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Pericentriolar material analyses in normal esophageal mucosa, Barrett's metaplasia and adenocarcinoma

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Summary. Barrett's esophagus metaplasia is a pre-cancerous condition caused by chronic esophagitis. Chromosomal instability (CIN) is common in Barrett's cells: therefore, we investigated the possible presence of centrosomal aberrations (a main cause of CIN) by centrosomal protein immunostaining in paraffined esophageal samples of patients who developed a Barrett's adenocarcinoma. In most (55%) patients, alterations of the pericentriolar material (PCM) signals were evident and consistently marked the transition between normal epithelium to metaplasia. The alterations could even be found in adjacent native squamous epithelium, Barrett's mucosa and submucosal gland cells, as well as in the basal/epibasal layers of the mucosa and submucosal gland duct, which are the regions hosting esophageal stem and progenitor cells. These findings strongly support the hypothesis that the three esophageal histotypes (one being pathological) can have a common progenitor. Surprisingly, PCM defective signal eventually decreased with neoplastic progression, possibly to enhance the genome stability of advanced cancer cells. Importantly, PCM altered signals in Barrett's mucosa and their apparent evolution in successive histopathological steps were correlated to adenocarcinoma aggressiveness, suggesting PCM as a possible prognostic marker for tumor relapse. Extending our observations in a prospective study might help in the development of new prevention protocols for adenocarcinoma patients.

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Introduction

Barrett's mucosa (BM) is a metaplastic intestinalization of the native (squamous) esophageal epithelium, due mainly to chronic esophagitis resulting from longstanding (duodeno)-gastroesophageal reflux. The increased risk of esophageal adenocarcinoma arising in BM is based on epidemiological, clinical and biological evidence, although a small minority of BM cases progress to cancer. Barrett's carcinogenesis is a multistep process consisting of a well documented sequence of phenotypic changes, including: i) Intestinal metaplasia (i.e. BM); ii) Low-Grade non invasive neoplasia (LG-NiN) arising in BM; iii) High-Grade non-invasive neoplasia (HG-NiN); iv) Barrett's adenocarcinoma (BAc) (Spechler, 2002; Wild and Hardie, 2003; Oberg et al., 2005; for a recent review see Shaheen and Richter, 2009). Inconsistent information is presently available on potential early and sensitive biological/clinical markers of BM cases prone to cancer progression (Atherfold and Jankowski, 2006; Tischoff and Tannapfel, 2008; Wang and Sampliner, 2008); further, the therapies applied to reduce BM can have a variable and temporary efficacy and a low cost-effectiveness (e.g. see Genta 2006; Wani et al., 2009). Therefore, any biomarker suitable to precociously prevent the arising of adenocarcinoma or even Barrett's metaplasia will represent a new key tool for the systematic and low-cost prevention of BAc (Kahrilas

2008).

Chromosomal instability (CIN) is considered a key driving force, capable of supporting the genetic variability needed for cancer progression (Lengauer et al., 1998; Storchova and Kuffer, 2008). It is intriguing that Barrett's cells already show a marked CIN (Garewal et al., 1989; Galipeau et al., 1996; Barrett et al., 1999) even in the absence of dysplasia or cancer (Chaves et al., 2007; Lai et al., 2007). The consequent aneuploidy has been proposed as a predictive marker of the onset of adenocarcinoma (Teodori et al., 1998), together with few other BM features (Wang and Sampliner, 2008).

CIN (and another very common cancer phenotype, i.e. loss of cell and tissue architecture) can be induced by defects of the centrosome, the main microtubule-organizing centers of the animal cell. Indeed, the centrosomes have a key role in determining both the mitotic spindle poles and the cell division plane. The organelle consists of two orthogonally arranged centrioles surrounded by pericentriolar material (PCM), which includes proteins responsible for microtubule nucleation (such as γ-tubulin) and anchoring. It also physically and functionally interacts with a large number of regulatory elements (Nigg, 2002; Schatten, 2008).

Centrosome aberrations are found in most sporadic tumors (Fukasawa, 2005; Nigg, 2006). The presence and prominence of centrosome alterations in cancer cells has been correlated with the degree of CIN, neoplastic development and invasiveness, and with clinical outcome (Boveri, 1914; Gustafson et al., 2000; Pihan et al., 2001; D'Assoro et al., 2002; Lingle et al., 2002; Yamamoto et al., 2004; Giehl et al., 2005). Centrosome anomalies were also detected in premalignant lesions (Pihan et al., 2003; Chng et al., 2006) but the evidence for the involvement of centrosomes in tumorigenesis remains mostly correlative and circumstantial. Only in a recent study in *D. melanogaster* have centrosomal dysfunctions been directly implied in tumorigenesis mechanisms (Basto et al., 2008). Therefore we investigated the early presence of centrosomal aberrations in patients who developed a Barrett's esophagus related adenocarcinoma. This idea was also supported by the fact that gastroesophageal reflux and chronic inflammation are a continuous cause of cell stress and damage and it is known that several environmental agents (including oxidative stresses) may cause centrosome derangement/dysfunction (Salisbury, 2001; Chae et al., 2005; Duensing, 2005).

In this study we retrospectively explored the presence of altered PCM in the whole spectrum of phenotypic lesions involved during carcinogenesis in Barrett's adenocarcinoma patients, via immunohistochemical analyses, also searching for possible relationships between PCM alterations and metaplastic or neoplastic features. The results clearly show that PCM altered signals (PCMas) are common in normal and metaplastic cells of these patients and can change as a function of tumor aggressiveness.

Materials and methods

Esophageal tissue samples and patient clinical data

Paraffin-embedded specimens were obtained from 28 cases (27 males; age range: 39-84 years; median age: 61.36 years) of chronic esophagitis followed by BM-derived adenocarcinoma. All patients underwent total esophagectomy and were regularly followed over time; clinical data were obtained under informed consent from the Padua University Hospital records (all cases available were analyzed; Table 1).

Histopathological analyses and immunological centrosome detection

Paraffin-embedded sections of each specimen were stained with hematoxylin and eosin (H&E) and analyzed independently by two pathologists.

γ-tubulin immunoperoxidase staining

Microtome sections were deparaffinized, rehydrated and heated in a pressure cooker for 25 min, treated with 3% hydrogen peroxide and saturated with a blocking solution containing 10% goat serum. They were then incubated with a primary antibody against γ-tubulin (diluted 1:600; GTU-88, Sigma Chemical Co., St. Louis, MO), followed by horseradish peroxidase-conjugated mouse-specific IgG (DakoCytomation Denmark A/S). Finally, DAB (3,3'-Diaminobenzidine tetrahydrochloride) substrate chromogen solution was added, followed by a light hematoxylin counterstaining. Samples were observed under a Leica-DRM microscope and pictures were taken with a Leica DFC480 camera, using the Leica IM500 program.

γ-tubulin and pericentrin double immuno-fluorescence

Monoclonal GTU-88 anti- γ-tubulin and polyclonal PRB-432C anti-pericentrin (1:150; Babco/Covance, Princeton, NJ, USA) antibodies were used for double immunofluorescence detection. The antibody-antigen complexes were detected by FITC-conjugate antimouse IgG (Roche Diagnostic GmbH, Germany) and Cy3-conjugate antirabbit IgG (Jackson ImmunoResearch) antibodies. Centrosome immunostaining was repeated at least 3 times for each case.

Centrin immunofluorescence staining

Deparaffinized sections were treated as explained above. After blocking, samples were incubated with the anti-centrin antibody (MC1, a generous gift from Prof. J. Salisbury, Mayo Clinic Cancer Center, Rochester, MN) dilution 1:2000), followed by a Cy3-conjugate anti-rabbit IgG antibody (Jackson ImmunoResearch

Laboratories Europe Ltd, Suffolk, UK CB8 7SY). Cell nuclei were stained with 0.2 μg/ml 4,6-Diamidino-2-phenylindole (DAPI, Sigma). Samples were observed under a Leica-DM 5000B Epifluorescence Microscope equipped with a Leica DFC300fx camera. Centrin immunostaining was performed 1-2 times on each analysed sample.

Analyses of the incidence of PCMas among cells in different histopathological tissues

PCMas (pericentriolar material altered signal) definition

We established that PCM signals (from γ-tubulin and/or pericentrin) were altered by comparing them to normal signals (exemplified in Fig.1A) and following these criteria: (i) signal size at least twice that of normal (in most cases PCMas were actually much larger) and/or (ii) there were more than 2 PCM signals per cell (in positive PCMas we often counted >10 signals per cell); in general we noted an inverse relationship between the size and number of PCMas but the above features were often found together. Importantly, in Barrett's mucosa the altered signal were no longer located in a cell apical position but always became perinuclear, as in non-epithelial cells. Finally, PCMas always showed an irregular shape.

Analyses of PCMas incidence

In all H&E stained samples, the histopathological regions corresponding to BE, LG-NiN, HG-NiN or cancer (G1 to G4) were accurately and comprehensively identified. The boundaries of each region corresponding to an unequivocally recognized histopathological tissue were traced on the corresponding digital images in adjacent sections stained for the centrosomes. To quantify the frequency of cells showing PCM altered signals (PCMas) in the different histopathological tissues, we used two independent methods (I and II). (I) We assumed that there was a constant cell density per unit area of the histopathological region being analyzed within the digital images and validated this approach by counting nuclei in randomly-chosen samples. The sum of the areas positive for PCMas in each histopathological tissue was then determined and divided by the value of the total tissue area considered for this analysis. This

Table 1. Histopathology, clinical data and presence of PCMas in Barrett's cells of esophageal adenocarcinoma patients.

Pt. No.	Age	Histological grade	Pathological stage	TNM	Adjuvant CR-T	Tumor relapse (months)	Survival (months)	PCMas	Altered centrin
1	68	G2	1A	T1N0M0	-	-	48	+	+
2	54	G2	1A	T1N0M0	-	-	30	+	+
3[a]	59	G2	1A	T1N0M0	-	4	7†	+	+
4	74	G1	1A	T1N0M0	-	13	39	+	nd
5	74	G3	1A	T1N0M0	-	-	45	-	-
6	57	G2	1A	T1N0M0	-	-	81	-	+[d]
7	70	G1	1A	T1N0M0	-	-	55	-	nd
8	55	G3	1A	T1N0M0	-	-	60	-	+
9	57	G3	1B	T2N0M0	-	15	21†	+	+
10	58	G2	1B	T2N0M0	-	-	39	-	-
11	48	G2	2	T3N0M0	-	-	60	+	+
12	64	G1	2	T3N0M0	-	-	49	+	nd
13	56	G4	2	T2N1M0	-	-	54	-	nd
14	65	G3	2	T3N0M0	-	-	62	-	-
15	71	G1	2	T3N0M0	-	-	49	-	nd
16	64	G3	3A	T3N1M0	-	8	26†	+	nd
17	56	G2	3A	T3N1M0	+	-	37	+	nd
18	72	G2	3A	T3N1M0	-	5	9†	+	nd
19	49	G3	3A	T3N1M0	+	-	81	-	+[d]
20	81	G4	3A	T3N1M0	-	9	11†	-	nd
21[b]	49	G3	3B	T3N2M0	+	15	55	+	+
22	52	G3	3B	T3N2M0	+	14	26†	-	nd
23[b]	66	G2	4	T3N1M1	-	1	3†	+	+
24	39	G3	4	T3N1M1	+	7	17†	-	nd
25	58	G2	1A	T1N0M0	-	nd	6	+	+
26	84	G3	1B	T2N0M0	-	nd	45	-	-
27	72	G2	2	T3N0M0	-	4	12†	+c	-
28	46	G2	3	T3N1M0	+	nd	7	+	+

Months to tumor relapse and months of survival were counted as of the esophagectomy. Abbreviations: +, prominent PCMas; CR-T, chemo- and/or radiotherapy. nd, not determined. †, deceased. In all cases we could analyse normal squamous epithelium regions (except #18 and 28), metaplastic and dysplastic histopathological regions (except #27) and invasive cancer (except #4, 18 and 27). Notes: (a) patient with both esophageal adenocarcinoma and non-Hodgkin lymphoma, (b) the only patients given neoadjuvant CR-T, (c) no BM in the available bioptic samples, but prominent PCMas in the squamous epithelium, (d) only found in submucosal glands.

normalized value, given here as the "percentage of tissue", was considered as a quantitative estimate of the incidence in PCMas in BE, NiN or cancer. Areas were calculated using the Leica IM500 software. This approach allowed us to exhaustively analyze entire sections. (II) We individually scored ~10^3 to 10^4 randomly-chosen cells per tissue and then simply determined the percentage of PCMas+ve cells.

Statistical analyses

The parametric and non-parametric Mann-Whitney tests were used to evaluate the differences in the mean incidence of PCMas. The multicomparison problem was taken into account by using a statistical technique based on the false discovery rate (FDR), defined as the expected number of false positives in the entire test set (Benjamini and Hochberg, 1995). We used a log-rank test to compare non-relapsing patient curves in PCMas-positive or -negative cases. A p-value of 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS 11.0 and R statistical software (http://www.r-project.org).

Results

PCM abnormalities in esophagitis/BM/BAc patients

Analyses were performed on 28 cases of esophagitis/BAc (Table 1). Adjacent sections of biopsies were scored for histopathology and pericentriolar matrix (PCM) proteins, by the *in situ* staining of γ-tubulin and pericentrin. PCM altered signals (PCMas) (defined in Material and Methods) were found in 55% of the patients in both Barrett's metaplasia and adjacent - histopathologically normal - squamous epithelium, and

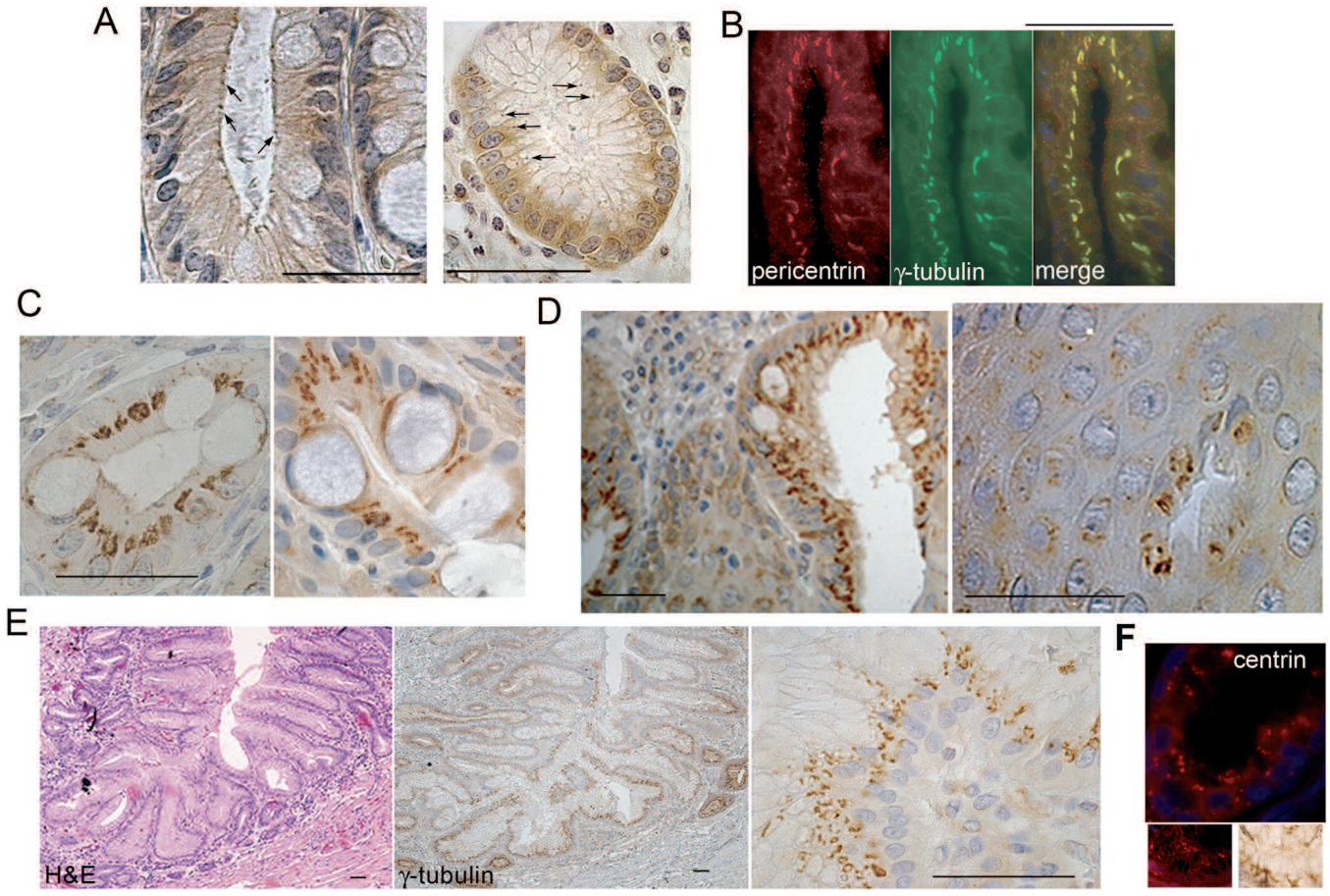

Fig. 1. Prominent and widespread PCMas of Barrett's cells in BM/BAc patients. **A**. Representative normal γ-tubulin signals observed in BM samples after *in situ* immunoperoxidase staining. Arrows indicate normal centrosomes. **B.** PCMas in BM cells seen with double immunofluorescence labeling of pericentrin (red) and γ-tubulin (green). Overlapping γ-tubulin and pericentrin signals are yellow in the merged image. DAPI stained nuclei are blue. **C.** Immunoperoxidase staining of PCM aberrations in Barrett's cells. **D.** PCMas marking the transition from squamous epithelium to intestinal metaplasia. **E.** Adjacent sections stained with H&E or for γ-tubulin (immunoperoxidase reaction). PCMas were found in most or even all BM cells in each PCMas positive case. Scale Bars: 50 μm. **F.** Upper part: centriole altered signals in BM cells observed after *in situ* immunofluorescence labeling of centrin (red). Lower part: PCMas (γ-tubulin immunoperoxidase) and altered centrin signals (IF) in adjacent BM sections.

clearly marked the transition from the normal mucosa to small intestine metaplasia (Fig. 1A,D). Abnormalities in the number, the size, the shape and the position of PCM signals with respect to normal centrosomes were quite common (Fig. 1A,C,E). The systematic co-localization of γ-tubulin and pericentrin (in a set of 4 unselected cases) confirmed the quality of the immunohistochemical reactions, as shown by double immunofluorescence analyses (Fig. 1B)

Incidence of PCMas in different histopathological stages

In general, the patients with defective PCM consistently showed the altered signals in the native mucosa, BM, NiN, and Bac. In the remaining 45% of cases evident PCMas were not found either in normal mucosa or in BM or any of the other cancerogenic steps (with two limited exceptions; see Discussion). Surprisingly, the PCM signals appeared less altered in frank cancer (becoming more elusive or almost disappearing) than in Barrett's cells (Fig. 2A,B). The Mann-Whitney test on the mean incidence of the lesions indicated that a global loss of prominent PCMas with the apparent progression from BE (or low-grade NiN) to adenocarcinoma (with G1, G2 or G3 grading) was of strong statistical significance (Fig. 2C,D and Table 2-all cases).

Correlation between PCMas in Barrett's cells and tumor aggressiveness

PCM aberrations detected in 12/24 cases were also statistically assessable in relation to clinical parameters but did not correlate significantly with the patient

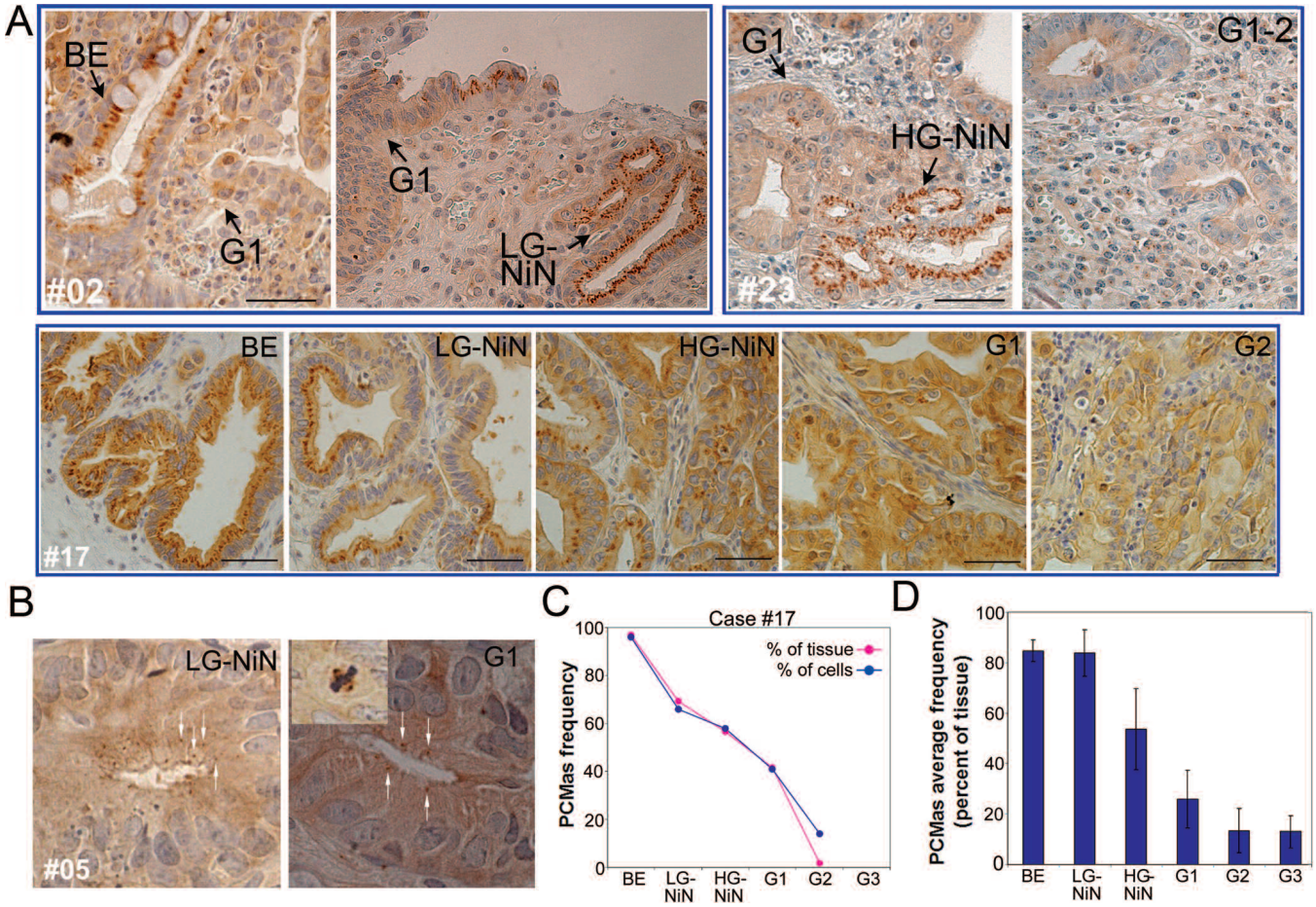

Fig. 2. Decrease in the incidence of PCMas from BE to cancer. **A.** Centrosome aberrations seen with γ-tubulin staining in intestinal metaplasia (marked in figure as BE, Barrett's Esophagus), LG-NiN, HG-NiN and Barrett's adenocarcinoma (G1 and G2 grade). Representative fields from cases 2, 17 and 23 (Table 1) are shown. Metaplastic and/or dysplastic lesions could always be found in close vicinity of the invasive adenocarcinoma. Scale Bars: 50 μm. **B.** Two representative fields of PCMas negative neoplasms of a sample with PCMas-ve BM and normal mucosa. Inset: bipolar mitotic spindle and metaphase plate of a G1-grade cancer cell of the same sample **C.** PCMas diffusion evaluated in different histopathological tissues of patient #17 samples by using two quantitative methods. The relative extent of PCMas+ve areas (pink line, method #1 in Material and Methods) and the percentage of PCMas+ve cells (blue line) were measured in each tissue. **D.** Mean incidence of PCMas in different histopathological tissues calculated as the average percentage of each tissue (method #1) on all the positive cases.

survival rate (data not shown). Instead, there was a suggestive increase in BAc relapse rate among all cases showing alterations of PCM, becoming statistically significant when the subgroup with a low pathological tumor stage (pTMN≤2) was considered (Fig. 3A). Therefore we also scored the *incidence* of PCMas amongst histopathologically homogeneous cells (i.e. in BE, NiN and EA stages) as a function of the tumor's estimated (pTMN) and actual (ability to relapse) aggressiveness. Although there was an incidence

Table 2. Non-parametric statistical tests performed on the difference in the mean incidence of PCMas between any pair of histopathological tissues in all samples, within pTNM subgroups and relapsing and non-relapsing subgroups (R and NR, respectively).

Histopathological step	p-values				
	All cases	pTNM ≤ 2	pTNM ≥ 3	Relapsing	Non Relapsing
BE→LG-NiN	0.4026 (0.46455)	0.47619	0.83252	0.061	0.33
BE→HG-NiN	0.2573 (0.35094)	0.06667	1	0.102	0.017*
BE→G1	0.0018* (0.00905)	0.00797*	0.11111	0.22	0.001*
BE→G2	0.0004* (0.00636)	0.01306*	0.01587	0.038°	0.0001*
BE→G3	0.0054* (0.01648)	nd	0.09524	0.001*	nd
LG-NiN→HG-NiN	0.1120 (0.21014)	0.11429	0.76423	0.74	0.11
LG-NiN→G1	0.0026* (0.0098)	0.03655*	0.06506	0.1	0.055°
LG-NiN→G2	0.0011* (0.00879)	0.02652*	0.01945	0.023°	0.037°
LG-NiN→G3	0.0416 (0.10401)	nd	0.07864	0.007*	nd
HG-NiN→G1	0.2200 (0.33011)	0.53339	0.22857	0.11	0.57
HG-NiN→G2	0.0874 (0.18746)	0.2186	0.11429	0.025°	0.2
HG-NiN→G3	0.2086 (0.34769)	nd	0.2	0.002*	nd
G1→G2	0.2951 (0.36892)	0.22725	0.38363	0.61	0.18
G1→G3	0.7776 (0.77769)	nd	0.53333	0.32	nd
G2→G3	0.7481 (0.80155)	nd	1	0.502	nd

(*) p-values considered significant or (°) verging on significance; false discovery rate is reported in brackets only for analyses on all cases; nd: not determined

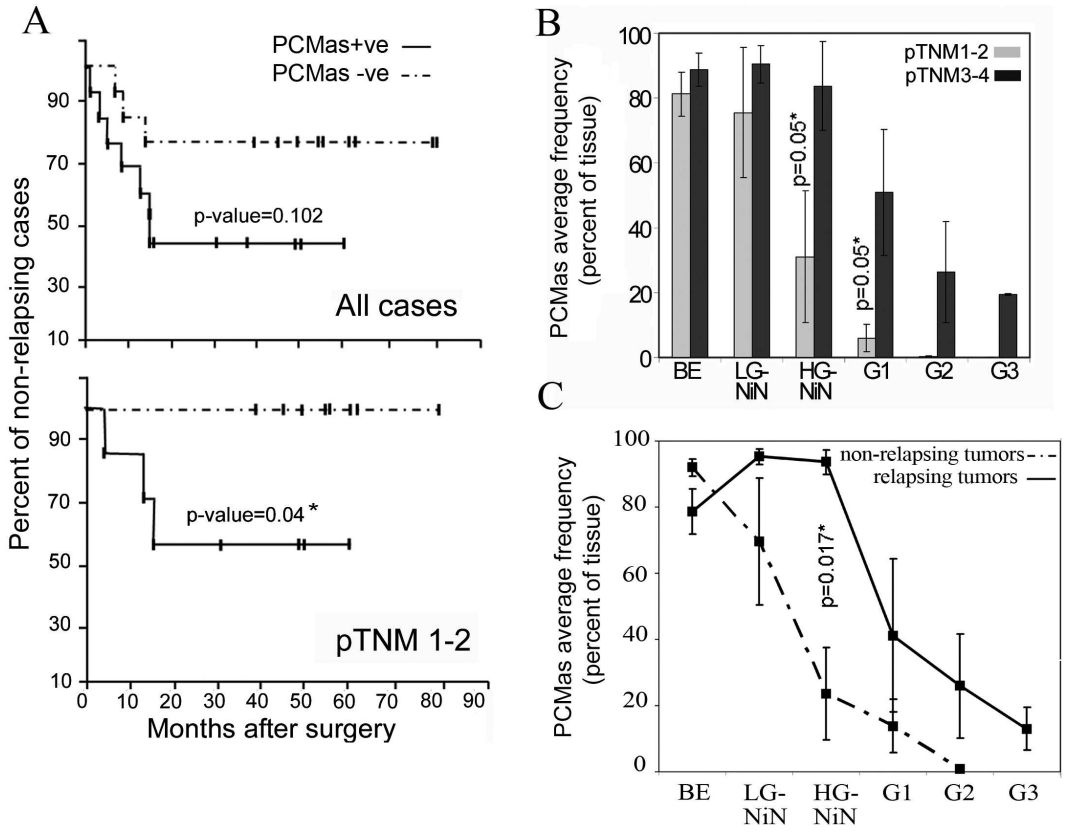

Fig. 3. Correlation between the presence and evolution of PCMas and tumor pathological staging or relapse. **A.** Tumor recurrence index in relation to presence (continuous line) or not (dotted line) of PCMas. The lower panel shows data for the patients sub-grouped by pTNM≤ 2 stage. Time to relapse or total relapse-free follow-up are given. **B.** Average overall frequency of PCMas in different histopathological tissues, plotted by pTNM cancer stage: gray and black bars refer to pTNM≤ 2 and pTNM≥ 3 cases, respectively. Statistically significant differences between the two subgroups are shown. **C.** Analyses as in panel B but comparing relapsing (continuous line) or non-relapsing (dotted line) cases.

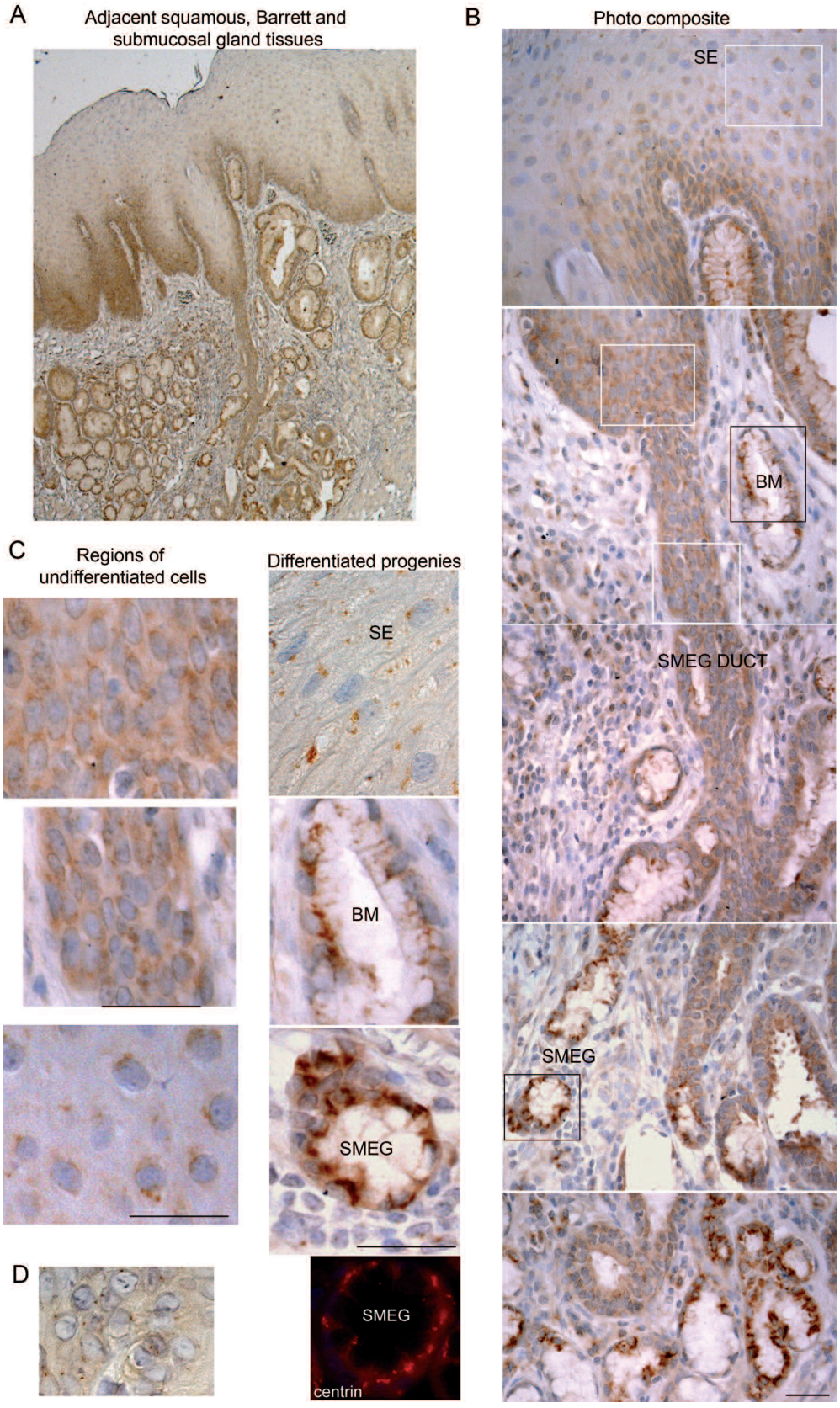

Fig. 4. Three contiguous esophageal mature histotypes and regions of more undifferentiated cells showing aberrant PCM (γ-tubulin immunostaining). **A.** A small magnification image showing the strict contiguity among the tissues. **B**. The composite photo of the central part of the sample shown in panel A. It comprises part of the three divergently differentiated tissues, the papillary and interpapillary basal/epibasal layers of the squamous epithelium and the adjacent duct region of the submucosal glands. **C.** PCMas of different cell types seen at higher magnifications (the corresponding regions are boxes in panel B. SMEG, submucosal esophageal glands; SE, squamous epithelium; BM, Barrett's Esophagus mucosa. Immunofluorescence: prominent alterations of SMEG centrin signals (red). **D.** A representative example of normal γ-tubulin signals in SE cells from a PCMas negative case. Scale Bars: 50μm.

Table 3. Statistical analyses of differences in the frequency of CeAb between the pTNM≤ 2 and pTNM≥ 3 or relapsing and non-relapsing subgroups for each histopathological tissue.

Histopathological tissue	p-values			
	pTNM≤ 2 vs.	pTNM≥ 3	Relapsing vs.	Non relapsing
BE	0.46	0.2	0.14	0.11
LG-NiN	0.46	0.26	0.22	0.27
HG-NiN	0.07°	0.05*	0.03*	0.017*
G1	0.13	0.05*	0.43	0.34
G2	0.09	0.09	0.53	0.209

(*) p-values considered significant or (°) bordering on significance; Non-parametric (left columns) and parametric tests (right columns) were used for each comparision.

decrease in more advanced neoplastic stages (see previous paragraph), this observation was statistically supported in pTNM≤ 2 cases but not in pTNM≥ 3 cases (Table 2). Indeed, these latter ones kept more widespread PCMas in HG-NiN and G1 cancer than the pTNM≤ 2 ones, and the difference was statistically significant (Fig. 3B and Table 3). Similarly, widespread and prominent PCMas were still found in HG-NiN (93% of cells) in relapsing cases but not in non-relapsing patients (Fig. 3C and see comparison of relapsing and non relapsing cases in Table 3). Indeed, in relapsing cases the PCMas reduction with respect to BM was statistically significant only in G2/G3 tumors (Table 2-relapsing), whereas in non-relapsing cases the decrease in the mean incidence of PCMas was already highly significant when BE was compared with HG-NiN (Table 2-non relapsing; see also Fig.3C: 91% of Barrett's cells but only 23% of HG-NiN cells showed aberrant γ-tubulin signals).

PCM as a possible marker of the common origin of different esophageal histotypes

In PCMas positive cases, alterations of the γ-tubulin signal were consistently found not only in differentiated native esophageal mucosa and metaplasia, but also in contiguous tissue regions of less differentiated cells; that is, PCMas can be seen in the putative transit amplifying cell compartment and sometimes even in the basal and epibasal layers, remaining in post-mitotic suprabasal cells undergoing terminal differentiation. In addition, the same lesions were always found in cells of the submucosal and mucosal esophageal glands (SMEG) and in the submucosal gland duct epithelium (Fig. 4). The systematically concomitant presence of PCMas in cells of the native squamous epithelium at different stages of maturation, BM, NiN, and Bac, as well as in SMEG and SMEG duct clearly suggests that certain multipotent cells might be shared by normal and pathological tissues.

Discussion

Normal centrosomes in esophageal mucosa give 1-2, small, round signals per cell. PCMas are γ-tubulin (and/or pericentrin) signals quite different from those derived from normal centrosomes: they are oversized, misshaped, mislocalized (in BM and SMEG) and, quite often, abnormally numerous. Size/shape changes were so prominent in all cases classified as PCMas+ve that they cannot fall within the well known cell cycle-driven PCM changes. So, the present study on esophagitis/BM/BAc samples indicates that at least the pericentriolar material could undergo profound alterations before any neoplastic or even metaplastic transformation takes place in esophageal mucosa. Structural and/or numerical centrosome aberrations, in particular supernumerary centrosomes, can be found in frank cancer and in premalignant tumor stages (see Introduction). Our findings are consistent with the possibility that centrosome defects can originate even earlier, as observed in normal or hyperplastic mammary glands after tumorigenic methylnitrosourea treatment (Goepfert et al., 2002) or carcinogenic ovarian hyper stimulation (Milliken et al., 2008), respectively. This idea is also supported by the finding of aberrant centrosomes in the histologically normal margins of head and neck carcinomas (Gustafson et al., 2000), inflamed bronchi of lung tumor patients (Lothschutz et al., 2002), and inflammation/hepatocirrhosis lesions (Chen and Kong, 2009).

It has been discussed how centrosomes often appear altered in size and/or shape or centrosome-related bodies (CRB) can even form at ectopic sites because of excessive PCM accumulation and propensity of PCM coiled coil proteins to form intracellular aggregates (Nigg, 2006). Although we frequently scored multiple intracellular PCM signals (our microscopic observations were also validated through confocal microscopy), not every particle staining positive with anti-PCM antibodies necessarily represented a complete centrosome because of possible PCM fragmentation and CRB formation. The exact counting of *bonafide* centrosomes, each defined as harboring 2 centrioles, is a demanding task, subject to many errors (Nigg, 2006), and our technical condition prevented the possibility of such an accurate counting. Nevertheless, in about 60% of the cases we could preliminarily analyze centrin, a centriole marker, by immunofluorescence staining: only a minority of Barrett's cells positive for PCMas also showed abnormal centrin signals. Notwithstanding this difference in incidence, 9 out of the 10 analyzed PCMas+ve samples also had at least some Barrett's cell groups with altered centrin signals (see Fig. 1F and Table 1). Although no abnormal centrin signals were found in the native differentiated squamous epithelium, the centriolar abnormalities were, however, frequent and quite evident in SMEG cells (Fig. 4C and unpublished data). We concluded that the presence of aberrant centriole structures is not necessarily associated with PCMas, perhaps because different proteins otherwise respond to refluxate and chronic inflammation (in this regard see Souza et al., 2008) as well as to different cell differentiation contexts.

Importantly, enlarged centrosomes or CRBs, looking similar but with different composition, can show different functional properties; therefore detailed analysis of structural centrosome aberrations, might have diagnostic or prognostic utility (discussed in Nigg, 2006). We performed preliminary γ-tubulin WB analyses on few frozen BM/BAc samples: intriguingly, although we could not microdissect the samples prior to analyses, PCMas positive cases showed an apparently different pattern of γ-tubulin isoforms with respect to the negative samples (data not shown). So, this protein might be rather sensitive to the pathological condition under study. It has already been shown that centrosomes can be altered in relation to cancer-related genetic, epigenetic and environmental changes, in conditions also induced by injuring agents and inflammation (see Introduction).

Numerical centrosome aberrations often correlate with genome instability and loss of tissue differentiation, and have also been proposed to have prognostic value (Gustafson et al., 2000; D'Assoro et al., 2002; Lingle, 2002; Nigg, 2002, 2006; Yamamoto et al., 2004; Duensing, 2005; Fukasawa, 2005). Our findings are not inconsistent with those higher centrosomal alterations described in more advanced cancer disease. First, centrosomes were never analyzed in the BM-NiN-BAc system and it is well established that the pathways of progression may be unique to each type of cancer (Merlo et al., 2006). Second, we described the progressive loss of evident alterations of the pericentriolar material during tumor progression, rather than changes of centrosome derangements always involving centrioles. Third, in two PCMas-negative cases we could exceptionally observe clear PCMas in a very small subpopulation of advanced cancer cells (data not shown), recalling those cases reported in literature for other tumors. Interestingly, within purely retrospective analyses we established that PCMas presence in pre-invasive populations could be correlated with BAc recurrence. In addition, the circumstantial evidence of a linkage between tumor aggressiveness and PCMas persistence in HG-NiN might be of clinical importance, since dysplasia is still the most predictive marker for risk of BAc. Indeed, in all cases the persistence of this morphological marker in most HG-NiN cells was well correlated with tumor recurrence, even at individual patient level (in all cases, as determined by two independent approaches; unpublished results). Since many PCMas-negative cases do exist among the analyzed patients, it is clear that the dramatic PCMas described here are not needed to generate either BM or BAc. If they are present, however, they might enhance the carcinogenic potential of the system until their reduction is likely to be beneficial to give a more stable phenotype to selected advanced cancer cells (Nigg, 2002; Merlo et al., 2006)

Finally, PCMas were concomitantly found in: (a) native esophageal mucosa, mucosal and submucosal glands and metaplastic cells that were strictly contiguous, (b) cells showing intermediate phenotypic features with respect to mature hystotypes (i.e. squamous mucosa and Barrett's esophagus) and located between them, (c) more undifferentiated cells located in regions known to host pluripotent stem cells or progenitor cells. This consistent presence supports the hypothesis that the normal and the metaplastic histotypes can have a common progenitor (Wild and Hardie, 2003; Souza, 2008; Shaheen and Richter, 2009) (also explaining why PCMas were often distributed over discrete areas of the squamous epithelium or SMEG; data not shown).

The putative cell that might first be affected by PCM lesions needs to be precisely identified and characterized. Our observations are compatible with this cell originating from the basal/epibasal layers of the mucosa (Seery and Watt, 2000) and/or the submucosal gland duct; indeed, the epithelium of the latter has been proposed as a source of the stem cells that may be involved in the pathogenesis of Barrett's esophagus (Coad et al., 2005). Further investigations and the identification of centrosome dysfunctions in the context of a prospective study on esophagitis/BM patients may reveal this intriguing organelle as a new tool for adenocarcinoma prevention.

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