of immunohistochemical expression of MMR proteins (Table 2). Nine cases (five colorectal and four gastric cancers) showed concomitant loss of MLH1 and PMS2 proteins, a colorectal NEC showed PMS2 negativity, while a duodenal NEC exhibited loss of both MSH2 and MSH6 expression. By contrast, all MSS-NECs showed MLH1, MSH2, PMS2, and MSH6 immunohistochemical expression.

The mean age of patients with MSI-NEC/MANECs was 74 years vs 65 years for patients with MSS-NEC/MANEC and there was a slight female prevalence. Six out of 11 cases were of the large-cell subtype and, interestingly, no carcinomas showed distant metastases at the time of diagnosis, being classified as stage II or III depending on the nodal status. The main morphological and immunohistochemical findings are summarized in Table 3. Among the various parameters evaluated, it is worth noting that about 54% of MSI-carcinomas showed prominent intraperitumoral lymphoid infiltration (Fig. 1) (against 25% of MSS cases), large-cell subtype (against 45% of MSS), and vascular invasion (against 74% of MSS), and that they were all negative for CD117 and TTF1. CDX2 was expressed in 3/4 gastric and in 4/6 colorectal MSI-cancers. Interestingly, intra and peritumoural lymphoid infiltration did not differ between MSI and MSS cases when evaluated in H&E stained sections. Using CD3-immunostained sections, intratumoral lymphoid infiltration was statistically higher (P=0.01) in MSI-cases (mean value of CD3-positive lymphocytes: 20.40$\pm$5.580) than in MSS cases (mean value: 7.13$\pm$1.914). Similarly, peritumoral lymphoid infiltration was also statistically higher (P=0.0002) in MSI-cases (mean value of CD3-positive lymphocytes 117.6+12.41) than in MSS ones (mean value: 55.53$\pm$8.05). In MSI-MANECs, the exocrine component was represented by a mucinous adenocarcinoma in two cases and by an adenocarcinoma not otherwise specified (NOS) in the other two. In such cases the intra and peritumoral lymphoid infiltrate was found close to both exocrine and neuroendocrine neoplastic components. The Ki67 index showed a comparable distribution between MSI- and MSS-cancers without any statistical significant difference between small- and large-cell subtypes. Carcinomas were divided into two groups using a cut-off value of 55% that was calculated with a model-based cluster algorithm (Fraley & Raftery 2002). This threshold value corresponded exactly to that used by Sorbye *et al.* (2013). More precisely, 80% of MSI- and 72.3% of MSS-cancers showed a Ki67 $\geq$55%; the mean value of the Ki67 index was 68.4% for MSI and 63.6% for MSS NEC-MANECs.

MMR gene methylation To investigate whether the loss of MMR proteins expression was associated with aberrant methylation, we first checked the promoter

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Table 2 Immunohistochemical and molecular characterization of mismatch repair genes and proteins in MSI-NEC/MANECs

			IHC				Promoter methylation		
							MS-MLPA		Pyrosequencing
MSI cases	Anatomic site	Type	MLH1	MSH2	MSH6	PMS2	MLH1	MSH2 MSH6 PMS2	MLH1
Case#25	Stomach	NEC	−	+	+	−	Met	U	31%
Case#31	Stomach	MANEC	−	+	+	−	Met	U	26.5%
Case#40	Stomach	MANEC	−	+	ne	−	Met	U	14%
Case#44	Stomach	NEC	−	+	+	−	Met	U	16.6%
Case#50	Duodenum	NEC	+	−	−	+	U	U	U
Case#58	Colon–rectum	NEC	−	+	+	−	Met	U	ne
Case#62	Colon–rectum	NEC	−	+	+	−	Met	U	ne
Case#63	Colon–rectum	NEC	−	+	+	−	Met	U	12.3%
Case#74	Colon–rectum	NEC	−	+	+	−	Met	U	26.3%
Case#100	Colon–rectum	MANEC	+	+	+	−	U	U	U
Case#101	Colon–rectum	MANEC	−	+	+	−	Met	U	45.9%

ne, not evaluable; Met, methylated; U, unmethylated.

methylation status of the *MMR* genes using MS-MLPA. As given in Table 2 , all the nine cases lacking *MLH1* expression showed methylation at the *MLH1* promoter. In the two cases showing MLH1 immunoreactivity (cases #50 and #100) *MLH1* methylation was not found. *MSH2*, *MSH6* and *PMS2* gene methylation was never observed in the 11 MSI-carcinomas. To validate the MS-MLPA results, we performed pyrosequencing analysis addressing the Deng-C region, which has recently been identified as a critical sequence for MLH1 hypermethylation (Gausachs *et al*. 2012). Pyrosequencing confirmed the MS-MLPA results in all nine cases, with percentages of methylated alleles ranging from 13% to 46% (Table 2).

Among the MSI-cases, four MANECs exhibited *MLH1* loss (three cases) or *PMS2* loss (one case). In these cases, loss of the immunohistochemical expression and MSI was observed in both the exocrine and the neuroendocrine components. Accordingly, *MLH1* methylation was always

Table 3 Morphological and immunohistochemical features of unstable (MSI) and stable (MSS) neuroendocrine carcinomas

	MSI 11 cases	MSS 78 cases	Total 89 cases
Large-cell subtype	6 (54%)	34 (45%)	40 (46%)
Vascular invasion	6 (54%)	57 (78%)	63 (75%)
Perineural invasion	6 (54%)	55 (74%)	61 (72%)
Necrosis	9 (90%)	62 (79%)	71 (80%)
Intratumoral lymphoid infiltration (mean value[a] ±S.D.)	20.40±5.580	7.13±1.914	11.7±13
Peritumoral lymphoid infiltration (mean value[a] ±S.D.)	117.6±12.41	55.53±8.05	77±45
Mitoses (×10 HPF)[b]	37.8	50.8	49.2
Ki67 index[b]	68.4	63.6	64.1
Ki67 index (>55%)	8 (80%)	55 (72.3%)	63 (73.2%)
Chromogranin A	6[c] (55%)	62[d] (79%)	68[e] (76%)
Synaptophysin	10 (91%)	76 (97%)	86 (97%)
CD117 positive	0	28 (37%)	28 (32%)
PDX1 positive	1 (10%)	8 (11%)	9 (11%)
CDX2 positive	7 (64%)	34 (45%)	40 (46%)
TTF1 positive	0	16 (21%)	16 (18%)
Cytokeratin 20	1 (9%)	6 (8%)	7 (8%)
Cytokeratin 7	2 (18%)	22 (29%)	24 (27%)

HPF, high power field.
[a]Mean value of CD3 positive cells.
[b]Mean value.
[c]Three cases with <10% of positive cells.
[d]26 cases with <10% of positive cells.
[e]29 cases with <10% of positive cells.

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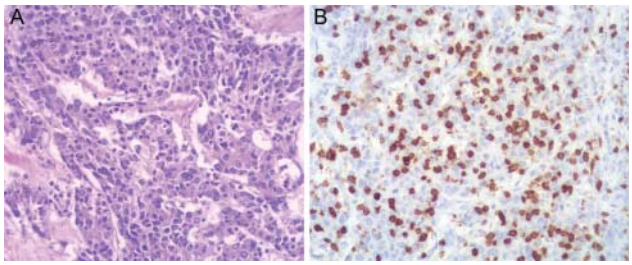

Figure 1
Unstable neuroendocrine carcinoma showing abundant intraepithelial lymphocytes. Although they can be recognized in the H&E-stained section (A), they become much more evident in the CD3-immunostained section (B) (original magnification 250×).

found in both tumor areas in the three MANECs showing *MLH1* loss (Fig. 2).

Mutation analyses Mutation analyses of codon V600 of *BRAF* and codons 12 and 13 of *KRAS* genes were possible for 88 samples. *BRAF V600E* substitutions were identified in six cases (7%) and all of them were found in colorectal cases. *KRAS* mutations were identified in 15 cases (17%): 13 in colorectal and two in gastric NECs. The mutations were the following types: G13D (40%), G12D (33%), G12A (20%), and G12V (7%). No cases showed mutations in both genes.

A strong association between *BRAF* mutation and MSI was observed, with 66% of MSI-cases showing *BRAF* V600E substitution vs 8% of MSS-cancers ($P<0.0008$). By contrast, no significant correlation was detected between *MSI* and *KRAS* mutations.

Gene methylation profiles and correlation with MSI status

Methylation analysis of the 34 promoters listed in Supplementary Table 2 was performed in all 89 GEP-NEC/MANECs by MS-MLPA. Overall, the percentage of methylated genes ranged from 0% to 74%. We used a model-based cluster algorithm (Fraley & Raftery 2002) to appropriately categorize different subsets of NEC/MANECs based on gene hypermethylation levels. With this method, it was possible to define three main subgroups (Fig. 3A): 42% of the tumors (37/89 cases) had a very low level of methylation (L-MET, less than five methylated genes), 26% (24/89) of cases exhibited intermediate levels of methylation (I-MET, more than five and less than eight methylated genes), and 31% (28/89) showed extensive gene hypermethylation (H-MET, more than eight methylated genes). H-MET NEC/MANECs were localized in the stomach, intestine, and gallbladder (17, ten, and one tumors respectively). No significant differences were observed in the methylation frequencies of cancers from different sites. However, more than half of H-MET cancers fell into the gastric group (Fig. 3B).

With regard to MSI status, all 11 unstable NEC/MANECs fell into either the H-MET or the I-MET group and showed higher methylation levels compared with MSS-cases, with a mean value of methylated genes per case equal to $40.6\%\pm5.2$ vs $20.2\%\pm1.9$ in MSS cases ($P<0.001$). The genes most frequently methylated in MSI group were *MLH1*, *P16*, *PAX6*, *PAX5*, *THBS1*, *TP73*, *DAPK1*, *MGMT*, *PYCARD*, *CDH13*, *HIC1* and *TIMP3* (for all $P<0.01$). No genes were significantly methylated at high frequency in MSS cancers.

Survival analyses

Survival analyses were carried out to test the prognostic value of all the molecular and clinicopathologic features examined in this study. Kaplan–Meier curves of all statistically significant variables at univariate analysis are shown in Fig. 4. The MSI phenotype was associated with a more favorable prognosis than MSS status, with a MS of

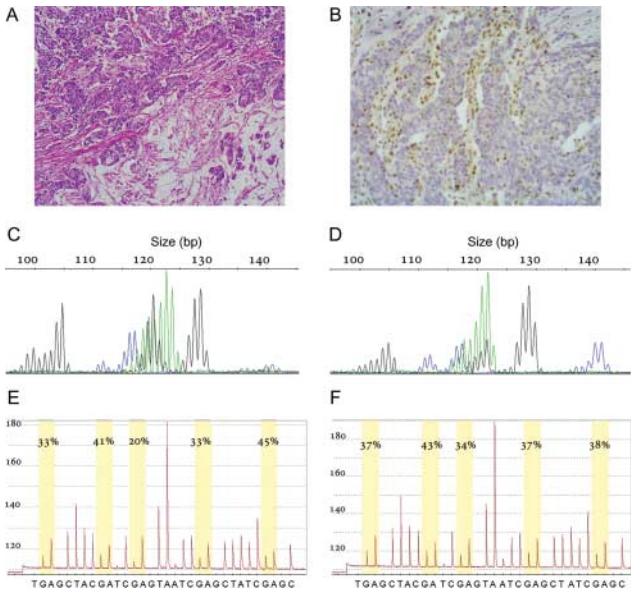

Figure 2
Example of unstable colorectal MANEC showing loss of MLH1 protein expression, presence of microsatellite instability, and methylation of *MLH1* gene. (A) Hematoxylin–eosin stain shows the association of a large-cell NEC component (upper left) with a mucinous adenocarcinoma component (bottom right). (B) MLH1 immunostaining demonstrates complete loss of MLH1 nuclear expression in tumor cells, while lymphocytes serve as positive control. (C and E) Presence of microsatellite instability and of *MLH1* methylation in the DNA sample from the adenocarcinoma component. (D and F) Presence of microsatellite instability and of MLH1 methylation in the DNA sample from the NEC component.

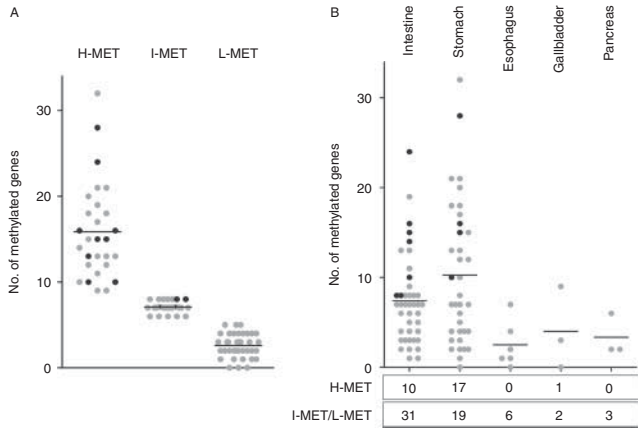

Figure 3
Distribution of NECs/MANECs based on methylation levels and anatomical sites. (A) Scatter plot showing three distinct subgroups of tumors with high levels (H-MET), low levels (L-MET), and intermediate levels (I-MET) of gene methylation, as determined by the model-based cluster method. (B) Scatter plot showing the distribution of H-MET and I-/L-MET cases in gastro-enteropancreatic sites. Black lines indicate the mean values, black dots represent MSI-NECs/MANECs while gray dots indicate MSS cases.

60 vs 5.5 months respectively ($P=0.0048$). GEP-NEC/MANECs showing aberrant methylation of the *MLH1* gene were associated with a better outcome, with a MS of 61.5 months compared with 6 months for MLH1-unmethylated cases ($P=0.01$). The methylation status of any of the other genes did not correlate with prognosis. Clinicopathological characteristics associated with worse prognosis included the presence of vascular invasion (MS: 5 vs 48 months; $P<0.0001$) and CD117 immunoreactivity (MS: 2 vs 14 months; $P=0.0027$). As the Ki67 index has been shown to subdivide NECs into two distinct prognostic groups (Sorbye *et al.* 2013), we performed survival analysis categorizing patients by using the Ki67 index threshold of 55%, which was identified by a model-based cluster algorithm (Fraley & Raftery 2002). Interestingly, this corresponded to that used by Sorbye *et al.* (2013). In all 23 cases with <55% Ki67 index (11 NECs and 12 MANECs), the neuroendocrine component was morphologically poorly differentiated of either small cell (13 cases) or large-cell (ten cases) type. The Ki67 index failed to prognosticate NEC/MANECs when applied to the whole series including both MSI- and MSS-cases. However, it successfully identified two different prognostic groups when only MSS carcinomas were considered, after excluding MSI-carcinomas from the analysis ($P=0.049$).

Multivariate analysis was performed including all the variables that were significant at univariate analyses (Table 4). Vascular invasion and MSI phenotype were identified as the only independent prognostic factors ($P=0.0003$ and $P=0.0084$ respectively). A trend was observed for the Ki67 index using a threshold of 55% ($P=0.063$).

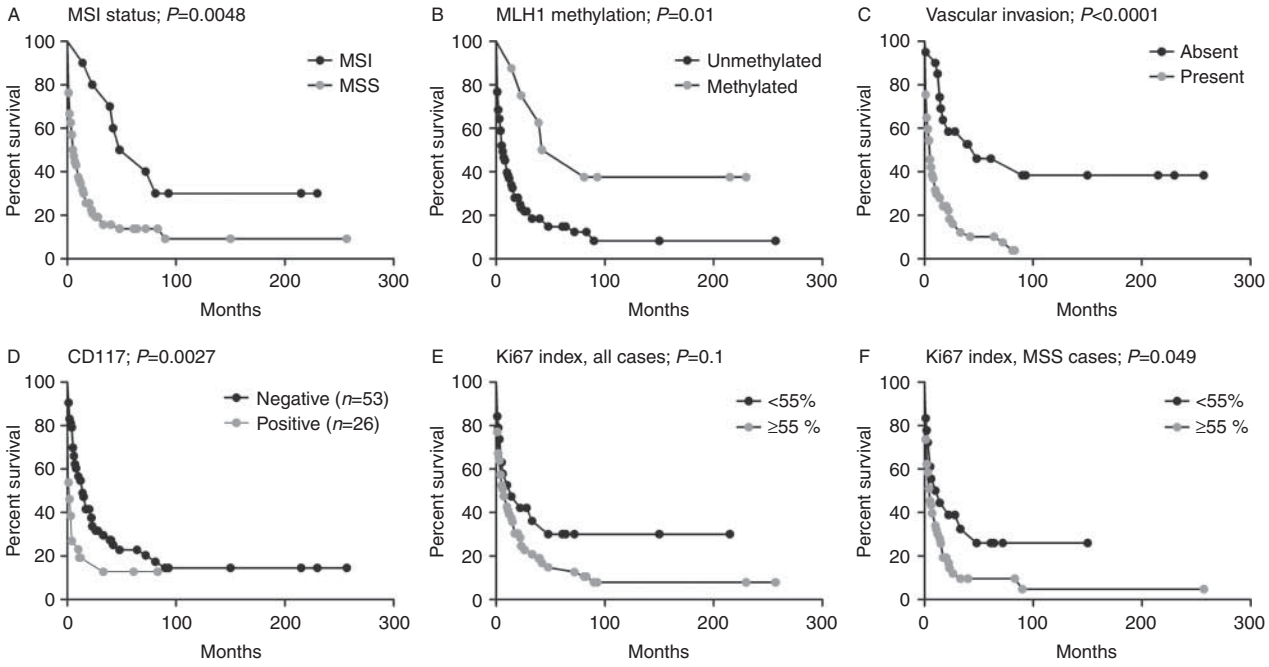

Figure 4
Molecular and clinicopathological features that were statistically significant at univariate analysis: (A) MSI status; (B) *MLH1* methylation; (C) vascular invasion; (D) CD117 immunostaining; (E) Ki67 index in the whole series and (F) Ki67 index considering only MSS NEC/MANEC cases.

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Table 4 Multivariable analysis

	HR	95% CI	*P* value
Vascular invasion	1.96	1.3631–2.813	0.0003
MSI phenotype	1.81	1.1639–2.814	0.0084
Ki67 index (55%)	1.36	0.9839–1.878	0.063

HR, hazard ratio.

Discussion

This study demonstrates for the first time that the analysis of MMR defects in GEP-NEC/MANECs allows the identification of a new clinicopathological entity characterized by an unexpected, relatively favorable prognosis and by distinct genetic and epigenetic features.

The MSI phenotype was observed in 12.4% of the GEP-NEC/MANECs located in the stomach or intestine, confirming and extending our recently published results obtained in a cohort of colorectal NEC/MANECs (La Rosa *et al.* 2012*a*,*b*).

In the present investigation, we demonstrate that the main pathogenetic mechanism leading to the onset of MSI-NEC/MANECs is the methylation-mediated silencing of the *MLH1* gene, similar to that which has been widely reported in sporadic exocrine gastrointestinal adenocarcinomas (Kane *et al.* 1997, Bevilacqua & Simpson 2000). Accordingly, several other observations suggest similarities between MSI-neuroendocrine and -exocrine cancers in these anatomic sites. Firstly, the incidence of the MSI phenotype in NEC/MANECs is comparable with that reported for colorectal and gastric adenocarcinomas (Ionov *et al.* 1993, Gologan & Sepulveda 2005). Secondly, MMR defect seems to occur early in the tumorigenic pathway of MSI-NEC/MANECs, if the large size of the allelic shifts observed in the five microsatellites examined is considered. Indeed, it is well known that the progressive shortening of mononucleotide tracts may be used as a simple molecular clock of MSI tumor evolution (Percesepe *et al.* 2000, Duval *et al.* 2001). In this study, we observed microsatellite deletions ranging from 5.4 to 9.7 bp that were very similar to those previously reported in advanced gastric and colorectal adenocarcinomas showing MSI (Furlan *et al.* 2002). In agreement with this hypothesis, the presence of MSI was found in both exocrine and neuroendocrine areas of all four MSI-MANECs examined, suggesting that the MMR defect occurs before the divergent differentiation starts. Thirdly, MSI-NEC/MANECs are associated with widespread gene methylation. This finding confirms the close association between MSI and the CpG island methylator phenotype (CIMP) already described in gastric and colorectal adenocarcinomas (Toyota *et al.* 1999, Samowitz *et al.* 2005). In these sites, genome-wide methylation studies have confirmed that CIMP and MSI are a homogeneous subset of cancers harboring aberrant DNA methylation mechanisms (Park *et al.* 2011, Hinoue *et al.* 2012, Xu *et al.* 2012). Fourthly, we demonstrated that the well-known relationship reported in colorectal adenocarcinomas between MSI phenotype and *BRAF* V600E mutation (Boland *et al.* 2009) is also found in MSI-NEC/MANECs of the colon–rectum. Fifth, at least one case of MLH1-negative large-cell NEC arising in a MLH1-negative sessile serrated adenoma, which is recognized to be a precursor of colorectal carcinoma as a part of the MSI pathway of colorectal carcinogenesis, has been reported (Naert & Dupre 2012).

It is worth noting that morphology alone did not help to identify MSI-NEC/MANECs. Indeed, none of the histologic features considered predicted the MSI status. On the contrary, immunohistochemistry for neuroendocrine markers and MMR proteins allowed us to identify MSI-NEC/MANECs, which were subsequently confirmed by MSI analysis.

A very important finding of our study regards the unexpected, more favorable prognosis of MSI-carcinomas compared with MSS-NEC/MANECs. Indeed, the presence of a MMR defect allowed us to identify a subset of 11 patients with MSI-carcinomas showing a MS of 60 months; much longer than the MS of 5.5 months observed in the patients with MSS-NEC/MANECs. Moreover, MSI status remains the only independent prognostic factor ($P=0.0084$) at multivariable analysis together with vascular invasion ($P=0.0003$). Interestingly, the MS of MSI-NEC/MANECs is similar to that of MSI-adenocarcinomas (Guidoboni *et al.* 2001).

The identification of MSI status may be also useful for therapeutic purposes. It is well known that MSI predicts poor response to 5-fluorouracil and oxaliplatin (Zaanan *et al.* 2010). On the contrary, MSI colorectal cancers are sensitive to irinotecan (Bras-Goncalves *et al.* 2000) and patients with MSI tumors have a better survival after adjuvant therapy that includes irinotecan (Bertagnolli *et al.* 2009). It remains to be demonstrated if this different response to therapeutic agents is similar in MSI-NEC/MANEC.

Regarding the prognostic value of the Ki67 index in our study, we observed an unexpected finding. The 55% threshold index failed to prognosticate NEC/MANECs when applied to the whole series, while it was a significant prognostic predictor in the group of 78 MSS-NEC/MANECs. It is worth noting that a high Ki67 index was not associated with a worse survival in colorectal MSI adenocarcinomas (Kim *et al.* 2007), indicating that the Ki67 index has a different prognostic value in MSI-positive and in MSI-negative neoplasms.

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In conclusion, this study demonstrates that immunohistochemical expression of MMR protein and MSI analysis, two simple tests routinely available in most laboratories of surgical pathology, can identify a subgroup of MSI-NEC/MANECs which are associated with a significantly more favorable prognosis than that of NEC/MANECs without a MMR defect. MSI-NEC/MANECs are observed in gastric and colorectal sites with very similar frequency to that reported for MSI gastrointestinal adenocarcinomas. The pathogenetic mechanisms as well as the clinicopathologic and the molecular profiles of MSI-NEC/MANECs closely resemble those described for sporadic gastric and colorectal MSI-adenocarcinomas.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0410.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
N Sahnane performed the experiments, analysis and interpretation of data, and drafting of the manuscript; D Furlan performed study concept and design, analysis and interpretation of data, drafting of the manuscript; M Monti performed the experiments; analysis and interpretation of data; C Romualdi performed statistical analysis; A Vanoli performed analysis and interpretation of data. E Vicari performed the experiments, analysis and interpretation of data; E Solcia and F Sessa critical revision of the manuscript; C Capella analysis and interpretation of data, critical revision of the manuscript; S L Rosa study concept and design, analysis and interpretation of data, drafting of the manuscript.

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