

PDCD4/miR-21 dysregulation in inflammatory bowel disease-associated carcinogenesis

Kathrin Ludwig · Matteo Fassan · Claudia Mescoli · Marco Pizzi ·
Mariangela Balistreri · Laura Albertoni · Salvatore Pucciarelli ·
Marco Scarpa · Giacomo Carlo Sturniolo · Imerio Angriman ·
Massimo Rugge

Received: 30 May 2012 / Revised: 9 October 2012 / Accepted: 18 November 2012 / Published online: 8 December 2012
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Abstract Inflammatory bowel diseases (IBDs; both ulcerative colitis [UC] and Crohn's colitis [CC]) are well-established predisposing pathological conditions for colorectal cancer (CRC) development. In IBDs, both the endoscopy and the histology assessment of CRC precursors (i.e., dysplasia, also defined as intraepithelial neoplasia) are associated with low interobserver consistency, and no reliable dysplasia-specific biomarker is available. The programmed cell death 4 (PDCD4) tumor suppressor gene is involved in sporadic colorectal oncogenesis, but scanty information is available on its involvement in IBD-associated colorectal oncogenesis. One hundred twenty tissue samples representative of active and inactive IBD and of flat dysplasia were obtained from 30 cases of UC and 30 of CC who undergone colectomy. Twenty additional biopsy samples obtained from patients with irritable bowel syndrome acted as normal controls. PDCD4 expression was assessed by immunohistochemistry; the expression of

miR-21 (a major PDCD4 regulator) was investigated by quantitative real-time PCR and in situ hybridization in different series of a hundred samples. Tissue specimens from both controls and inactive IBD consistently featured strong PDCD4 nuclear immunostain; conversely, lower PDCD4 nuclear expression was featured by both active IBD and IBD-associated dysplastic lesions. Significant PDCD4 down-regulation distinguished IBD-associated dysplasia ($p < 0.001$) versus active IBD. In both active IBD and dysplasia, PDCD4 down-regulation was significantly associated with miR-21 up-regulation. PDCD4 nuclear down-regulation (which parallels miR-21 up-regulation) is involved in the molecular pathway of IBD-associated carcinogenesis. PDCD4 nuclear expression may be usefully applied as ancillary maker in the histological assessment of IBD-associated dysplastic lesions.

Keywords PDCD4 · IBD · Dysplasia · MicroRNA

Kathrin Ludwig and Matteo Fassan contributed equally to this work.

K. Ludwig · M. Fassan · C. Mescoli · M. Pizzi · M. Balistreri ·
L. Albertoni
Department of Medicine (DIMED), Surgical Pathology and
Cytopathology Unit, University of Padua, Padua, Italy

M. Fassan
Department of Surgical Oncology and Gastroenterological
Sciences (DiSCOG), General Oncology Unit, University of Padua,
Padua, Italy

S. Pucciarelli
Department of Surgical Oncology and Gastroenterological
Sciences (DiSCOG), Clinica Chirurgica II, University of Padua,
Padua, Italy

M. Scarpa · I. Angriman
Oncological Surgery Unit,
Veneto Institute of Oncology (IOV–IRCCS), Padua, Italy

G. C. Sturniolo
Department of Surgical Oncology and Gastroenterological
Sciences (DiSCOG), Gastroenterology Unit, University of Padua,
Padua, Italy

M. Rugge
Surgical Pathology Unit,
Veneto Institute of Oncology (IOV–IRCCS),
Padua, Italy

M. Rugge (✉)
Department of Medical Diagnostic Sciences and Special Therapies,
University of Padua, Via Aristide Gabelli, 61,
35121 Padua, Italy
e-mail: massimo.rugge@unipd.it

Abbreviations

| | |
|-------|----------------------------|
| IHC | Immunohistochemistry |
| IBD | Inflammatory bowel disease |
| PDCD4 | Programmed cell death 4 |
| UC | Ulcerative colitis |
| CC | Crohn's colitis |

Introduction

Ulcerative colitis and Crohn's disease are the two major forms of idiopathic inflammatory bowel disease (IBD) [1]. Colorectal cancer (CRC) is a well-established adverse outcome of longstanding IBDs (both ulcerative colitis [UC] and Crohn's colitis [CC]), accounting for approximately 10–15 % of all IBD deaths [2, 3]. In colitis-associated carcinogenesis, both the histology phenotypes and the molecular events are basically similar to those of sporadic CRCs, but the sequence of the molecular deregulations consistently differ [4, 5].

Secondary prevention of IBD-associated CRC depends on the endoscopy and the histology detection of precancerous lesions (i.e., dysplasia, recently defined as intraepithelial neoplasia). As for endoscopy, IBD-associated dysplasia may arise from both raised (dysplasia-associated lesions or masses) or flat mucosal lesions, which distinction has been proven to rely on subjective criteria [6–8]; moreover, dysplastic lesions can blend with inflammatory abnormalities, which represent an additional confounding gross feature even for experienced endoscopists. As for histology, the reliability of dysplasia assessment is largely affected by the pathologist's experience and frequently limited by intrinsic characteristics of the biopsy samples (high-grade active inflammation, shrinkage, fragmentation, and "burning"); both situations result in a low rate of interobserver agreement [9]. Because of all these reasons, dysplasia assessment is inconsistent, which prompts to the priority of dysplasia-specific tissue biomarkers.

Programmed cell death 4 (PDCD4) is a newly characterized tumor suppressor gene (TSG) involved in the apoptotic machinery, cell transformation and invasion, and tumor progression by interacting with translation initiation factors eIF4A and eIF4G [10–20]. PDCD4 protein expression is consistently down-regulated in both human cancers and cancer cell lines [19, 21–28]. Different mechanisms are involved in PDCD4 dysregulation; among others, the oncogenic microRNA miR-21 has been shown to specifically target the PDCD4 3' untranslated region, which negatively regulates PDCD4 expression [21, 25]. In active IBDs, miRNA expression profiling consistently features disease-specific signatures, and miR-21 has been pinpointed as one of the most dysregulated genes [29–31]. PDCD4 involvement in IBD-related carcinogenesis has never been addressed.

Thus, the aim of this study was to investigate both PDCD4 expression (as assessed by immunohistochemistry)

and miR-21 levels/expression (as assessed by real-time PCR and in situ hybridization) in a series of inflammatory and dysplastic IBD surgical specimens in the search of additional diagnostic tools to be used in daily pathology routine.

Materials and methods

Patients

The cases considered in the present study were retrospectively collected from the archives of the Surgical Pathology and Cytopathology Unit at the University of Padua. The institute's ethical regulations on research conducted on human tissues were followed. A total of 60 consecutive IBD patients [UC=30; CC=30] who underwent colectomy (total or sub-total) for histologically proven dysplastic lesions were considered. The surgical specimens were grossly assessed according to a standardized local protocol: the surgical specimens were opened anteriorly, pinned to a cork board, and fixed in an adequate volume of formalin (10 %) for 24 h; subsequently, the specimens were semi-serially sliced into 3–4-mm thick transverse gross sections at intervals of 2 cm.

The assessment of IBD (UC and CC) acute inflammation (a) versus non-active inflammation was based on microscopic criteria, activity being defined as the presence of neutrophils or unequivocal damage of the surface and crypt epithelium typically in conjunction with neutrophils [32, 33].

In all cases, the histology assessment of low-grade dysplasia (LGD) versus high-grade dysplasia (HGD) was done according to the current histology criteria [6, 34]. Original slides (4–6-microns thick) obtained from archival paraffin-embedded tissue samples (H&E) were jointly reassessed by two pathologists (KL and MF); in cases where their opinions differed, a third expert GI pathologist (CM) was involved.

From the 60 considered cases, a total of 120 tissue specimens were selected. The tissue specimens were the representatives of the following histology lesions: (1) non-active IBD (UCna=20 and CCna=20 samples); (2) active IBD (UCa=20 and CCa=20 samples); (3) flat LGD (UC/LGD=15 and CC/LGD=15 samples); and (5) flat HGD (UC/HGD=5 and CC/HGD=5 samples).

Twenty additional normal (N) colonic mucosa biopsy samples obtained at colonoscopy from patients with irritable bowel syndrome were also included (Clinica Chirurgica II, University of Padua).

PDCD4 immunohistochemistry

The histological sections were stained with hematoxylin and eosin, and the original diagnosis was histologically confirmed in all cases. Immunohistochemistry (IHC) staining was

automatically performed (Ventana Benchmark XT system; Touchstone, AZ, USA) for PDCD4 (catalog #HPA001032; Atlas Antibodies, Stockholm, Sweden; 1:100) [21, 27, 35] according to the manufacturer's instructions. Sections were lightly counterstained with hematoxylin. Appropriate positive and negative controls were run concurrently. PDCD4 nuclear expression was jointly scored by two pathologists (KL and MF) unaware of the patients' clinical history. In case of discordance between the two pathologists, the case was reviewed by a third expert gastrointestinal pathologist (CM). As described previously [19, 21], nuclear PDCD4 staining was scored on a four-tiered scale (score 0, no nuclear staining; score 1, $\geq 1 \leq 30$ %—positive nuclear staining; score 2, $>30 < 70$ %—positive nuclear staining; score 3, ≥ 70 %—positive nuclear staining). Cytoplasmic PDCD4 expression was scored by staining intensity (score 0, none; score 1, weak; score 2, intermediate; score 3, strong). In the statistical analysis, only PDCD4 nuclear staining was considered. In all the considered tissue samples, non-epithelial cells (fibroblasts, lymphocytes, smooth muscle cells, and endothelia) always featured PDCD4 nuclear expression and were assumed as positive internal control (not considered in IHC score) [19, 21, 27, 28, 36].

Quantitative real-time polymerase chain reaction

Formalin-fixed paraffin-embedded biopsy samples were deparaffinized with xylene at 50 °C for 3 min. Total RNA extraction was done using the RecoverAll kit (Ambion Inc, Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using the GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA, USA), and gene expression levels were quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), as previously described [37]. Primers' sequences for PDCD4 were forward 5'-GGCCTCCAAGGAGTAA

GACC-3' and reverse 5'-AGGGGTCTACATGGCAACTG-3'. GAPDH was used as the internal control gene (forward 5'-AAGGGAAGGTTGCTGGATAG-3'; reverse 5'-CACATCCACCTCCTCCACATC-3'). The NCode™ miRNA qRT-PCR method (Invitrogen, Carlsbad, CA, USA) was used to detect and quantify mature miR-21 (primer sequence: 5'-CGGTAGCTTATCAGACTGATGTTGA-3') according to the manufacturer's instructions. Normalization was done with the small nuclear RNA U6B (Invitrogen). PCR reactions were run in triplicate, including no-template controls. The data were analyzed using the comparative CT method. Additional tissue samples obtained from 20 N, 20 CCa, 15 CC/LGD, 5 CC/HGD, 20 UCa, 15 UC/LGD, and 5 UC/HGD were considered for the qRT-PCR study.

In situ hybridization

To confirm qRT-PCR data, miR-21 expression was further investigated by in situ hybridization (ISH). ISH was performed using the GenPoint™ Catalyzed Signal Amplification System (DakoCytomation) according to the manufacturer's protocol. Briefly, slides were incubated at 60 °C for 30 min and deparaffinized as previously described [26, 37]. Sections were treated with Proteinase K (DakoCytomation) for 30 min at room temperature, rinsed several times with dH₂O, and immersed in 95 % ethanol for 10 s before air-drying. The slides were prehybridized at 49–56 °C for 1 h with mRNA ISH buffer (Ambion) before overnight incubation at 49–56 °C in buffer containing the 5'-biotin-labeled miR-21 miRCURY™ LNA detection probe (Exiqon, Woburn, MA, USA) or the scrambled negative control probe (U6, Exiqon) at a final concentration of 200 nM. The slides were washed in both Tris-buffered saline with Tween (TBST) and GenPoint™ stringent wash solution (54 °C for 30 min), then exposed to H₂O₂ blocking solution (DakoCytomation) for 20 min, and then further blocked in a

Fig. 1 PDCD4 is down-regulated in IBD carcinogenesis models. Immunohistochemical scores of nuclear PDCD4 in the different histological lesions (*white*—score 0, *light gray*—score 1, *dark gray*—score 2, *black*—score 3). Nuclear PDCD4 staining was significantly lower in active disease (UCa and CCa) and associated dysplasia (LGD and HGD) specimens than in normal tissues ($p < 0.001$)

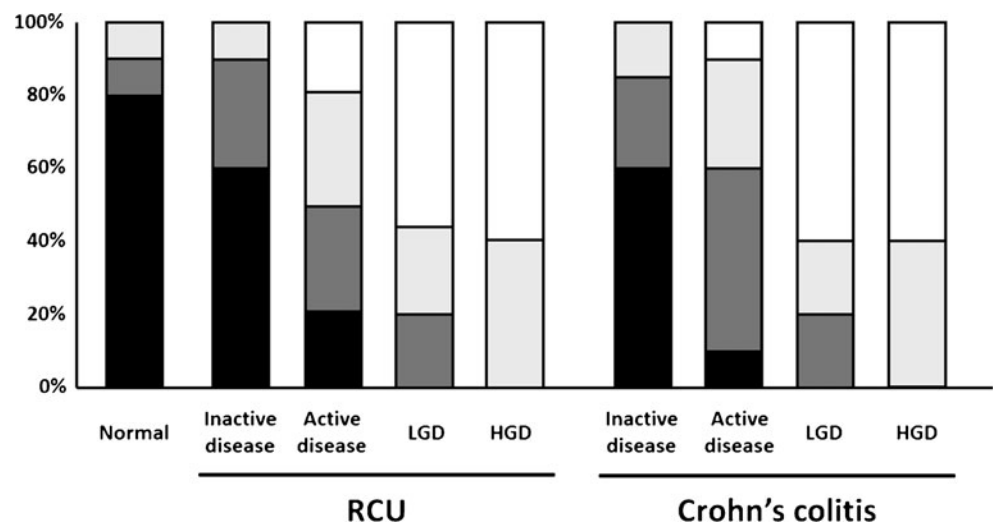


Table 1 PDCD4 nuclear expression in normal colonic mucosa and IBD (UC and CC) specimens with inactive and active diseases and associated dysplastic lesions

| | | PDCD4: nuclear IHC score | | | | <i>p</i> value |
|----|-----|--------------------------|----------|-----------|-----------|----------------|
| | | 0 | 1 | 2 | 3 | |
| N | N | 0 (0 %) | 2 (10 %) | 2 (10 %) | 16 (80 %) | – |
| UC | Na | 0 (0 %) | 2 (10 %) | 6 (30 %) | 12 (60 %) | <0.001 |
| | A | 4 (20 %) | 6 (30 %) | 6 (30 %) | 4 (20 %) | |
| | LGD | 8 (53 %) | 4 (27 %) | 3 (20 %) | 0 (0 %) | |
| | HGD | 3 (60 %) | 2 (40 %) | 0 (0 %) | 0 (0 %) | |
| CC | Na | 0 (0 %) | 3 (15 %) | 5 (25 %) | 12 (60 %) | <0.001 |
| | A | 2 (10 %) | 6 (30 %) | 10 (50 %) | 2 (10 %) | |
| | LGD | 9 (60 %) | 3 (20 %) | 3 (20 %) | 0 (0 %) | |
| | HGD | 2 (40 %) | 3 (60 %) | 0 (0 %) | 0 (0 %) | |

N normal, *UC* ulcerative colitis, *CC* Crohn's colitis, *Na* non-active, *A* active, *LGD* low-grade dysplasia, *HGD* high-grade dysplasia

blocking buffer (DakoCytomation) for 30 min before they were exposed to primary streptavidin–horseradish peroxidase (HRP) antibody, biotinyl tyramide, secondary streptavidin–HRP antibody, and DAB chromogen solutions, following the manufacturer's protocol. The slides were then briefly counterstained in hematoxylin and rinsed with TBST and water before mounting. Out of the initial series of biopsy samples, a total of 5 N, 5 UC, 5 CC, 5 UC/LGD, and 5 CC/LGD were considered for the ISH study.

Statistical analysis

Differences and correlations between groups were tested by applying the modified Kruskal–Wallis nonparametric test for trend, the chi-square test, Pearson's correlation, and the *t* test. *P* values <0.05 were considered significant. All statistical assessments were performed with STATA 8.0 software (Stata Corporation, College Station, TX, USA).

Results

PDCD4 protein is down-regulated in active UC/CC and in IBD-associated dysplastic lesions

Normal mucosa (i.e., non-IBD tissue samples) consistently featured strong PDCD4 nuclear expression coexisting with moderate/strong cytoplasm immunostain (Fig. 1; Table 1).

Overall, in both UC and CC, PDCD4 nuclear expression decreased progressively and significantly from inactive IBD to IBD-associated intraepithelial neoplastic lesions (Kruskal–Wallis: $p < 0.001$; Figs. 1 and 2; Table 1). Inactive IBDs (both UC and CC) retained PDCD4 strong nuclear expression (score 3, never featured by the crypts' proliferative compartment) and moderate cytoplasm immunostain (Fig. 2).

Tissue specimens obtained from active IBD (both UC and CC) consistently showed PDCD4 nuclear down-regulation

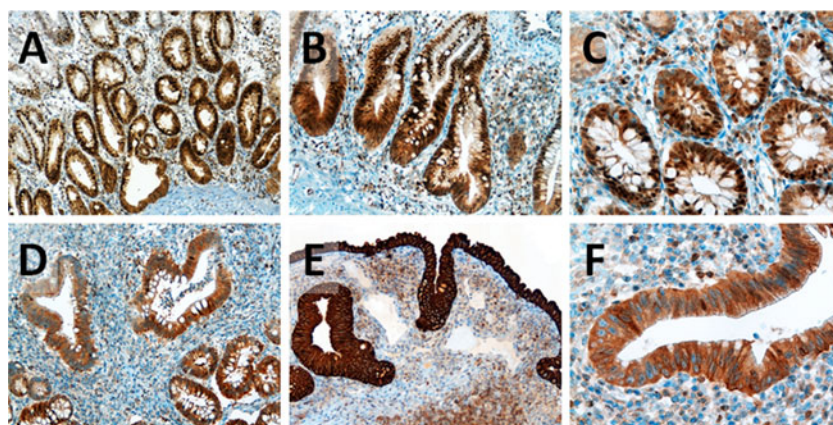


Fig. 2 Representative PDCD4 immunostaining of normal colonic tissues, UC and CC inactive/active disease, and UC/CC-associated dysplasia. **a** In native mucosa, colocytes featured strong nuclear and moderate/strong cytoplasm stain. **b** Non-active UC maintains strong nuclear and weak cytoplasm immunostain. Note the nuclear PDCD4 loss in the proliferative basal compartment of the glands. **c–d** Active CC and active UC tissues

showed a significant down-regulation of PDCD4 nuclear expression. Note the presence of cryptic abscess in active UC (**d**). **e** A minority of active UC specimens throughout featured a strong cytoplasmic expression with no nuclear immunoreaction. **f** Almost complete nuclear PDCD4 loss in CC-associated dysplastic lesion. Original magnifications $\times 10$, $\times 20$, and $\times 40$

(scores 2/3=10/20 and 12/20, respectively; Figs. 1 and 2c, d; Table 2). As for the cytoplasm immunostain, active lesions retained weak to moderate positivity. Five cases of active UC throughout featured a strong cytoplasmic expression coexisting with no nuclear immunoreaction (Fig. 2e).

Dysplasia (low and high grade, from both UC and CC cases) showed null or weak nuclear immunostain (scores 0/1=17/20, for both) associated to weak to moderate cytoplasm protein expression (Figs. 1 and 2f; Table 2).

Of note, by merging UC and CC cases, a significant lower PDCD4 nuclear expression distinguished LGD from active IBD (nuclear scores 0/1 assessed in 24/30 cases (80 %) of dysplasia versus 18/40 (45 %) cases of active IBD; chi-square: $p=0.003$).

miR-21 is overexpressed in active CC/UC and associated dysplastic lesions

A significant miR-21 up-regulation (Fig. 3) was found in samples of active UC (with a 6.3-fold increase over normal colonic mucosa samples; t test: $p<0.001$), active CC (with a 5.8-fold increase; t test: $p<0.001$), UC/LGD (with a sevenfold increase; t test: $p<0.001$), CC/LGD (with a sixfold increase; t test: $p<0.001$), UC/HGD (with a 7.3-fold increase; t test: $p<0.001$), and CC/HGD (with a 6.1-fold increase; t test: $p<0.001$) (Fig. 3a). Of note, there was a significantly higher expression of miR-21 in UC/LGD and UC/HGD tissue samples compared with active UC (t test: $p=0.025$ and $p=0.021$; Fig. 3a). No significant difference was observed between LGD and HGD lesions in both UC and CD.

Discussion

Colorectal cancer is an adverse late outcome of both ulcerative and Crohn's colitis [3]. Dysplasia (currently named as intraepithelial neoplasia) is the major risk factor for IBD-associated CRC, but its low interobserver

Table 2 PDCD4 nuclear expression in active IBD (UC and CC) versus IBD-associated dysplastic specimens

| | | PDCD4: nuclear IHC score | | <i>p</i> value |
|-------|-----|--------------------------|-----------|----------------|
| | | 0–1 | 2–3 | |
| UC | A | 10 (50 %) | 10 (50 %) | 0.018 |
| | D | 17 (85 %) | 3 (15 %) | |
| CC | A | 8 (40 %) | 12 (60 %) | 0.003 |
| | D | 17 (85 %) | 3 (15 %) | |
| UC+CC | A | 18 (45 %) | 22 (55 %) | 0.003 |
| | LGD | 24 (80 %) | 6 (20 %) | |

UC ulcerative colitis, CC Crohn's colitis, A active, D dysplasia

agreement brought about efforts to identify ancillary biomarkers of cancer-prone histology.

The PDCD4 tumor suppressor gene is consistently down-regulated in sporadic CRC, but its value as marker of IBD-associated oncogenesis is still unfathomed [19, 25, 38]. By exploring the IHC expression of PDCD4 in a series of UC and CC (with and without coexisting dysplastic lesions), two main results were established: (1) normal mucosa and inactive IBD feature strong PDCD4 nuclear immunostain (associated with moderate/strong cytoplasm expression) (Fig. 2) and (2) both

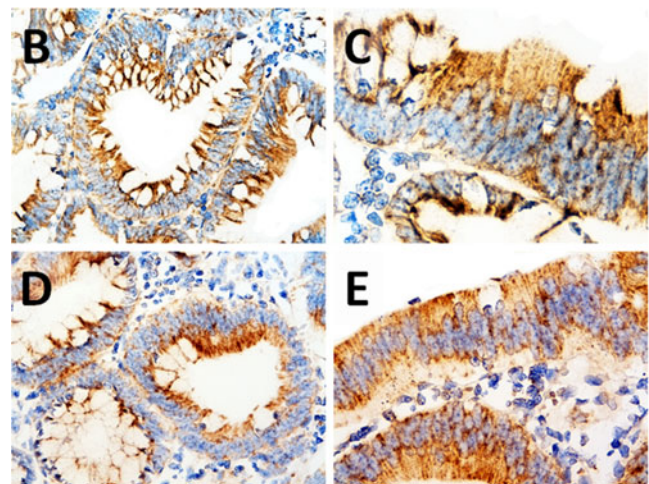
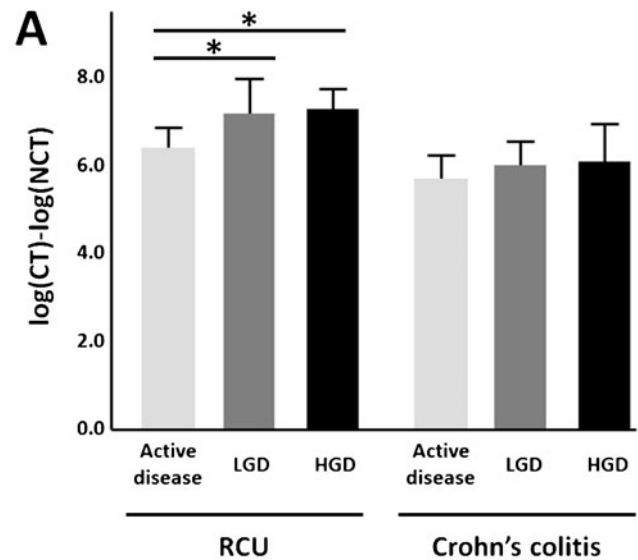


Fig. 3 miR-21 is overexpressed in UC and CC active disease and in UC/CC-associated dysplasia. **a** Altered miR-21 expression between active UC/CC and UC/dysplasia and CC/dysplasia (CT: active disease and dysplasia) and normal tissue (NCT) in IBD patients by qRT-PCR analysis. miR-21 was significantly up-regulated in active UC and CC, and IBD-associated dysplastic lesions ($p<0.001$). Note the significantly higher expression of miR-21 in UC/LGD and UC/HGD when compared to active UC (values are mean \pm SD). Representative examples of miR-21 overexpression by epithelial cells in active UC (**b**) and UC/LGD (**c**), and active CC (**d**) and CC/LGD (**e**). The presence of miR-21 is revealed by a granular brown stain (original magnifications, $\times 20$ and $\times 40$)

active IBDs and IBD-associated dysplasia consistently show PDCD4 nuclear down-regulation (paralleling with weak/moderate cytoplasm stain) (Fig. 1).

The reasons behind these (nuclear/cytoplasm) immunophenotypes are only partially understood. Similar patterns of PDCD4 expression have already been reported in both sporadic colorectal [20, 38] and in Barrett's carcinogenesis [21]. In vitro studies have shown that, under normal growth conditions, PDCD4 is located mainly in the nucleus, moving to the cytoplasm on serum deprivation in vitro [10]. The PDCD4 nuclear overexpression may identify a proliferative "attitude" shared by both "reparative" reactions (to inflammation) and epithelial dedifferentiation. Such observation is consistent with the absence of any PDCD4 nuclear stain in the proliferative compartment of normal colonic crypts. In this study, the nuclear expression found in active IBD and associated dysplasia supports active inflammatory disease as the "cancerization field" of the IBD-associated carcinogenesis [39, 40].

Because of the histological similarity between hyperplastic/regenerative lesions (coexisting with active inflammation) and low-grade intraepithelial lesions, PDCD4 nuclear expression was specifically compared in such gray-zone histology phenotypes. In both UC and CC, the nuclear protein down-regulation significantly prevailed in associated dysplasia versus active IBDs. PDCD4 nuclear loss could be used in combination with other dysplasia-specific IHC markers when active inflammation acts as confounding variable in the histology assessment. In fact, several other specific markers have been proposed for the prediction of colorectal cancer development and dysplasia assessment in the IBD context [41]. Among the others, p53 and ki-67 IHC assessments are the most widely used in routine practice to differentiate dysplastic epithelia within inflamed colonic mucosa [40, 41]. However, mutations and loss of heterozygosity of p53 and cell proliferation (as assessed by ki-67 labeling index) are early events in IBD mucosa, often occurring before dysplasia is detected. In this respect, PDCD4 down-regulation did not show a superior diagnostic performance by comparing it to previously described IHC markers. These data further underlines the absence of a real dysplasia-specific marker and the need of a multi-marker immunohistochemical panel (in which PDCD4 could be reasonably included) to identify colorectal dysplasia in inflamed mucosa in IBD patients.

In esophageal squamous carcinoma cell lines, Hiyoshi and colleagues recently demonstrated that PDCD4 is regulated by miR-21 and such interplay affects both cancer cell proliferation and invasion [43]. In the CRC setting, evidence has been provided on the inverse relationship between miR-21 and PDCD4, suggesting that miR-21 post-transcriptionally modulates PDCD4 via mRNA degradation [39]. The present in vivo study supports the miR-21 regulatory activity on PDCD4 and consistently demonstrates a significant up-regulation of miR-

21 in both active IBD (UC and CC) and UC/CC-associated dysplasia. MicroRNA expression profiling in active IBD has been addressed by various studies, which featured both a disease-specific miRNA signature [29–31, 44] and a miRNA role in the onset/relapse of active inflammation [44]. Some miRNAs have been already indicated as specific markers of IBD-related dysplasia [45, 46], and the different expressions of miR-21 in active ulcerative colitis versus UC dysplasia further support the specific role of the oncomir in UC-associated oncogenesis.

In conclusion, PDCD4 is involved in IBD-associated colon carcinogenesis. Nuclear down-regulation of PDCD4 expression is featured by both active IBD and IBD-associated intraepithelial neoplasia; in histology doubtful cases (differential diagnosis between regenerative and dysplastic lesions), nuclear protein down-regulation can be considered as supporting information in favor of a neoplastic intraepithelial lesion. miR-21 up-regulation significantly parallels the PDCD4 down-regulation supporting the current knowledge that the oncomiR post-transcriptionally modulates the TSG activity via mRNA degradation.

Acknowledgments We wish to acknowledge the continuous support of the "G. Berlucci" and the "G.B. Morgagni" Foundations. This work was partially supported by an AIRC Regional grant (2008). We also thank Dr. Cristiano Lanza, Dr. Vincenza Guzzardo, and Dr. Vanni Lazzarin for their excellent technical support. All authors of this research paper participated directly in the planning and execution of the study and in the analysis of the results.

Conflict of interest The authors have no competing interests to declare.

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