Mixed Adenoneuroendocrine Carcinomas (MANECs) of the Gastrointestinal Tract: Targeted Next Generation Sequencing Suggests a Monoclonal Origin of the Two Components

Neuro endocrinology

Neuroendocrinology (DOI:10.1159/000369071)
(Accepted, unedited article not yet assigned to an issue)

Advanced Release: October 20, 2014

Received: July 17, 2014
Accepted after revision: October 14, 2014

Mixed Adenoneuroendocrine Carcinomas (MANECs) of the gastrointestinal tract: targeted next generation sequencing suggests a monoclonal origin of the two components

Maria Scardoni[1,2], Emanuele Vittoria[1], Marco Volante[3], Borislav Rusev[1,2], Samantha Bersani[1,2], Andrea Mafficini[1], Marisa Gottardi[1], Valeria Giandomenico[4], Giuseppe Malleo[5], Giovanni Butturini[5], Sara Cingarlini[6], Matteo Fassan[1,2], Aldo Scarpa[1,2]

1 ARC-Net Research Centre, University and Hospital Trust of Verona, 37134 Verona, Italy
2 Department of Pathology and Diagnostics, University and Hospital Trust of Verona, 37134 Verona, Italy
3 Department of Oncology, University of Turin at San Luigi Hospital, 10034 Turin, Italy
4 Department of Medical Sciences, Endocrine Oncology, Uppsala University, 75105 Uppsala, Sweden
5 Department of Surgery, General Surgery B, University of Verona, 37134 Verona, Italy
6 Department of Medical Oncology, University and Hospital Trust of Verona, 37134 Verona, Italy

Short title: MANEC & NGS

Tables: 1

Figures: 3

Supplementary files: 1

Word count: 2243

Authors' contributions: VC, MF, AS: ideation of the study, planning, primary writers. BR, MF, VG, AS: histopathological and immunohistochemical study and evaluation. BR, MF: sample microdissection. MS, EV, SB, AM, MG: preparation of DNA, quality control and sequencing including data analysis and interpretation. AM: bioinformatic analysis of next-gen sequencing. MS, EV, MV, AM, MF: sample choice and preparation, collection and assembly of clinical-pathological data. MV, GM, GB, SC, AS: clinical-pathological data analysis and interpretation of results related to the peculiar tumor type and clinical implications. All authors participated in writing and approved the final, submitted manuscript.

Competing interests: The authors have no competing interests to declare.

Funding: Italian Cancer Genome Project grant from the Italian Ministry of Research (FIRB - RBAP10AHJB) and Fondazione Italiana Malattie Pancreas – Ministry of Health (J33G13000210001), AIRC grant n. 12182, and FP7 EU project CAM-PaC no: 602783.

Correspondence to:
Dr. Matteo Fassan, MD, PhD
ARC-NET Research Centre, Department of Pathology & Diagnostics
University of Verona, Policlinico GB Rossi, Piazzale L.A. Scuro, 10
37134 Verona (VR), Italy
Tel.: +39-045-8124043; Fax: +39-045-8127432; Email: matteo.fassan@gmail.com

ABSTRACT

Background. Mixed AdenoNeuroEndocrine Carcinomas (MANECs) of the gastrointestinal tract are rare neoplasms characterized by coexisting exocrine and neuroendocrine neoplastic components. MANECs' histogenetic classification and molecular characterization remain unclear, significantly affecting the identification of innovative therapeutic options for these tumors. **Methods.** In this study, the exocrine and neuroendocrine components of 6 gastrointestinal MANECs were microdissected and subjected to the simultaneous mutation assessment in selected regions of 54 cancer-associated genes, using Ion Torrent semiconductor-based next-generation sequencing (NGS). Sanger sequencing and immunohistochemistry were used as validation of the mutational status. **Results.** A total of 20 driver gene somatic mutations were observed among the 12 neoplastic components investigated. In 11 of 12 (91.7%) samples at least one mutation was detected; 7 samples (58.3%) were found to have multiple mutations. *TP53* gene mutations were the most frequent genetic alterations observed in the series, occurring in 11/12 samples (91.7%). Somatic mutations in other genes were detected at lower frequencies: *ATM, CTNNB1, ERBB4, JAK3, KDR, KRAS, RB1*. **Conclusions.** Five of the six MANECs presented an overlapping mutational profile in both components, suggesting a monoclonal origin of the two MANEC components.

Keywords: mixed adenoneuroendocrine carcinomas; next generation sequencing; WHO 2010 classification; gastrointestinal tract.

INTRODUCTION

Mixed AdenoNeuroEndocrine Carcinomas (MANECs) of the gastrointestinal tract are rare and heterogeneous neoplasms characterized by coexisting exocrine and neuroendocrine neoplastic components [1,2]. According to WHO 2010 classification [3], either tumor component should represent at least 30% of the entire lesion. By the clinical point of view, MANECs are considered as carcinomas since both components are histologically malignant; tumor behavior is dictated by the most aggressive component, which is usually the neuroendocrine one [1,2].

MANEC histogenetic definition represents a current controversial issue among pathologists [1,2,4-8]. Two main theories have been formulated: (i) these tumors might arise independently in a synchronous or methacronous fashion, or (ii) they might derive from a common, multipotent stem/progenitor cell. The presence in most MANECs of amphicrine cells characterized by the simultaneous presence of mucin droplets and neuroendocrine secretory granules strongly sustain the hypothesis of a common precursor cell capable of divergent differentiation [9,10]. This has also been supported by the majority of molecular studies, which suggested a possible multistep progression from a common precursor lesion, especially in those cases in which the neuroendocrine component is represented by a poorly differentiated carcinoma [1,7,11-13]. However, a comprehensive molecular characterization of these tumors is still lacking, and this significantly affects the introduction of innovative and targeted therapeutic options, which are currently left to subjective choices [1,14].

Understanding the molecular basis of MANEC carcinogenesis and lineage commitment would be of fundamental importance in the prognostic and therapeutic stratification of these patients. To address this point, the exocrine and neuroendocrine components of 6 gastrointestinal MANECs were microdissected and subjected to the simultaneous mutational assessment in selected regions of 50 cancer-associated genes, using Ion Torrent semiconductor-based next-generation sequencing (NGS). The neuroendocrine components were also characterized by an AmpliSeq custom panel exploring the genes most frequently altered in pancreatic neuroendocrine neoplasms.

MATERIALS AND METHODS

Cases

Six surgically-treated gastroenteropancreatic MANECs, (2 gastric, 2 pancreatic, 1 jejunal, 1 rectal) were retrieved from the FFPE archives of the ARC-Net biobank at Verona University Hospital under the local ethics committee approval (n. prog. 1959).

DNA extraction and qualification

DNA was obtained from formalin-fixed paraffin-embedded (FFPE) tissues after enrichment for neoplastic cellularity. Suitable areas for microdissection were marked on archival haematoxylin and eosin (H&E) slides, which serve as templates. The corresponding tissue blocks were serially cut to 5-µm-thin sections. Unstained sections were therefore deparaffinized, and sligthly counterstained with haematoxylyn. Tumor cells were dissected manually using a sterile syringe needle, and at least 70% of neoplastic cells were collected from both exocrine and neuroendocrine components. Normal peritumoral tissues (i.e., non-tumor gastrointestinal mucosa or pancreatic parenchyma) were microdissected and used to determine the somatic or germline nature of mutations. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen). DNA was quantified and its quality assessed using NanoDrop (Invitrogen) and Qubit (Invitrogen) platforms [15]. The quality of DNA was further evaluated by PCR using the BIOMED 2 PCR multiplex protocol [16].

Deep Sequencing of Multiplex PCR Amplicons

Two multigene NGS panels were used: (1) the 50-gene Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) was tested on the two tumor components and the normal counterpart, (2) an Ampliseq neuroendocrine-custom panel investigating hotspots regions in *MEN1*, *ATRX*, *DAXX*, and *TSC2* genes, which are frequently mutated in pancreatic neuroendocrine neoplasms [17-19], that was applied only on the neuroendocrine component of the MANEC and the normal sample. Details of the target regions for both panels are in Supplementary Tables 1 and 2.

The first panel explores selected regions of the following 50 genes: *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, JAK2, JAK3, IDH2, KDR/VEGFR2, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL*.

Forty nanograms of DNA were used for multiplex PCR amplification. Emulsion PCR

was performed with the OneTouch2 systems (Life Technologies). The quality of the obtained library was evaluated by the Agilent 2100 Bioanalyser on-chip electrophoresis (Agilent Technologies). Sequencing was run on the Ion Torrent Personal Genome Machine (PGM, Life Technologies) loaded with 316 (50-gene panel) or 318 chips (custom panel). Data analysis, including alignment to the hg19 human reference genome and variant calling, was done using the Torrent Suite Software v.3.2 and v.3.6 (Life Technologies). Filtered variants were annotated using the SnpEff software v.3.1 and the IonReporter software v.1.6 (Life Technologies). Alignments were visually verified with the Integrative Genomics Viewer; IGV v.2.2, Broad Institute.

DNA Sanger Sequencing

KRAS (exon 2) and *TP53* (exons 5, 6, 7, 8) specific PCR fragments were analysed by Sanger sequencing. PCR products were purified using Agencourt AMPure XP magnetic beads (Beckman Coulter), labeled with Big Dye Terminator v3.1 (Applied Biosystems). Agencourt CleanSEQ magnetic beads (Beckman Coulter) were used for post-labeling purification. Sequence analysis was performed on an Applied Biosystems 3130xl Genetic Analyser.

Immunohistochemistry

The immunohistochemical expression of p53 (clone DO-1; prediluted; Immunotech) and ß-catenin (clone 15B8; 1:150; Sigma) was tested as a surrogate validation of deep sequencing results, and performed as described elsewhere [20,21].

RESULTS

Prevalence of driver genes mutations in gastrointestinal MANECs

The clinico-pathological information of the six cases are summarized in **Table 1**. All patients were male, with a mean age of 72±10 years. Four tumors arose within the gastrointestinal tract (2 stomach, 1 jejunum, 1 rectum) and two were from the pancreas. All the exocrine components were represented by an adenocarcinoma; 5 cases were moderately differentiated, one was poorly differentiated. All the neuroendocrine components were poorly differentiated neuroendocrine carcinomas, characterized by a Ki67 index >20%. According to WHO 2010 classification, all morphologically and immunophenotypically-proven components represented at least 30% of the tumor. In all cases, collision (**Figure 1A**) and/or variably combined (**Figure 1B**) areas of the two components were observed.

DNA from the microdissected components and from the adjacent normal tissue were subjected to deep sequencing of mutational hotspots of 54 genes. The mean read length was 101 base pairs and a mean coverage of 866x was achieved, with 96.8% target bases covered more than 100x by using the 50-genes hotspot Panel; the mean read length was 108 base pairs and a mean coverage of 1,068x was achieved, with 75.4% target bases covered more than 100x by using the 4-genes neuroendocrine-custom panel. A minimum coverage of 20x was obtained in all samples for both panels.

A total of 20 somatic mutations were observed among the 50 cancer genes investigated by the Ion AmpliSeq Cancer Hotspot Panel v2. No mutation was observed among the custom panel-specific genes *ATRX*, *DAXX*, *MEN1*, and *TSC2*.

In 11 of 12 (91.7%) neoplastic samples - two components for each tumor - at least one somatic mutation was detected (Table 1); 7 samples (58.3%) showed multiple gene somatic mutations.

TP53 somatic mutations were the most frequent alterations observed in the series, occurring in all exocrine and 5/6 neuroendocrine components. Somatic mutations in other genes were detected at lower frequency: *ATM, CTNNB1, ERBB4, JAK3, KDR, KRAS, RB1*.

Most of the observed mutations were missense mutations. A stop mutation was observed in *RB1* (R358*) and *TP53* (R209Kfs*6); one deletion in *ATM* (S1923del) was found in the neuroendocrine component of a gastric MANEC (case #1). *KRAS* and *TP53* gene mutations detected by deep sequencing were confirmed by PCR amplification of appropriate fragments and conventional Sanger sequencing.

Mutation profile in matched MANEC components

Five cases presented an overlapping mutational profile in both components (cases #1, #2, #3, #4, #5; Table 1, **Figure 2**). One of the pancreatic MANECs (case #6) presented no mutation in the 54 analyzed genes in the neuroendocrine component, while the exocrine component presented missense mutations in the *CTNNB1* and *TP53* genes (**Figure 3**).

Six mutations observed in the *KRAS*, *RB1*, and *TP53* (*n*=4) genes were shared by both components of the same MANEC. Somatic point mutations in the *CTNNB1* gene were found exclusively in exocrine components; somatic mutations in the *ATM, ERBB4, JAK3*, and *KDR* genes were found in neuroendocrine components.

B-catenin and p53 immunohistochemistry was used as a valid surrogate confirmation of the mutational status. As expected a nuclear/cytoplasmic ß-catenin immunoreaction was observed in the exocrine component of case #6, which was characterized by a S45F

missense mutation in the *CTNBB1* gene (Figure 2). This mutation has already been linked to the nuclear localization of the protein in desmoid tumors [22].

Five of the six exocrine components and four of the five neuroendocrine components presenting *TP53* somatic mutations showed a strong p53 nuclear immunostaining in more than 50% of neoplastic cells. In the negative case (case #4), the immunohistochemical negativity may be explained by the fact that the R209 stop mutation likely prevents p53 stabilization, as reported for other similar mutations in *TP53* [20]. An example of *TP53* mutational results from paired samples is shown in Figure 2.

DISCUSSION

The results of our mutational survey on a series of 6 routinely formalin-fixed paraffin-embedded gastro-entero-pancreatic MANECs can be summarized as follows: (i) in most tumors, the two diverse components share similar molecular profiles, which supports an origin from a common progenitor cell of the tumor; (ii) the vast majority (91.7%) of neoplastic components harbor a driver-gene mutation; (iii) *TP53* is a key gene in the carcinogenetic process of gastrointestinal MANECs; (iv) NGS of multiple genes is applicable to routinely-processed tissues.

The concept of human cancers displaying a combination of exocrine (glandular or squamous; also urothelial in the genitourinary tract) and neuroendocrine features has been a matter of debate and still represents a controversial issue [1,3,5]. MANEC can present as composite neoplasms with exocrine and neuroendocrine components occurring in separate areas of the same lesion (i.e., collision tumor), or as combined neoplasms, when the two components are intimately and diffusely admixed [1]. Moreover, in amphicrine tumors exocrine and neuroendocrine features are present in the same neoplastic cell, which shows a double immunophenotype [1,3,5]. Thus, two main histogenetic theories have been proposed: the simultaneous proliferation of multiple cell lineages or the proliferation of stem/progenitor cells capable of differentiating along multiple cell lineages.

Most studies using different molecular techniques (i.e., loss of heterozygosity, mutational analysis, clonality analysis) claim that a common genetic origin of the two tumor components is more probable [1,7,11-13,23]. Shared LOH at various chromosomes suggest a multistep progression from a common precursor lesion, with a higher frequency of chromosomal abnormalities in the NEC than in the adenocarcinoma component [1].

In our series, 5 of 6 cases presented similar mutational profiles in the two tumor components. Of interest, in cases #1 and #5 a common *TP53* gene mutation coexisted with multiple driver gene mutations in the neuroendocrine component (*KDR/VEGFR2*, *JAK3*,

ERBB4, *ATM*). This further supports the theory of a common precursor lesion that underwent divergent clonal evolution. Only case #6 showed a mutational profile compatible with the collision tumor theory, but it is also possible that the common ancestor mutation of this case is in a gene that is not included in the 54 genes explored herein.

Overall these histogenetic data are compatible with what is observed in clinical practice. A recent study on colorectal MANECs pinpointed that the type of cancer cell population in the metastatic site is largely unpredictable, being either mixed or pure [24]. Moreover, it did not necessarily correspond to the most prevalent or most aggressive neoplastic component [24]. By the prognostic point of view, it is generally considered that the clinical behavior of the tumor is dictated by the most aggressive component [1]. In the stomach, mixed tumors follow a behavior intermediate between pure large cell NEC, which is the most aggressive subgroup, and conventional gastric adenocarcinoma [25]. In the colon and rectum, the extent of the neuroendocrine component is not a predictor of behavior, and even a minor high-grade neuroendocrine cell population was found able to metastasize [24,26]. Very few cases of hepatobiliary MANEC have been reported so far and clinical comparisons are still missing [6].

By deep sequencing 54 cancer-related genes, we observed that most neoplastic samples (11 of 12) presented at least one somatic mutation. *TP53* gene mutations were the most frequent alterations observed in the series, and this was further confirmed by the strong p53 immunostaining observed in most samples. The presence of *TP53*, *CTNBB1*, *KRAS*, and *RB1* alterations has already been observed in pancreatic NECs [27], supporting the aggressiveness of this tumor component. On the other hand, the lack of mutations in *ATRX*, *DAXX*, *MEN1*, and *TSC2* supports a distinct molecular evolution of the NEC component from those reported for well-differentiated neuroendocrine tumors [28,29].

As previously described for other tumor types [20,30,31], our results further support the clinical impact of targeted NGS on routinely processed samples. The integration of the mutational profiles with morphological and immunophenotypic data depicts a next-generation type of histopathological diagnosis. This approach allows both the description of cancer heterogeneity in a diagnostic report and the identification of potential therapeutic targets for which agents are currently in clinical trials. In this respect, our series showed mutations in genes that could have a clinical impact such as *KRAS*, *ATM*, *JAK3*, *KDR/VEGFR2*, and *ERBB4*. This information might represent the biological ground for planning future personalized target therapies in these patients.

In conclusion, the present NGS data are strongly suggestive to consider gastrointestinal MANECs as biphenotypic stem/progenitor cell tumors. Similar molecular

approaches should investigate MANECs' genomic landscape, which will improve the clinical strategies for these rare and underestimated tumors.

REFERENCES

1 La Rosa S, Marando A, Sessa F, Capella C: Mixed adenoneuroendocrine carcinomas (manecs) of the gastrointestinal tract: An update. Cancers (Basel) 2012;4:11-30.

2 Volante M, Rindi G, Papotti M: The grey zone between pure (neuro)endocrine and non-(neuro)endocrine tumours: A comment on concepts and classification of mixed exocrine-endocrine neoplasms. Virchows Arch 2006;449:499-506.

3 Bosman FT, World Health Organization, International Agency for Research on Cancer: Who classification of tumours of the digestive system, ed 4th. Lyon, International Agency for Research on Cancer, 2010.

4 Volante M, Birocco N, Gatti G, Duregon E, Lorizzo K, Fazio N, Scagliotti GV, Papotti M: Extrapulmonary neuroendocrine small and large cell carcinomas: A review of controversial diagnostic and therapeutic issues. Hum Pathol 2014;45:665-673.

5 Capella C, La Rosa S, Uccella S, Billo P, Cornaggia M: Mixed endocrine-exocrine tumors of the gastrointestinal tract. Semin Diagn Pathol 2000;17:91-103.

6 Harada K, Sato Y, Ikeda H, Maylee H, Igarashi S, Okamura A, Masuda S, Nakanuma Y: Clinicopathologic study of mixed adenoneuroendocrine carcinomas of hepatobiliary organs. Virchows Arch 2012;460:281-289.

7 Paniz Mondolfi AE, Slova D, Fan W, Attiyeh FF, Afthinos J, Reidy J, Pang Y, Theise ND: Mixed adenoneuroendocrine carcinoma (manec) of the gallbladder: A possible stem cell tumor? Pathol Int 2011;61:608-614.

8 Yamashina M, Flinner RA: Concurrent occurrence of adenocarcinoma and carcinoid tumor in the stomach: A composite tumor or collision tumors? Am J Clin Pathol 1985;83:233-236.

9 Lewin K: Carcinoid tumors and the mixed (composite) glandular-endocrine cell carcinomas. Am J Surg Pathol 1987;11 Suppl 1:71-86.

10 Cheng H, Leblond CP: Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial cell types. Am J Anat 1974;141:537-561.

11 Furlan D, Cerutti R, Genasetti A, Pelosi G, Uccella S, La Rosa S, Capella C: Microallelotyping defines the monoclonal or the polyclonal origin of mixed and collision endocrine-exocrine tumors of the gut. Lab Invest 2003;83:963-971.

12 Huang J, Behrens C, Wistuba, II, Gazdar AF, Jagirdar J: Clonality of combined tumors. Arch Pathol Lab Med 2002;126:437-441.

13 Kim KM, Kim MJ, Cho BK, Choi SW, Rhyu MG: Genetic evidence for the multi-step progression of mixed glandular-neuroendocrine gastric carcinomas. Virchows Arch 2002;440:85-93.

14 Ramage JK, Ahmed A, Ardill J, Bax N, Breen DJ, Caplin ME, Corrie P, Davar J, Davies AH, Lewington V, Meyer T, Newell-Price J, Poston G, Reed N, Rockall A, Steward W, Thakker RV, Toubanakis C, Valle J, Verbeke C, Grossman AB: Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours (nets). Gut 2012;61:6-32.

15 Simbolo M, Gottardi M, Corbo V, Fassan M, Mafficini A, Malpeli G, Lawlor RT, Scarpa A: DNA qualification workflow for next generation sequencing of histopathological samples. PLoS One 2013;8:e62692.

16 Zamo A, Bertolaso A, van Raaij AW, Mancini F, Scardoni M, Montresor M, Menestrina F, van Krieken JH, Chilosi M, Groenen PJ, Scarpa A: Application of microfluidic technology to the biomed-2 protocol for detection of b-cell clonality. J Mol Diagn 2012;14:30-37.

17 Corbo V, Dalai I, Scardoni M, Barbi S, Beghelli S, Bersani S, Albarello L, Doglioni C, Schott C, Capelli P, Chilosi M, Boninsegna L, Becker KF, Falconi M, Scarpa A: Men1 in pancreatic endocrine tumors: Analysis of gene and protein status in 169 sporadic neoplasms reveals alterations in the vast majority of cases. Endocr Relat Cancer 2010;17:771-783.

18 Missiaglia E, Dalai I, Barbi S, Beghelli S, Falconi M, della Peruta M, Piemonti L, Capurso G, Di Florio A, delle Fave G, Pederzoli P, Croce CM, Scarpa A: Pancreatic endocrine tumors: Expression profiling evidences a role for akt-mtor pathway. J Clin Oncol 2010;28:245-255.

19 Jiao Y, Shi C, Edil BH, de Wilde RF, Klimstra DS, Maitra A, Schulick RD, Tang LH, Wolfgang CL, Choti MA, Velculescu VE, Diaz LA, Jr., Vogelstein B, Kinzler KW, Hruban RH, Papadopoulos N: Daxx/atrx, men1, and mtor pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science 2011;331:1199-1203.

20 Fassan M, Simbolo M, Bria E, Mafficini A, Pilotto S, Capelli P, Bencivenga M, Pecori S, Luchini C, Neves D, Turri G, Vicentini C, Montagna L, Tomezzoli A, Tortora G, Chilosi M, De Manzoni G, Scarpa A: High-throughput mutation profiling identifies novel molecular dysregulation in high-grade intraepithelial neoplasia and early gastric cancers. Gastric Cancer 2013

21 Amato E, Molin MD, Mafficini A, Yu J, Malleo G, Rusev B, Fassan M, Antonello D, Sadakari Y, Castelli P, Zamboni G, Maitra A, Salvia R, Hruban RH, Bassi C, Capelli P, Lawlor RT, Goggins M, Scarpa A: Targeted next-generation sequencing of cancer genes dissects the molecular profiles of intraductal papillary neoplasms of the pancreas. J Pathol 2014

22 Hamada S, Futamura N, Ikuta K, Urakawa H, Kozawa E, Ishiguro N, Nishida Y: Ctnnb1 s45f mutation predicts poor efficacy of meloxicam treatment for desmoid tumors: A pilot study. PLoS One 2014;9:e96391.

23 Furlan D, Cerutti R, Uccella S, La Rosa S, Rigoli E, Genasetti A, Capella C: Different molecular profiles characterize well-differentiated endocrine tumors and poorly differentiated endocrine carcinomas of the gastroenteropancreatic tract. Clin Cancer Res 2004;10:947-957.

24 Li Y, Yau A, Schaeffer D, Magliocco A, Gui X, Urbanski S, Waghray R, Owen D, Gao ZH: Colorectal glandular-neuroendocrine mixed tumor: Pathologic spectrum and clinical implications. Am J Surg Pathol 2011;35:413-425.

25 Jiang SX, Mikami T, Umezawa A, Saegusa M, Kameya T, Okayasu I: Gastric large cell neuroendocrine carcinomas: A distinct clinicopathologic entity. Am J Surg Pathol 2006;30:945-953.

26 La Rosa S, Marando A, Furlan D, Sahnane N, Capella C: Colorectal poorly differentiated neuroendocrine carcinomas and mixed adenoneuroendocrine carcinomas: Insights into the diagnostic immunophenotype, assessment of methylation profile, and search for prognostic markers. Am J Surg Pathol 2012;36:601-611.

27 Yachida S, Vakiani E, White CM, Zhong Y, Saunders T, Morgan R, de Wilde RF, Maitra A, Hicks J, Demarzo AM, Shi C, Sharma R, Laheru D, Edil BH, Wolfgang CL, Schulick RD, Hruban RH, Tang LH, Klimstra DS, Iacobuzio-Donahue CA: Small cell and large cell neuroendocrine carcinomas of the pancreas are genetically similar and distinct from well-differentiated pancreatic neuroendocrine tumors. Am J Surg Pathol 2012;36:173-184.

28 Oberg K, Casanovas O, Castano JP, Chung D, Delle Fave G, Denefle P, Harris P, Khan MS, Kulke MH, Scarpa A, Tang LH, Wiedenmann B: Molecular pathogenesis of neuroendocrine tumors: Implications for current and future therapeutic approaches. Clin Cancer Res 2013;19:2842-2849.

29 Capelli P, Fassan M, Scarpa A: Pathology - grading and staging of gep-nets. Best Pract Res Clin Gastroenterol 2012;26:705-717.

30 Simbolo M, Fassan M, Ruzzenente A, Mafficini A, Wood LD, Corbo V, Melisi D, Malleo G, Vicentini C, Malpeli G, Antonello D, Sperandio N, Capelli P, Tomezzoli A, Iacono C, Lawlor RT, Bassi C, Hruban RH, Guglielmi A, Tortora G, de Braud F, Scarpa A: Multigene mutational profiling of cholangiocarcinomas identifies actionable molecular subgroups. Oncotarget 2014;5:2839-2852.

31 Scarpa A, Sikora K, Fassan M, Rachiglio AM, Cappellesso R, Antonello D, Amato E, Mafficini A, Lambiase M, Esposito C, Bria E, Simonato F, Scardoni M, Turri G, Chilosi M, Tortora G, Fassina A, Normanno N: Molecular typing of lung adenocarcinoma on cytological samples using a multigene next generation sequencing panel. PLoS One 2013;8:e80478.

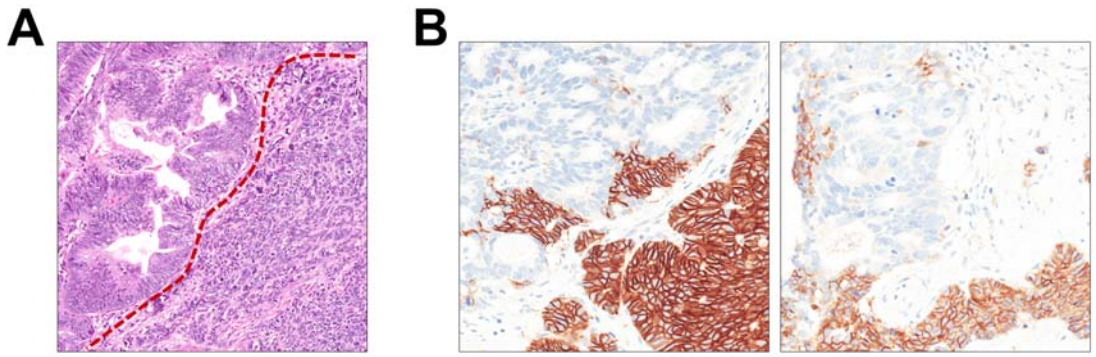

Figure 1. Representative histomorphological features of gastrointestinal MANECs. A tumor presenting areas in which the adenocarcinoma and neuroendocrine components are arranged as collision (**A**; H&E) or combined tumors (**B**; CD56 immunohistochemical staining in two areas of the same tumor). Original magnifications, 10x and 20x.

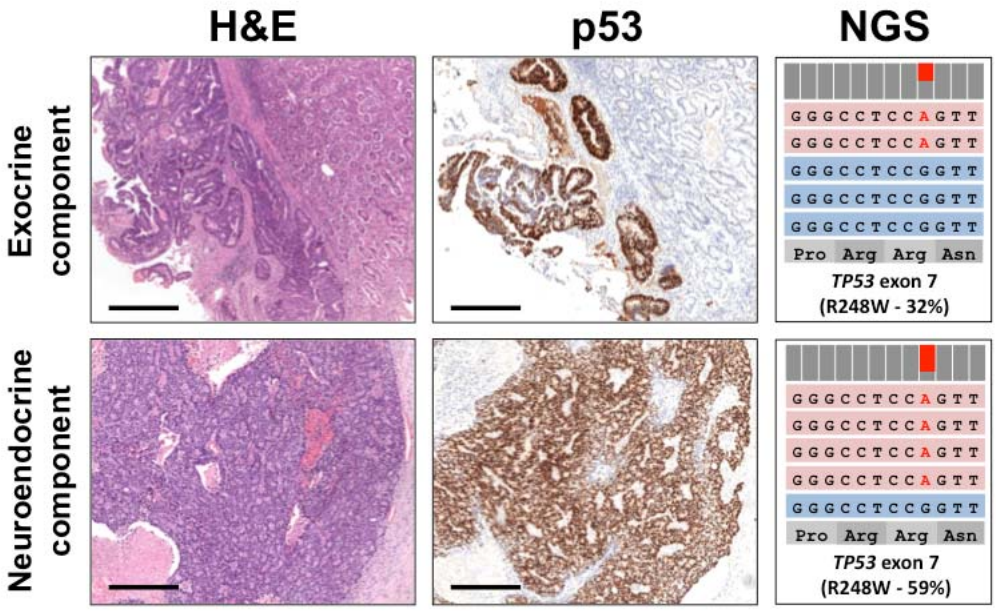

Figure 2. MANEC exocrine and neuroendocrine components share similar mutational profiles. A jejunal MANEC showing a *TP53* R248W mutation in both the exocine and neuroendocrine components. Representative p53 immunohistochemical images of the lesions are shown (original magnifications, 20x), and confirm that *TP53* mutational status corresponds to p53 protein nuclear accumulation. On the right there is the representation of the reads aligned to the reference genome as provided by the Integrative Genomics Viewer (IGV v.2.1, Broad Institute) software.

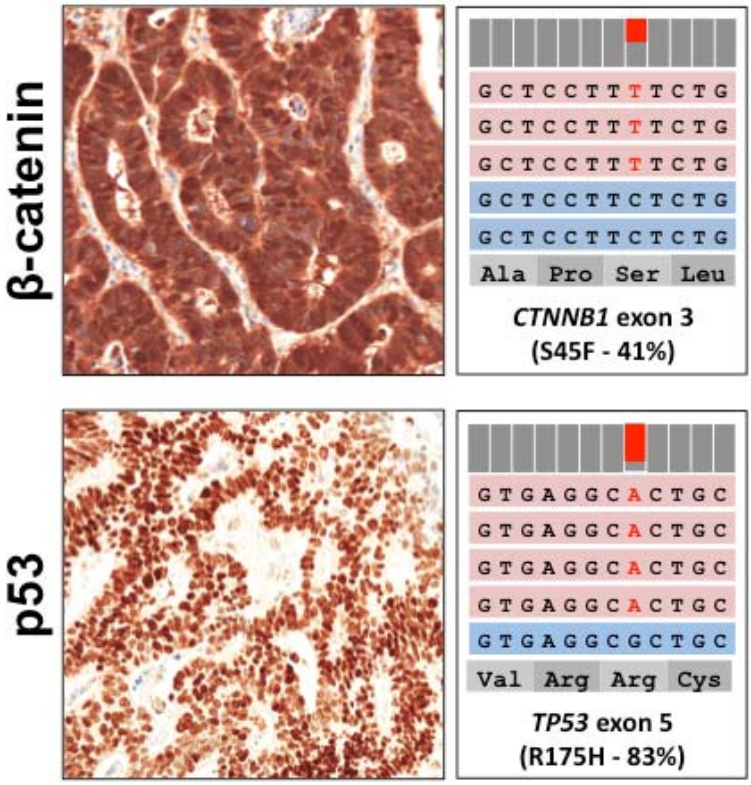

Figure 3. Immunohistochemistry is a valid surrogate of ß-catenin and p53 mutational status. A pancreatic MANEC harboring a *CTNNB1* and a *TP53* mutation in the exocrine component that were absent in the neuroendocrine component. Representative ß-catenin and p53 immunohistochemical images of the exocrine lesion are shown (original magnifications, 20x). A nuclear and cytoplasmic ß-catenin immunoreaction and a p53 nuclear accumulation were observed. The neuroendocrine component had a membranous ß-catenin and a negative p53 immunostaining (not shown). On the right there is the corresponding representation of the gene sequence reads aligned to the reference genome as provided by the Integrative Genomics Viewer (IGV v.2.1, Broad Institute) software.

Table 1. Clinico-pathological features and mutational status of 6 gastroenteropancreatic MANECs

Case	Gender	Age	Location	Grading adenocarcinoma*	Grading NEN*	Gene mutations (% of mutated alleles)	
						Exocrine component	Neuroendocrine component
1	Male	89	Stomach	G2	NEC G3 (Ki67: 68%)	*TP53* R273H (52%)	*TP53* R273H (88%) *ERBB4* F247S (20%) *ATM* S1923del (26%)
2	Male	80	Stomach	G2/3	NEC G3 (Ki67: 72%)	*RB1* R358* (63%) TP53 R273C (63%)	*RB1* R358* (82%) *TP53* R273C (77%)
3	Male	66	Jejunum	G2	NEC G3 (Ki67: 45%)	*TP53* R248W (32%)	*TP53* R248W (59%)
4	Male	64	Rectum	G3	NEC G3 (Ki67: 55%)	*KRAS* G13D (21%) *TP53* R209Kfs*6 (25%)	*KRAS* G13D (35%) *TP53* R209Kfs*6 (64%)
5	Male	70	Pancreas	G2	NEC G3 (Ki67: 73%)	*TP53* D281Y (53%)	*TP53* D281Y (95%) *KDR* Q472H LOH *JAK3* V722I LOH
6	Male	63	Pancreas	G2	NEC G3 (Ki67: 82%)	*CTNNB1* S45F (41%) *TP53* R175H (83%)	wt

* According to WHO 2010 classification [3].

Neuroendocrinology (*International Journal for Basic and Clinical Studies on Neuroendocrine Relationships*)
Journal Editor: Millar R.P. (Edinburgh)
ISSN: 0028-3835 (Print), eISSN: 1423-0194 (Online)
www.karger.com/NEN