Adenomatous polyposis coli alteration in digestive endocrine tumours: correlation with nuclear translocation of β-catenin and chromosomal instability

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Abstract

The role of Wnt pathway in digestive endocrine tumours is debated. The aim of this work is to investigate key players in Wnt pathway by a multimodal approach. Sixty cases (49 well-differentiated and 11 poorly differentiated) were investigated for methylation of *adenomatous polyposis coli* (*APC*) and *E-cadherin* promoters, the loss of heterozygosity (LOH) at *APC* locus and *β-catenin* and *E-cadherin* expression by immunohistochemistry. Tumours showing altered β-catenin localization were tested for *β-catenin* and *APC* mutations. *APC* promoter methylation was restricted to gastroduodenal tumours (21 out of 59, 36%), prevalent in poorly differentiated carcinomas ($P=0.042$) and correlating with aggressive features (high histology grade, $P<0.02$; tumour death, $P=0.026$; high fractional allelic loss, $P=0.002$, in turn correlating with short survival, $P=0.017$). LOH at *APC* locus was found in 14 out of 53 cases (26%, 10 gastroduodenal and 4 colorectal), prevalent in poorly differentiated carcinomas ($P=0.002$) and correlating with histology grade ($P=0.012$). *β-catenin* abnormal expression was found in 41 out of 54 cases (76%), with nuclear staining correlating with *APC* alteration ($P=0.047$) and short survival ($P=0.006$). *APC*, but not *β-catenin*, gene mutations were found (7 out of 35 tumours), 4 of which in the midgut. *E-cadherin* promoter methylation was rarely detected (2 out of 52 cases), with cytoplasmic expression in 18 out of 43 cases (42%), not correlating with any clinico-pathological feature. In conclusion, Wnt pathway alterations, as represented by abnormal β-catenin localization, are common events in digestive endocrine tumours, but only nuclear expression correlates with tumour aggressiveness. Though with different alteration mechanisms according to anatomical site, APC plays a major role in Wnt pathway activation and in determining the high chromosomal instability observed in aggressive endocrine carcinomas.

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Introduction

The *adenomatous polyposis coli* (*APC*) tumour suppressor gene was originally identified as the gene responsible for familial adenomatous polyposis (FAP; see (Bright Thomas & Hargest 2003) and reference herein). The *APC* gene, located on chromosome 5q21-22, is mutated in the majority of colon cancers and acts as antagonist of the Wnt signalling pathway. The loss of APC function leads

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to the stabilization of β-catenin in the cytoplasm, followed by its migration in the nucleus, where β-catenin acts as co-activator of the T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factor family, inducing the expression of cell proliferation regulator genes, such as c-myc and cyclin D1. β-catenin is normally localized at the cell membrane, where it anchors the E-cadherin adhesion protein to the cytoskeleton. In normal conditions, free β-catenin is rapidly degraded by proteasomes after phosphorylation and ubiquitination following the binding to a complex comprising APC, axin and glycogen syntase kinase-3β (Doucas *et al*. 2005).

Generally, *APC* and *β-catenin* mutations are mutually exclusive in cancer, but *β-catenin* mutations are more frequent in small adenomas than in large or invasive colon cancers (Samowitz *et al*. 1999). This suggests that *APC* mutations confer greater selective advantage than *β-catenin* mutations, likely due to tumour suppressor functions of *APC* other than the mere control of the Wnt pathway. Indeed, APC is a multifunctional protein with roles in cell migration and adhesion (dependent or not of β-catenin/E-cadherin system and Wnt pathway), chromosome segregation, spindle assembly, apoptosis and neuronal differentiation (Hanson & Miller 2005). Mutations of the *APC* gene are quite rare in cancers outside the gut, while *β-catenin* gene mutations are common in desmoid, gastric cancer, hepatocarcinoma, medulloblastoma, melanoma, ovarian cancer, pancreatic cancer and prostate cancer (Kikuchi 2003).

In digestive neuroendocrine tumours, abnormal β-catenin expression was frequently found, though mutation of *β-catenin* exon 3 was reported by some and not by others, most often in the absence of *APC* mutations (Gerdes *et al*. 1999, Semba *et al*. 2000, Fujimori *et al*. 2001, Barshack *et al*. 2002, Li *et al*. 2002, Hervieu *et al*. 2006, Su *et al*. 2006). Additionally, *APC* promoter methylation was reported in digestive endocrine tumours (House *et al*. 2003, Arnold *et al*. 2004*a*).

The aim of this work is to assess the status of major players involved in Wnt pathway in a series of digestive endocrine tumours, investigating with a multimodal approach: i) *APC* and *E-cadherin* promoter methylation; ii) *APC* chromosomal locus alterations; iii) β-catenin and E-cadherin expression and iv) *β-catenin* and *APC* mutation.

Methods

Patients

This study was approved by the ethic committee of the University of Parma and informed consent was obtained from all patients and/or guardians. Sixty benign and malignant endocrine tumours of the gastroenteropancreatic (GEP) tract from 59 patients were routinely formalin-fixed, paraffin-embedded and classified according to WHO criteria, with histology grade and stage as recently proposed (Table 1; Solcia *et al*. 2000, Pizzi *et al*. 2005, Rindi *et al*. 2006, 2007).

DNA extraction

DNA from tumours and normal adjacent mucosa were isolated by manual microdissection, resulting in 80% tumour cell enrichment as described (D'Adda *et al*. 2002).

Methylation

DNA methylation status of the CpG islands of *APC* and *E-cadherin* gene promoters were determined by chemical modification of genomic DNA with sodium bisulphite and subsequent methylation-specific PCR (MSP) as described (Pizzi *et al*. 2005). Primer sequences and annealing temperatures for amplification of methylated (m) and unmethylated (u) alleles of *APC* and *E-cadherin* are as previously reported (Herman *et al*. 1996, Tsuchiya *et al*. 2000).

Loss of heterozygosity (LOH) analysis

The highly polymorphic microsatellite marker D5S346 in 5q22-23 on the *APC* locus (Zauber *et al*. 2003) was investigated with primer sequences and amplification conditions (http://www.gdb.org) with fluorescently labelled 5′-primers (WellRed dyes, Research Genetics, Huntsville, AL, USA) as previously described (D'Adda *et al*. 2002). PCR products were analyzed by the CEQ 2000XL DNA analysis systems (Beckman Coulter Inc., Fullerton, CA, USA). The following ratio was calculated on the basis of peak height data: $(\text{lower allele/higher allele})_{tum}/(\text{lower allele/higher allele})_{norm}$. The values ≤ 0.6 (allelic imbalance $\geq 40\%$) were considered as indicative of LOH.

Fractional allelic loss (FAL) index calculation

Most of the samples here investigated have been previously characterized for LOH at the following microsatellite markers: PYGM, D11S4946 and D11S913 (D'Adda *et al*. 1999*a*, 2002, Pizzi *et al*. 2003), DXS989, DXS1100 and DXS1192 (D'Adda *et al*. 1999*b*, Pizzi *et al*. 2002), TP53, D3S1478, D3S1481, D18S58 and D18S61 (Pizzi *et al*. 2003), D3S1100 and D3S1621 (Pizzi *et al*. 2005) and D9S157 and D9S171 (manuscript in preparation). A mean FAL index was calculated from the ratio: (number of markers with LOH)/(total number of informative markers).

Table 1 Clinico-pathological features of the gastroenteropancreatic (GEP) endocrine tumours analyzed

				Sex						Grade			Stage			
Site	**Type**	***n***	**Age median** (range)	**M**	**F**	**Size** (cm) **median** (range)	**Meta**	**Mitoses 10HPF median** (range)	**Ki67% median** (range)	**G1**	**G2**	**G3**	**n/stage**		**T-death**	**Obs. time person-months**
Stomach	WDET	8	60 (47–77)	5	3	0.50 (0.2–1.2)	0/8	0 (0–2)	1.60 (0.1–5)	5	3	0	6	I	1	634
													1	IIA		
	WDEC	9	47 (29–66)	7	2	6.00 (2.8–16)	9/9	7 (0–84)	25.00 (0.1–30)	2	2	5	4	IIIB	7	330
													5	IV		
	PDEC	7	63 (44–83)	6	1	3.00 (0.4–10)	6/7	51.5 (23–103)	40.00 (5–80)	0	0	7	4	IIIB	4	77
													2	IV		
Duodenum	WDEC	5	47 (30–62)	4	1	1.20 (0.7–4)	5/5	0 (0–12)	0.10 (0.1–7)	4	1	0	2	IIIB	4	240
													3	IV		
Pancreas	WDET	5	54 (52–87)	1	4	1.60 (0.7–3)	0/5	0.5 (0–2)	0.46 (0.04–0.9)	4	1	0	4	I	1	166
													1	IIA		
	WDEC	4	58 (51–67)	3	1	4.00 (3–6)	4/4	2 (1–2)	5.00 (0.1–11.5)	1	3	0	3	IIIB	2	217
													1	IV		
Ileum	WDEC	8	59 (45–75)	2	6	1.80 (1.2–4)	7/8	2 (1–3)	0.10 (0.1–2)	4	4	0	1	IIA	2	593
													3	IIIB		
													4	IV		
Appendix	WDET	6	30 (22–41)	1	5	0.90 (0.5–1.4)	0/6	0 (0–2)	0.10 (0.1–0.28)	5	1	0	4	I	0	633
													2	IIA		
Colon	PDEC	4	70 (63–81)	2	2	7.00 (4–8)	3/4	38 (22–81)	40.00 (40–40)	0	0	3	3	IIIB	2	80
Rectum	WDET	3	62 (56–68)	2	1	1.00 (0.9–2)	0/3	0 (0–1)	0.30 (0.1–0.5)	3	0	0	1	IA	0	240
													2	IB		
	WDEC	1	67	1	0	–	1/1	2	5	0	1	0	1	IIIB	0	19

WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; M, male; F, female; Meta, presence of metastases; T-death, tumour-related death; Obs.time, observation time. Gastric tumours were 7 type I (in chronic atrophic gastritis), 1 type II (in MEN1 syndrome) and 9 type III carcinoids plus 7 non-functioning PDECs. Duodenal WDECs were two gastrinomas, one VIPoma and two non-functioning carcinomas (two cases were in MEN1 syndrome). Pancreatic tumours were four insulinomas and five non-functioning neoplasms. Ileal tumours were six non-functioning, one with typical carcinoid syndrome and two cases with unknown clinical data. Appendicular and colorectal tumours were all non-functioning.

Immunohistochemistry (IHC)

General neuroendocrine markers, hormones and Ki-67 were investigated as described (Bordi *et al.* 1991). β-catenin and E-cadherin IHC was performed after thermal antigen retrieval using the mouse clones β-catenin-1 (1:400 Dako, Glostrup, Denmark) and HECD-1 (1:100 Zymed Laboratories, Inc., San Francisco, CA, USA). The cells of normal epithelial tissue were used as positive controls. Negative controls consisted of omission of the primary antibody. The fraction of tumour cells expressing β-catenin and/or E-cadherin was assessed, as well as the staining distribution (membranous, cytoplasmic and nuclear) according to Aust *et al.* (2001). Areas with stronger protein expression were evaluated in tumours with zonal staining pattern.

β-Catenin and APC gene mutation analysis

Samples showing altered β-catenin expression were further analysed for mutations in exon 3 of the *β-catenin* gene and in the mutation cluster region (MCR) of exon 15 of the *APC* gene (approximately codons 1300–1500 (Miyoshi *et al.* 1992)). The primers used for *β-catenin* gene were: CAT3F 5′-ATGGAACCAGACA-GAAAAGC-3′ and CAT3R 5′-GCTACTTGTTCT-GAGTGAAG-3′ (fragment size: 200 bp; Gerdes *et al.* 1999). *APC* mutation analysis was performed using four sets of primers, amplifying two overlapping portions of exon 15: 1) APCGn 5′-AAGAAACAATA-CAGACTTATTGT-3′ and APC-Gc 5′-ATGAGTG-GGGTCTCCTGAAC-3′ (fragment G: codons 1256–1381, 377 bp; Doglioni *et al.* 2003); 2) APC-Hn 5′-ATCTCCCTCCAAAAGTGGTGC-3′ and APC-Hc 5′-TCCATCTGGAGTACTTTCCGT G-3′ (fragment H: codons 1359–1499, 421 bp; Doglioni *et al.* 2003); 3) APC-1f 5′-CATCAGCTGAAGATGAAATAGGA-3′ and APC-1r 5′-GCAATCGAACGACTCTCAAA-3′ (fragment APC-1: codons 1281–1402, 364 bp; Su *et al.* 2006) and 4) APC-2f 5′-ATGTTCAGGAGACCC-CACTC-3′ and APC-2r 5′-CACTCAGGCTGGAT-GAACAA-3′ (fragment APC-2: codons 1376–1508, 396 bp; Su *et al.* 2006). PCR was performed in a final volume of 50 μl, with 25 pmol of each primer, 0.8 mM total dNTPs, 1.5 mM $MgCl_2$ and 1.25 units AmpliTaq Gold (Applied Biosystem, Foster City, CA, USA). Conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C (for CAT3, APC-1 and APC-2 primers) or 48 °C (for G and H fragments) for 45 s and elongation at 72 °C for 45 s. DNA sequencing was performed using Eurofins MWG Operon/M-Medical (Milano, Italy). Sequencing results were verified in our laboratory in both sense and antisense directions using DNA STAR PC software (Lasergene, Madison, WI, USA). Mutations were determined through alignment with normal sequences as reported in NCBI/Blast Human Genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi: ref|NT_034772.5|Hs5_34934 for APC, ref|NT_022517.17|Hs3_22673 for β-catenin). Mutations were named according to the nomenclature proposed by the Human Genome Variation Society (http://www.hgvsorg/) and submitted to the Human Gene Mutation Database, Cardiff (http://www.hgmd.cf.ac.uk).

Statistical analysis

The frequencies of specific gene alterations in different tumour groups were analyzed using two-tailed Fisher's exact test. Overall, survival curves were calculated using the method of Kaplan & Meier (1958). The log-rank test (Mantel 1966) was used to compare survival distributions. $P<0.05$ were considered significant in all analysis. SPSS software (version 8.0, SPSS Inc., Chicago, IL, USA) was used in all analyses.

Results

APC promoter methylation is restricted to gastroduodenal tumours and correlates with unfavourable prognostic factors

APC promoter methylation (as an example see Fig. 1A) was detected in 21 out of 59 (36%) of all cases and restricted to gastroduodenal tumours (75 and 60% of cases respectively; Table 2 and Fig. 2A, left). *APC* methylation was more frequent in poorly differentiated endocrine carcinomas (PDECs, occurring in 100% of gastric cases) than in well-differentiated neoplasms, either benign or malignant ($P=0.042$), in male than female patients ($P=0.015$), with no significant correlation with age (Table 3). Though not correlating with size, the presence of metastases nor mitotic count, *APC* promoter methylation strongly correlated with Ki67 index $>2\%$ ($P<0.001$), high histology grade ($P<0.02$) and stage ($P=0.047$), and poor outcome, being more frequent in patients dead of disease than in patients alive with or without evidence of disease ($P=0.026$) and barely missing statistical significance in survival analysis (median OS 100 vs 84 months, $P=0.097$; Fig. 2A, right and Table 3). *APC* methylation strongly correlated with chromosomal instability, as indicated by higher FAL index (≥ 0.3) in methylated tumours versus unmethylated ($P<0.001$, mean value 0.49 ± 0.27 vs 0.18 ± 0.26; Fig. 2B, left). In turn, FAL values ≥ 0.3 correlated with shorter survival (median OS: 24 months versus median not reached, $P=0.017$; Fig. 2B, right).

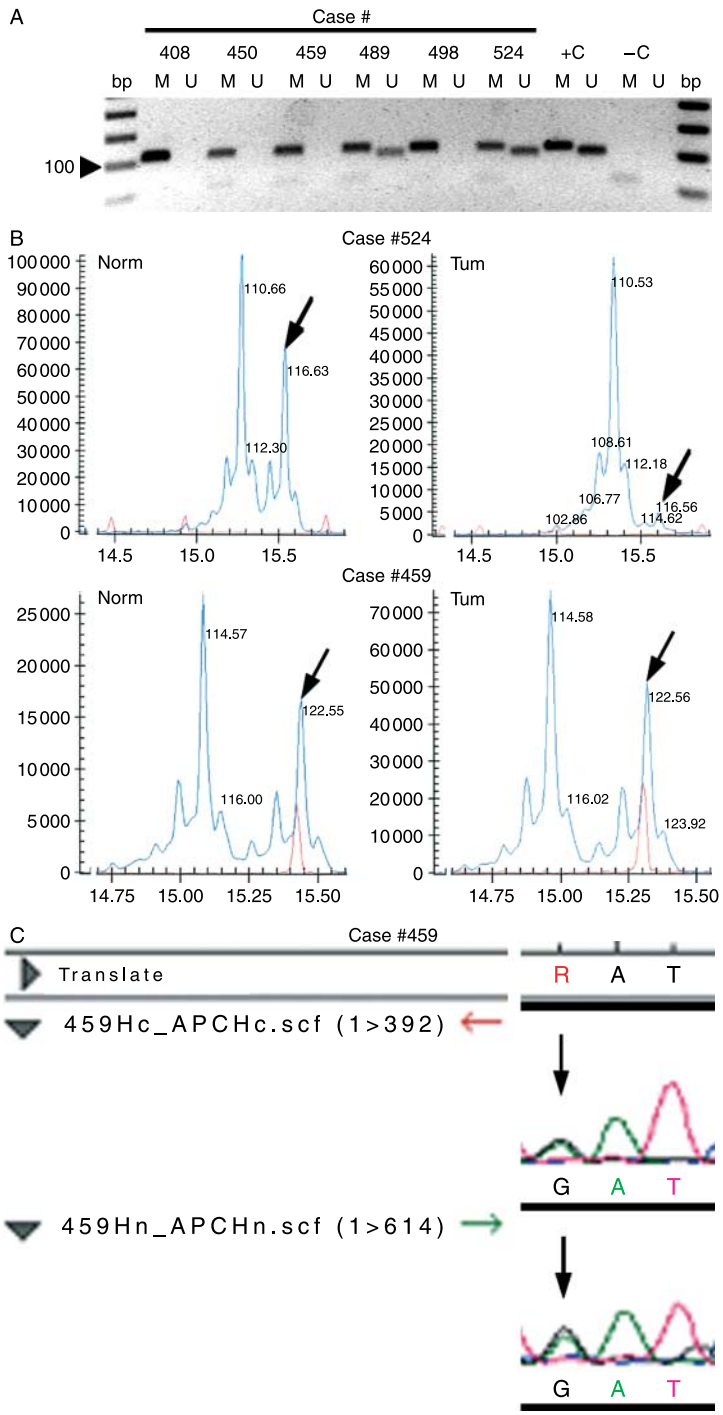

Figure 1 (A) *APC* promoter methylation-specific PCR in six endocrine carcinomas: case #408, ileum WDEC G2, stage IIIB; #450 ileum WDEC G3, stage IV (not used in the present study); #459, ileum WDEC G3, stage IV; #489, stomach PDEC G3, stage IIIB; #498, stomach WDEC G1, stage IV; #524, stomach WDEC G3, stage IV; in cases #489 and 524 both alleles are methylated; M, methylated allele; U, unmethylated allele; +C, positive control; −C, negative control. (B) Electrophoretic profiles for the microsatellite D5S346: case #524 (*APC* promoter methylation in (A)) shows the loss of the shorter allele (arrow) in tumour DNA when compared with normal, while case #459 (no *APC* promoter methylation in (A)) retains both alleles at the same length (no LOH); *x*-axis, size of the PCR fragments in bps; *y*-axis, intensity of fluorescence (peak heights); norm, normal tissue DNA; tum, tumour DNA. (C) Mutational analysis of *APC* gene MCR: case #459 (no *APC* promoter methylation in (A)) and no D5S346 LOH in (B)) shows mutation at codon 4247 (G>T) – GAT>AAT, as observed in both forward and reverse sequences (arrow).

Table 2 Summary of the molecular and immunohistochemical analyses results

Site	Type	*n*	FAL median (range)	APC			β-Catenin				E-Cadherin		
				MET	LOH	MUT	MUT	IHC			MET	IHC	
								mem	lom	cyt		mem	lom	cyt
Stomach	WDET	8	0.29 (0.00–0.60)	4/8	2/7	0/3	0/4	3/7	0/7	4/7	0/7	2/6	1/6	3/6
	WDEC	9	0.62 (0.00–0.90)	7/9	2/6	1/6	0/6	0/6	1/6	2/6	0/7	4/5	0/5	1/5
	PDEC	7	0.55 (0.40–0.70)	7/7	4/7	1/5	0/5	2/7	0/7	2/7	1/6	2/4	1/4	1/4
Duodenum	WDEC	5	0.30 (0.00–0.33)	3/5	2/5	0/2	0/2	2/4	0/4	1/4	1/5	4/4	0/4	0/4
Pancreas	WDET	5	0.00 (0.00–0.33)	0/5	0/5	0/2	0/2	2/4	1/4	1/4	0/3	2/4	0/4	2/4
	WDEC	4	0.38 (0.00–0.75)	0/4	0/3	0/1	0/3	1/4	0/4	2/4	0/3	0/4	0/4	4/4
Ileum	WDEC	8	0.00 (0.00–0.20)	0/8	0/6	2/6	0/6	2/8	0/8	4/8	0/8	2/5	0/5	3/5
Appendix	WDET	6	0.00 (0.00–0.00)	0/5	0/6	2/5	0/6	0/6	0/6	6/6	0/6	3/3	0/3	0/3
Colon	PDEC	4	0.58 (0.22–0.86)	0/4	3/3	1/2	0/3	0/3	0/3	1/3	0/3	2/3	0/3	1/3
Rectum	WDET	3	0.00 (0.00–0.67)	0/3	1/3	0/2	0/2	0/3	0/3	3/3	0/2	2/3	0/3	1/3
	WDEC	1	0.22	0/1	0/1	0/1	0/1	0/1	0/1	1/1	0/1	1/1	0/1	0/1

WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; FAL, fractional allelic loss; MET, promoter methylation; LOH, loss of heterozygosity; MUT, gene mutation; IHC, immunohistochemistry; mem, membranous staining without cytoplasmic expression; lom, loss of membranous staining without cytoplasmic expression; cyt, cytoplasmic staining with or without membranous expression; nu, nuclear expression.

APC locus deletions are extended to colorectal tumours

APC locus marker LOH (as an example see Fig. 1B) was found in 14 out of 53 (26%) informative cases, with similar distribution as for *APC* methylation, i.e. in gastroduodenal tumours plus colon PDECs and one rectal well-differentiated endocrine tumour (WDET; Fig. 2A, left and Table 2). *APC* LOH was significantly more frequent in PDECs than in well-differentiated neoplasms ($P=0.002$) and in tumours with high histology grade ($P=0.028$) and high FAL index ($P=0.001$; Table 3).

Nuclear accumulation of β-catenin correlates with tumour aggressiveness and *APC* alterations

Disruption of normal membranous pattern (Fig. 2C, left), including various combinations of cytoplasmic accumulation and nuclear migration, occurred in the majority of GEP endocrine neoplasms (41 out of 54, 76%), independently of type and site (Tables 2 and 3). Nuclear accumulation (Fig. 2C, left) was found in 12 out of 54 cases (22%) co-segregating with aggressiveness. It was significantly more frequent in well-differentiated carcinomas (WDEC) than WDET ($P=0.011$) and in PDECs than well-differentiated neoplasms ($P=0.033$), correlating with high size (≥ 2 cm; $P=0.002$), the presence of metastases ($P<0.001$), mitotic count ($P=0.011$), Ki67 index (>2; $P=0.011$), high histology grade ($P=0.005$) and stage ($P<0.001$), tumour-related death ($P=0.018$) and shorter survival (median OS: 13 months versus median not reached, $P=0.006$, Fig. 2C, right). Finally, any *APC* alteration (methylation and/or LOH) correlated with β-catenin nuclear expression ($P=0.047$).

E-cadherin abnormality does not correlate with clinical-pathological features

E-cadherin promoter methylation was found in 2 out of 52 analyzed cases only (Table 2). The loss of normal membranous staining pattern and concurrent cytoplasmic accumulation was found in 18 out of 43 cases (42%; Table 2). E-cadherin alteration was more frequent in pancreatic tumours than in other neoplasms (6 out of 8 cases, 75%, $P=0.044$), in the absence of statistically significant correlation with any clinical-pathological feature or any other alteration investigated here.

Mutations in APC but not in β-*catenin* gene are found in tumours with altered β-catenin expression

Tumours with altered β-catenin expression ($N=41$) were analysed for mutations in exon 3 of *β-catenin* gene and

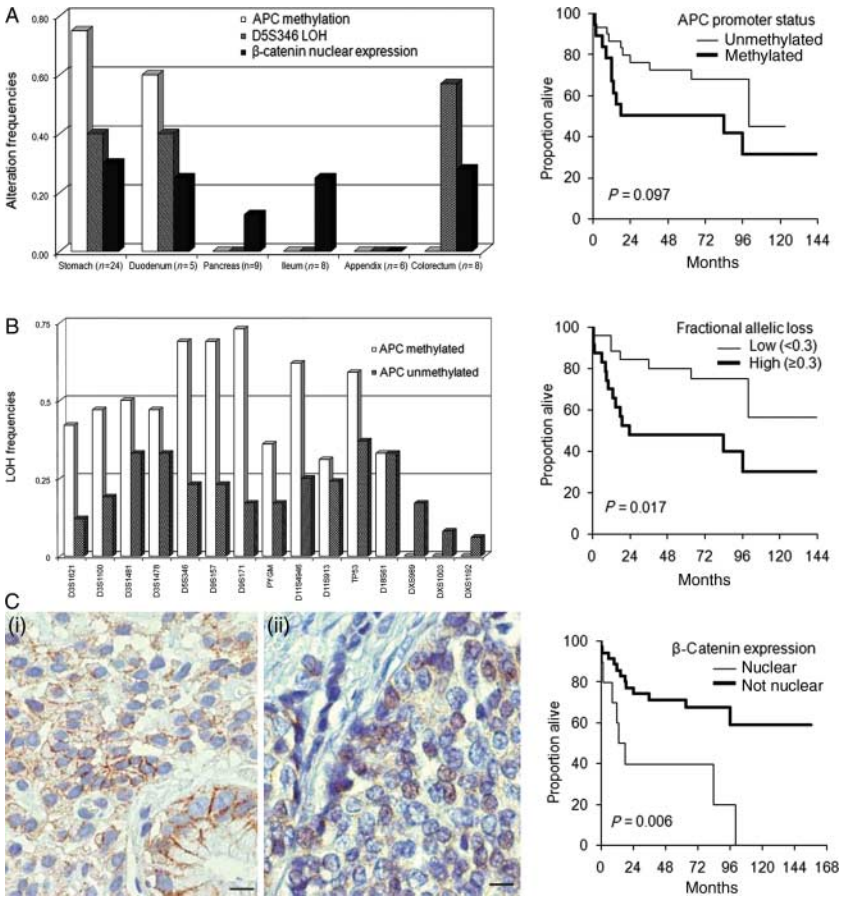

Figure 2 (A) Wnt pathway alterations in gut endocrine tumours. Left: distribution of *APC* alterations and β-catenin nuclear expression in tumours from different sites. Right: Kaplan–Meyer survival curve according to APC promoter status: APC methylation tends to correlate with shorter survival, though not reaching the statistical significance by log-rank test ($P=0.097$). (B) Correlation between aneuploidy and *APC* promoter methylation. Left: differential LOH frequencies at different microsatellite loci between tumours with or without APC promoter methylation; overall, tumours with promoter methylation showed higher FAL values ($P=0.00$). Right: Kaplan–Meyer survival curve according to FAL values: patients with tumours with FAL ≥ 0.3 showed a statistically significant shorter survival ($P=0.017$). (C) Alterations in β-catenin expression and tumour aggressiveness. Left: examples of immunohistochemical analysis of β-catenin in gastric endocrine tumours. (i) WDET with normal membranous β-catenin and (ii) PDEC showing the loss of membrane expression and migration of the protein in the nucleus (20× magnification, immunoperoxidase). Bar = 200 μm. Right: Kaplan–Meyer survival curve according to β-catenin alteration: patients with nuclear β-catenin expression showed a statistically significant shorter survival ($P=0.006$).

exon 15 MCR of *APC* gene. No mutation was found in *β-catenin* gene (0 out of 40). Of 35 cases, 7 (20%) showed mutations in the *APC* gene (as an example see Fig. 1C), 2 of which in PDECs and 5 in well-differentiated neoplasms (Table 4). Most mutations (4 out of 7) were found in the midgut (4 out of 11 cases investigated for, 36%). One mutation was a 2 bp frameshift deletion causing a stop codon, as described in the literature (Miyoshi *et al*. 1992). All other alterations were single-base substitutions, missense mutations, one of which previously reported (Saito *et al*. 2002). Additionally, two single nucleotide polymorphisms (SNP) were identified: 1493ACG > ACA (T1493T) in seven cases and 1478ACG > AGA (R1478R) in one case. Since located at the very end of the MCR, assessment of 1493 SNP was possible in 14 cases only.

No correlation was found between *APC* mutations and clinico-pathological features of the neoplasms and/or the presence of other molecular alterations.

Significance of multiple events cooperating in APC inactivation

A complete set of information on multiple/concurrent events of *APC* gene inactivation was available for 33 cases only, 32 of which with abnormal β-catenin expression (one undetermined; Table 5). In this series, a subset of nine cases displayed a double event (two hits), six with promoter methylation and LOH (all

Table 3 Summary of statistical analysis results (Fisher's exact test)

Variables	APC methylation	D5S346 LOH	Nuclear β-catenin expression
Tumour type			
WDETs versus *WDECs*	NS	NS	**0.011**
WDECs versus *PDECS*	NS	**0.013**	NS
WDET-Cs versus *PDECs*	**0.042**	**0.002**	**0.033**
Age			
≤50 vs >50 years	NS	NS	NS
Gender			
M versus F	**0.015**	NS	NS
Size			
≤2 vs >2 cm	NS	NS	**0.002**
Presence of metastases	*0.059*	NS	**0.000**
Mitoses			
<2 vs ≥2 (10HPF)	NS	NS	**0.011**
Ki67			
≤2 vs >2	**0.000**	NS	**0.006**
Grade			
G1 versus G2	NS	NS	NS
G2 versus G3	**0.012**	**0.000**	NS
G1 versus G3	**0.020**	**0.028**	**0.005**
Stage			
I+II versus III+IV	**0.047**	NS	**0.0007**
Tumour death	**0.026**	NS	**0.018**
FAL			
<0.3 vs ≥0.3	**0.000**	**0.004**	NS
APC methylation	–	NS	NS
D5S346 LOH	NS	–	**0.006**
β-catenin expression			
Nuclear versus other	NS	**0.005**	–
E-cadherin expression			
Membranous versus cytoplasmic	NS	NS	NS
APC inactivation			
versus *MET* and/or *LOH*	–	–	**0.047**

WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; M, male; F, female; FAL, fractional allelic loss; LOH, loss of heterozygosity; MET, promoter methylation; bold values, statistically significant *P* values; italic values, close to significant values.

gastric) and three with other combinations. Overall, two hits identified a subset of aggressive cases with significant correlation with tumour differentiation ($P=0.004$), size ($P=0.027$), the presence of metastases ($P=0.039$), Ki67 ($P=0.013$), high grade ($P=0.01$) and stage ($P=0.04$), tumour-related death (0.018), high FAL (0.013) and nuclear β-catenin ($P=0.011$).

Discussion

The major finding emerging from the above data is the important role played by APC in malignant gastrointestinal endocrine tumours. The mechanism of *APC* alteration varies between different cancer subsets: promoter methylation (with or without *APC* locus LOH) is restricted to gastroduodenal endocrine tumours while *APC* mutations are often found in the midgut. *APC* alteration associates with abnormal β-catenin expression and high chromosomal instability, which, in turn, correlates with shorter survival, supporting a double role for *APC* in both Wnt signalling and chromosomal segregation.

Mutational activation of Wnt signalling plays a key role in regulating intestinal epithelium fate and invariably initiates colorectal cancer, but it is also implicated in many other types of human tumours (Kikuchi 2003). The hallmark of Wnt pathway alterations is the stabilization of β-catenin, its migration in the nucleus where it determines the expression of various genes, involved in tumourigenesis including c-Myc and cyclin D1.

Data on Wnt pathway in GEP endocrine tumours are controversial. Some report suggest no role or some only as late event, others support its involvement although with limited role for *APC* (Fujimori *et al.* 2001, Hervieu *et al.* 2006). In specific, different frequencies of *β-catenin* mutations were reported in

Table 4 Mutations in the *adenomatous polyposis coli* (APC) gene

Case #	Site	Type	Codon	Nucleotide change	Amino acid change
173g	Stomach	WDEC	1439	CCT>CTT	Pro→Leu
370	Stomach	PDEC	1414	GTA>ATA	Val→Ile
459	Ileum	WDEC	1422	GAT>AAT	Asp→Asn
237	Ileum	WDEC	1464	GAG>AAG	Glu→Lys
351	Appendix	WDET	1482	CTT>TTT	Leu→Phe
265	Appendix	WDET	1416	GGC>GAC	Gly→Asp
487	Colon	PDEC	1465	GAGTG>GTG	AG deletion

WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma.

different series (Semba *et al*. 2000, Fujimori *et al*. 2001, Su *et al*. 2006). Nonetheless, abnormal β-catenin expression was frequently observed, often with concurrent loss of membranous E-cadherin expression, and usually associated with malignant features (Fujimori *et al*. 2001). The recent demonstration of *APC* promoter methylation in colorectal cancer and GEP endocrine tumours suggested an alternative mechanism for *APC* inactivation (Arnold *et al*. 2004*a*,*b*).

Here, we report frequent β-catenin alteration, with accumulation in the cytoplasm and/or nucleus, in GEP endocrine tumours (76% of cases), though the nuclear expression pattern only (22% of cases) correlated with biological parameters and co-segregated with aggressiveness. The nuclear localization of β-catenin was more frequent in PDECs than well-differentiated neoplasms and correlated with tumour size, mitotic count, Ki-67 index, grade, stage, presence of metastases, tumour-related death and shorter survival. Additionally, no *β-catenin* exon 3 mutation was observed in cases with altered β-catenin expression ($N=40$), suggesting alternative mechanism(s) for Wnt pathway activation. In contrast to previous studies (Semba *et al*. 2000, Fujimori *et al*. 2001, Li *et al*. 2002), we did not find statistically significant correlation between E-cadherin alterations and β-catenin expression and/or clinico-pathological features. The lack of correlation with E-cadherin alteration may find a possible explanation by the alternative role proposed for different β-catenin expression patterns (Doucas *et al*. 2005). The simple reduction or loss of membrane β-catenin indicates an alteration of the cell adhesion system through the cadherin pathway. By converse, the nuclear accumulation and concurrent increased cytoplasmic expression, as observed in our series, point to a transcriptional role for β-catenin in the Wnt pathway. Of the classical downstream transcriptional targets of β-catenin, cyclin D1 expression was previously investigated in the same series (Pizzi *et al*. 2005), proving not to correlate with β-catenin nuclear expression in a present analysis (unpublished). Alternative transcriptional targets of β-catenin should be sought for in endocrine cancer disease.

In our series, *APC* promoter methylation was almost restricted to gastroduodenal (36%) endocrine tumours, whereas it was consistently absent in pancreatic, ileal and colorectal neoplasms. This finding appears not to be part of the so-called CpG island methylation phenotype, since about 50% (11 out of 21) of cases with *APC* promoter methylation tested for showed no methylation for other genes (Pizzi *et al*. 2005 and unpublished). LOH at the *APC* locus in 5q21 largely reflected a similar distribution, with the exception of colonic PDECs and one rectal WDET. In the whole series, the presence of *APC* methylation and/or LOH correlated with β-catenin nuclear expression, indicating a central role for *APC* in Wnt pathway abnormality in gut endocrine tumours. Mutation of *APC* MCR, though conducted only in a subset of cases with altered β-catenin expression ($N=35$), was found in seven cases (20%). Mutations were frequently found in PDECs (2 out of 7) and midgut well-differentiated neoplasms (4 out of 11). The only other mutation was observed in a gastric WDEC in multiple endocrine neoplasia syndrome type 1 (MEN1). The possible association between FAP and MEN1 has been previously proposed (Sakai *et al*. 2002). Additionally, the 1493ACG>ACA SNP was often observed, its potential significance requiring further investigation.

The mechanism by which Wnt signalling is altered differs in distinct subset of GEP endocrine tumours, as shown by the restriction of *APC* promoter methylation to gastroduodenal tumours and frequent *APC* gene mutations in midgut neoplasms. These data provide further support to the hypothesis of different genetic background for different site endocrine tumours, as previously suggested (Rindi & Bordi 2003).

In our series, *APC* promoter methylation and/or LOH significantly correlated with various malignancy parameters including grade, stage and tumour death.

Table 5 *Adenomatous polyposis coli* gene inactivation hits (promoter methylation, LOH and mutation) investigated in a subset of 32 cases with evidence of altered β-catenin expression and one not determined: distribution and statistical analysis

A. Distribution per type and site		
0 hit	$n=14$	1 gastric WDET, 1 gastric WDEC; 2 pancreas WDETs, 1 pancreas WDEC; 3 ileum WDECs; 3 appendix WDETs; 2 rectum WDETs, 1 rectum WDEC
1 hit	$n=10$	4, mutation (2 appendix WDETs and 2 ileal WDECs) 3, LOH (1 gastric WDET, 1 duodenal WDEC and 1 colon PDEC) 3, promoter methylation (1 gastric WDET, 1 gastric PDEC and 1 duodenal WDEC)
≥2 hits	$n=9$	6, promoter methylation and LOH (1 gastric WDET, 2 gastric WDECs, 3 gastric PDECs) 1, promoter methylation and mutation (1 gastric WDEC) 1, mutation and LOH (1 colon PDEC) 1, promoter methylation, mutation and LOH (1 gastric PDEC)

	APC inactivation hits			
Variables	**0 vs 1**	**0 vs 2**	**1 vs 2**	**0 vs 1+2**
B. Statistical analysis (Fisher's exact test)				
Tumour type				
WDETs versus *WDECs*	NS	NS	NS	NS
WDECs versus *PDECS*	NS	**0.031**	NS	*0.061*
WDET-Cs versus *PDECs*	NS	**0.004**	NS	**0.013**
Age				
≤50 vs >50 years	NS	NS	NS	NS
Gender				
M versus F	NS	NS	NS	NS
Size				
≤2 vs >2 cm	NS	**0.027**	NS	*0.075*
Presence of metastases	NS	**0.039**	NS	*0.065*
Mitoses				
<2 vs ≥2 (10HPF)	NS	NS	NS	NS
Ki67				
≤2 vs >2	NS	**0.013**	*0.069*	NS
Grade				
G1 vs G2	NS	NS	NS	NS
G2 vs G3	NS	**0.010**	**0.045**	NS
G1 vs G3	NS	*0.050*	NS	**0.037**
Stage				
I+II versus III+IV	NS	**0.040**	NS	*0.066*
Tumour death	*0.056*	**0.018**	NS	**0.018**
FAL				
<0.3 vs ≥0.3	NS	**0.013**	NS	*0.073*
β-catenin expression				
Nuclear versus cytoplasmic	NS	**0.011**	NS	NS
E-cadherin expression				
Membranous versus cytoplasmic	NS	NS	NS	NS

WDET, well-differentiated endocrine tumour; WDEC, well-differentiated endocrine carcinoma; PDEC, poorly differentiated endocrine carcinoma; M, male; F, female; FAL, fractional allelic loss; LOH, loss of heterozygosity; bold values, statistically significant *P* values; italic values, close to significant values.

In addition, in the subset of cases investigated for molecular events cooperating in APC inactivation, the combination of multiple hits resulted in stronger statistical association. Moreover, *APC* alterations strongly correlated with tumour mean FAL values, an index of chromosomal instability, in turn, strikingly correlating with shorter patient survival. The correlation between malignancy and chromosomal instability is a well-known phenomenon at least in pancreatic endocrine neoplasms (Rindi & Bordi 2003, Jonkers *et al*. 2007). Our data suggest a role for APC in the control of chromosome segregation in digestive endocrine cancer, as demonstrated in colorectal adenocarcinoma (Hanson & Miller 2005). Finally,

the present findings support the effectiveness of the recently proposed histology grading and staging for endocrine tumours (Rindi *et al*. 2006, 2007).

In conclusion, in spite of methodology limits (namely the lack of direct demonstration of APC loss of expression), our data are proof of concept that Wnt pathway is often abnormal in aggressive GEP endocrine tumours, and this goes along with malignancy and survival. APC plays a major role in Wnt pathway activation, but the mechanisms of alteration differ depending on the anatomical site of origin: in gastroduodenal endocrine tumours via *APC* promoter methylation and in midgut through *APC* mutation. Additionally, APC alterations appear to play a central role in determining the high chromosomal instability in aggressive carcinomas.

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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