Low HtrA1 expression in patients with long-standing ulcerative colitis and colorectal cancer

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Abstract. The association between inflammatory bowel disease (IBD) and colorectal cancer (CRC) is being increasingly investigated. HtrA1 overexpression inhibits cell growth and proliferation by influencing apoptosis, invasiveness and migration of tumour cells. In the present study, HtrA1 expression was analysed in 228 colon tissue samples from patients with CRC, adenoma with high-grade dysplasia (AHD), adenoma with low-grade dysplasia (ALD), ulcerative colitis of >10 year duration (UCL), ulcerative colitis of <5 year duration (UCS) and colonic diverticulitis (D), and was compared with its expression in normal colon tissues (NCTs) collected 5 cm from the CRC lesion and in healthy colon mucosa (HC), to establish whether HtrA1 can serve as a biomarker for these conditions. All tissue specimens came from Italian Caucasian subjects. The main finding of the present study was that HtrA1 expression was significantly reduced in CRC and UCL tissues compared with that observed in both NCT and HC samples and with tissues from the other patients. In particular, a similar HtrA1 expression was detected in the stromal compartment of UCL and CRC samples. In contrast, the HtrA1 level was significantly lower (p=0.0008) in UCL compared with UCS tissues, suggesting an inverse relationship between HtrA1 expression and ulcerative colitis duration. HtrA1 immunostaining in the stromal compartment of AHD and ALD tissues showed no differences compared with the HC tissues. No data are available on the immunohistochemical localization of HtrA1 in CRC or IBD. The present findings suggest that HtrA1 could serve as a marker to identify UCL patients at high risk of developing CRC.

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Introduction

Colorectal cancer (CRC) is a highly common malignancy in European countries (1-3) and worldwide (3). According to GLOBOCAN data (3), 1.36 million new cases affecting 17.2/100,000 individuals (746,000 men and 614,000 women) are diagnosed worldwide each year; of these patients, 693,000 (373,000 men and 320,000 women) die, accounting for a yearly mortality rate of 8.4/100,000. Crucially, more than 95% of CRC patients may benefit from surgical treatment when diagnosed early (4). CRC is sporadic in 90% of patients, whereas in <10% of cases it is inherited (5,6) or is a complication of inflammatory bowel disease (IBD), either ulcerative colitis (UC) or Crohn's disease (CD) (7-9). In the majority of cases, CRC develops from adenoma, a preclinical benign precursor;

the progression from early adenoma to invasive cancer takes years (10,11).

A growing body of evidence supports the notion that inflammation and CRC are interrelated (12,13). The confirmed risk factors for the association between IBD and CRC are duration, severity, and extent of the inflammatory disease, concurrent primary sclerosing cholangitis (PSC), and a family history of CRC (14,15). Whereas the link between chronic inflammation and cancer is well recognized, the molecular mechanisms involved in the association between IBD and CRC are largely unknown. The need for a greater knowledge of their underlying molecular biology, immune pathobiology, and genetic processes has been driving an intense research effort (16-19).

High-temperature requirement serine protease A (*HtrA*) is a heat-induced gene, indispensable for bacterial survival at elevated temperatures, that was first identified in *Escherichia coli* (20). Subsequent studies have indicated that it degrades misfolded protein in the periplasm at high temperatures (21). HtrA1, also called PRSS11 or L56, is one of the 4 members of the human HtrA protein family (HtrA 1-4) that has been identified by Zumbrunn and Trueb (22) in human embryonic lung fibroblasts. As a serine protease, HtrA1 is a conserved protein that is widely expressed in normal tissue in several species (22).

HtrA1 plays a pivotal role in the fibroblast growth factor pathway, downregulating tumour progression (24). Changes in its expression have been reported in conditions such as osteoarthritis, age-related macular degeneration and cancer (25-27). It tends to be downregulated in the metastatic foci of various tumours compared with the primary tumour in a number of malignancies, such as melanoma, sarcoma, neuroblastoma and lung cancer (23,24), while its overexpression has been hypothesized to inhibit growth and proliferation processes by acting on tumour cell apoptosis, invasiveness and migration (28). There are currently no data on the immunohistochemical localization of HtrA1 in CRC, colorectal adenoma or IBD.

In the present study, HtrA1 concentrations were determined in colon mucosa from patients with CRC, adenoma with high-grade dysplasia (AHD), adenoma with low-grade dysplasia (ALD), UC of >10 year duration (UCL), UC of <5 year duration (UCS) and colonic diverticulitis (D), and compared with the expression found in normal colon tissues (NCTs) collected 5 cm from the CRC lesions and in colon tissues from healthy controls (HC), to test its ability to serve as a biomarker of these diseases.

Materials and methods

A total of 250 colon tissue specimens collected from July 1, 2014 to July 30, 2015, and fixed with haematoxylin and eosin according to a standard protocol were retrieved from the pathology archives of the Sections of Pathological Anatomy of the University of L'Aquila (L'Aquila, Italy), the Hospital of Teramo (Teramo, Italy) and the Università Politecnica Delle Marche (Ancona, Italy). Specimens were reviewed by 3 pathologists (G.C., G.Q. and R.M.) to confirm the histological diagnosis and select the more representative areas for immunohistochemical analysis. The study protocol was approved by the Ethics Board of Teramo Health Service (26 June 2013) and was in line with the ethical standards laid down in the 1964 Declaration of Helsinki.

Specimen characteristics. The 250 samples of colon mucosa examined are described in Fig. 1. Of these, 22 could not be evaluated for the reasons reported in the figure, leaving 228 CRC, AHD, ALD, UCL, UCS, D, NCT and HC specimens for the analyses.

All specimens came from Italian Caucasian subjects who suffered from a single disease. The 37 CRC tissue specimens, 17 (45.9%) from the right colon and 20 (54.1%) from the left colon, were obtained from patients subjected to radical surgical resection of the primary tumour. Two different tissue samples were obtained from these specimens; one representative of CRC and another of NCT. The latter samples were collected at a distance of 5 cm from the neoplastic lesion and their normal features were established by histology.

CRC specimens. Tumour staging was based on the TNM classification, the Dukes' system and histological grading. The latter included the following classification: well differentiated (G1), moderately differentiated (G2) and poorly differentiated (G3). Genetic and epigenetic alterations were not assessed in these specimens.

Adenoma specimens. The 49 specimens of conventional adenoma (tubular, villous, tubulovillous) were divided into 2 groups according to the grade of dysplasia: 22 (44.9%) were adenomas with high-grade dysplasia and focal low-grade dysplasia (AHD) and 27 (55.1%) were adenomas with low-grade dysplasia (ALD). All adenomas were removed by colonoscopy. Sessile serrated adenomas/polyps were not included in this series.

Ulcerative colitis specimens. The 37 endoscopic biopsies from patients with UC were divided according to disease duration. There were 18 (48.6%) samples from patients with disease duration >10 years (UCL) and 19 (51.4%) from patients with disease duration <5 years (UCS). All UC patients showed mild/moderate clinical activity and mild/moderate mucosal lesions on colonoscopy.

Colonic diverticulitis specimens. The 32 samples from D patients had an average length of 23 cm (sigmoid colon; range, 12-30 cm).

Healthy specimens. The HC mucosal biopsies were from 36 subjects without a family history of CRC or IBD who underwent colonoscopy due to clinical signs related to haemorrhoid disease or IBS. This group did not include macroscopic lesions of large bowel mucosa.

The same exclusion criteria were applied to all tissue samples. Subjects with a personal or family history of malignancy and those previously subjected to adjuvant therapy (chemotherapy or radiotherapy) were excluded. Subjects with a personal or family history of hereditary or familial CRC or familial adenomatous polyposis were also excluded, since the genetic and epigenetic mechanisms underpinning these forms are different from those of sporadic and UC-associated CRC.

Immunohistochemistry. Paraffin sections were processed for HtrA1 analysis as previously described (29). Briefly, they were deparaffinized and rehydrated via xylene and a graded series of ethyl alcohol, and treated with Tween 0.3% in phosphate-buffered saline (PBS) for 25 min at room

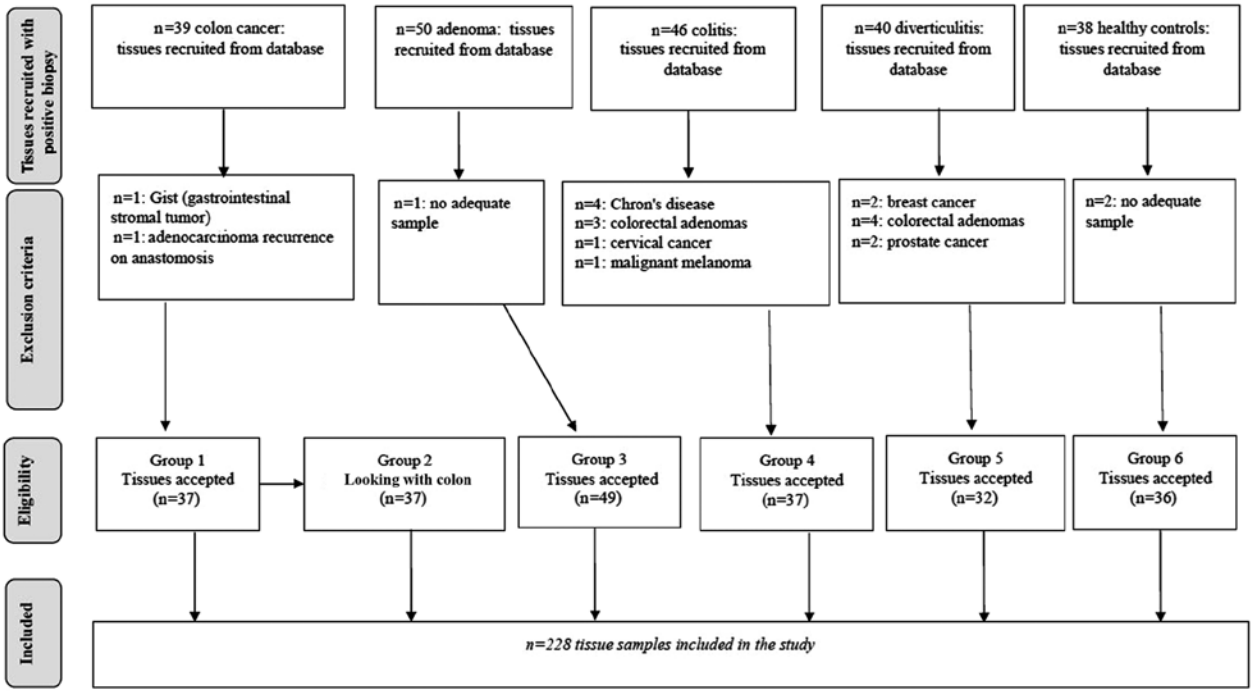

Figure 1. Flow chart of the tissue samples included in the present study.

temperature. Sections were incubated for 50 min with 3% hydrogen peroxide in deionized water to inhibit endogenous peroxidase activity. To block non-specific background, they were incubated for 1 h at room temperature with normal serum. Sections were then incubated overnight at 4˚C with rabbit polyclonal HtrA1 antibody diluted 1:20 (ab38610; Abcam PLC, Cambridge, UK). After washing in PBS, they were incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). The peroxidase ABC method (Vector Laboratories) was performed for 1 h at room temperature; 3',3'-diaminobenzidine hydrochloride (Sigma, St. Louis, MO, USA) was used as the chromogen. Sections were counterstained with Mayer's haematoxylin, dehydrated and mounted with Eukitt solution (Kindler GmbH and Co., Freiburg, Germany). Negative controls were performed by omitting the primary or the secondary antibody. Further negative controls were performed using an isotype control antibody (rabbit IgG, cat. ab27478; Abcam). Placenta tissue was used as a positive control.

Immunostaining evaluation. Immunohistochemical evaluations were independently performed by 3 histologists (M.M., D.M. and C.L.). Immunostaining was evaluated in mucosal glandular and surface epithelial and in mucosal stromal cells and mucosal extracellular matrix. HtrA1 staining was scored as positive when brown precipitate was detected in the epithelial or in the stromal compartment of the mucosa. The expression level of HtrA1-stained cells by light microscopy was evaluated in a semi-quantitative manner and ranked as follows: 0 (negative, 0% of positive cells), 1 (weak positive, 1-25% of positive cells), 2 (moderate positive, 26-60% of positive cells), and 3 (intense positive, 61-100% positive cells) at a magnification of x20, as previously described (30). A value 1 was used as the cut-off. Accordingly, samples ranked 0 and 1 were classified as 'low expression' and those ranked 2 and 3 were classified as 'high expression'.

Statistical analysis. Patient age is shown as the mean ± standard deviation (SD). As in our previous study of HtrA1 (31), the Kolmogorov-Smirnov test was performed in advance to check the normality of variables. Differences in HtrA1 expression levels among groups were evaluated using the non-parametric Kruskal-Wallis test. The inter-observer agreement of the immunohistochemical evaluations (colon mucosa) was calculated as follows: intensity, weak-high, of brown staining in epithelial cells in regards to the epithelial compartment, and intensity of the brown staining in cells and extracellular matrix (ECM) in regards to the stromal compartment (lamina propria). The inter-observer agreement was expressed using Cohen's κ statistic as follows: κ, <0.20 poor, 0.21-0.40 fair, 0.41-0.60 moderate, 0.61-0.80 good and 0.81-1.00 very good agreement. The agreement was required, since the HtrA1 antibody is not applied in routine diagnosis and cannot therefore be determined with an automatic analyzer. In addition, the observers were able to evaluate the background in the sections. The inter-observer agreement was calculated for the main groups (CRC, NCT, HC, UC, D and adenoma) not for the subgroups, since the histological elements that were evaluated were always the same.

Cohn's κ expresses the degree of agreement beyond chance. Cell counts were performed by 3 observers with different experience in light microscopy (LM) who were not aware of the histopathological diagnosis. Their LM experience was expressed by a score that took into account the number of years spent specializing in LM (score: 1, <5 years; 2, >5 years); their daily LM work (1, <3 h/day; 2, >3 h/day), and the number of workshops/seminars attended (1, <5; 2, >5). These criteria allowed classifying the observers into those

Table I. Characteristics and site of tissue samples studied.

Characteristics	CRC	AHD	ALD	UCL	UCS	D	NCT	HC
Total no. of cases	37	22	27	18	19	32	37	36
Age (years) (mean ± SD)	65.5±9.4	66.6±9.1	68.9±9.2	55.4±14.5	53.1±17.4	58.4±16.2	65.5±9.4	53.0±10.0
Sex, n (%)								
Female	15 (40.5)	6 (27.3)	8 (29.6)	10 (55.6)	12 (63.2)	13	15 (40.5)	23 (63.9)
Male	22 (59.5)	16 (72.7)	19 (70.4)	8 (44.4)	7 (36.8)	19	22 (59.5)	13 (36.1)
Anatomic site, n (%) sampling								
Right colon	17 (45.9)	5 (22.7)	7 (25.9)				17	18 (50)
Left colon	20 (54.1)	9 (40.9)	14 (51.9)				20	17 (47.2)
Transversum		2 (9.1)	5 (18.5)					1 (2.8)
Rectum		6 (27.3)	1 (3.7)					
Left colitis				11 (61.1)	12 (31.6)			
Sub-total colitis				1 (5.6)	1 (5.3)			
Total colitis				6 (33.3)	6 (63.1)			
Sigmoid colon						32		
Grading, n (%)								
G1	12 (32.4)							
G2	22 (59.5)							
G3	3 (8.1)							
TNM, n (%)								
pT1 (N0)	7 (19.0)							
pT2 (11 N0 and 2 N1)	13 (35.1)							
pT3 (10 N0, 3 N1, 1 N1c, 1 N2a, 1 N2b)	16 (43.2)							
pT4 (N2a)	1 (2.7)							
Dukes' stage								
A	18 (48.6)							
B	12 (32.4)							
C	7 (19.0)							

CRC, colorectal cancer; AHD, adenoma with high dysplasia; ALD, adenoma with low dysplasia; UCL, ulcerative colitis - duration of disease >10 years; UCS, ulcerative colitis - duration of disease <5 years; D, diverticulitis; NCT, normal colon tissues; HC, healthy controls.

with strong experience (A and B) and limited experience (C). All evaluations were performed in a blinded manner. Global agreement was estimated for each group of samples.

The statistical significance of contingency tables was verified by the χ^2 or Fisher's exact test as appropriate.

The present study had 91% power considering the following parameters: p=0.70 (expected proportion in CRC group), p=0.30 (expected proportion in HC group), sample size=37 (CRC group), sample=36 (HC group), α=0.05.

All p-values <0.05 were considered statistically significant. There were no missing data. All analyses were carried out using SAS/STAT statistical software.

Results

A total number of 228 tissue samples were examined and divided into 6 groups (Fig. 1): group 1, 37 samples from CRC patients; group 2, 37 NCT samples from group 1 patients; group 3, 49 adenoma samples subdivided into low-degree dysplasia (ALD) and high-degree dysplasia (AHD); group 4, 37 UC samples subdivided into short (UCS) and long disease duration (UCL); group 5, 32 samples from patients with D; group 6, 36 HC samples. The characteristics of each group are summarized in Table I. Since HtrA1 is a secreted protein, immunohistochemical HtrA1 staining was detected either in the cytoplasm of colonic epithelial or in stromal cells and ECM of the colon mucosa. HtrA1 staining was scored as low or high expression both in the epithelial and in the stromal compartment.

In HC and NCT specimens, HtrA1 expression was homogeneous in all segments of the colon and rectum, suggesting that its expression patterns in the different conditions are not affected by lesion site. HtrA1 expression did not differ in relation to age. HtrA1 was absent or weakly expressed

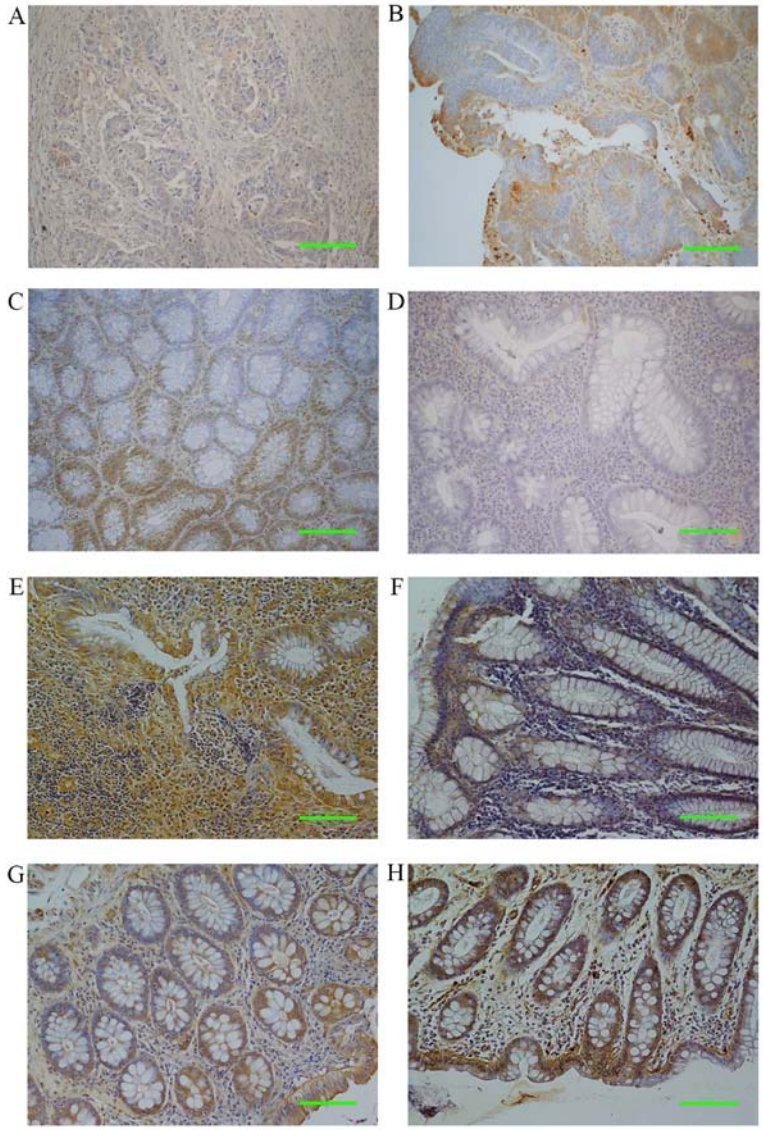

Figure 2. HtrA1 immunostaining in (A) CRC, (B) AHD, (C) ALD, (D) UCL, (E) UCS, (F) D, (G) NCT and (H) HC tissue. HtrA1 was weakly expressed in the epithelial and stromal compartment in (A) CRC compared with (G) NCT and (H) HC specimens. HC (H) shows strong HtrA1 immunostaining. Staining is clearly evident in the stroma compartment of (B) AHD and (C) ALD samples and is moderate and inhomogeneous in the epithelium. HtrA1 is weakly expressed in (D) UCL, whereas (E) UCS specimens show homogenous staining in the stroma and moderate staining in the epithelial compartment. (F) Moderate and homogeneous immunostaining is observed in diverticulitis samples (magnification, x20; green scale bar, 65 μm). CRC, colorectal cancer; AHD, adenoma with high-grade dysplasia; ALD, adenoma with low-grade dysplasia; UCL, ulcerative colitis of >10 year duration; UCS, ulcerative colitis of <5 year duration; D, colonic diverticulitis; NCT, normal colon tissues collected 5 cm from the CRC lesion; HC, healthy colon mucosa.

in the epithelium and in the stromal compartment of CRC sections (Fig. 2A), whereas NCT and HC samples showed a strong positive reaction in both compartments (Fig. 2G and H). In the adenoma samples, HtrA1 expression was weaker in the epithelium than in the stroma, without clear differences between AHD (Fig. 2B) and ALD (Fig. 2C) specimens. The immunostaining pattern of UCL tissue was very similar to that of CRC samples, with very weak staining in the epithelium and stroma (Fig. 2D), whereas UCS and D tissues showed moderate-high staining in the stroma and moderate-weak staining in the epithelium (Fig. 2E and F).

The results of HtrA1 expression in the tissue groups are summarized in Table II according to staining intensity (low/high). Statistically significant differences among the groups were found both in the stromal ($p<0.0001$) and the epithelial ($p<0.0002$) compartment. In the stromal compartment, low HtrA1 expression was most frequent in samples from CRC and UCL patients (83.8 and 83.3%) and least frequent in HC tissue (2.8%); the difference was significant ($p<0.0001$). In the epithelial compartment, low HtrA1 expression was most frequent in UC patients with disease duration >10 years (94.4%) and least common in HC samples (8.3%).

Testing for differences in HtrA1 expression between diseases highlighted a number of statistically significant differences. Stromal compartment: CRC vs. AHD ($p=0.0001$); CRC vs. ALD ($p=0.0001$); CRC vs. UCL ($p=1.0$); CRC vs. UCS ($p=0.0001$); CRC vs. D ($p=0.03$); CRC vs. NCT ($p=0.0001$); CRC vs. HC ($p=0.0001$); UCL vs. UCS ($p=0.0008$); UCL vs. HC ($p=0.0001$); UCL vs. AHD ($p=0.0001$); and UCL vs. ALD ($p=0.0001$) (Table III). Epithelial compartment: CRC

Table II. HtrA1 expression according to the pathologies considered.

	Low expression		High expression		
Groups	No.	(%)	No.	(%)	P-value[a]
Stromal compartment					
CRC	31	83.8	6	16.2	<0.0001
AHD	1	4.4	22	95.6	
ALD	1	3.9	25	96.1	
UCL	15	83.3	3	16.7	
UCS	5	26.3	14	73.7	
D	18	56.2	14	43.8	
NCT	10	27.0	27	73.0	
HC	1	2.8	35	97.2	
Epithelial compartment					
CRC	24	64.9	13	35.1	<0.0001
AHD	10	43.5	13	56.5	
ALD	6	23.1	20	76.9	
UCL	17	94.4	1	5.6	
UCS	10	52.6	9	47.4	
D	17	53.1	15	46.9	
NCT	9	24.3	28	75.7	
HC	3	8.3	33	91.7	

CRC, colorectal cancer; AHD, adenoma with high dysplasia; ALD, adenoma with low dysplasia; UCL, ulcerative colitis - duration of disease >10 years; UCS, ulcerative colitis - duration of disease <5 years; D, diverticulitis; NCT, normal colon tissues; HC, healthy controls; [a]Kruskal-Wallis test.

Table III. HtrA1 expression in stromal and epithelial compartments: comparison between the different groups investigated.

Disease	Stromal P-value	Epithelium P-value
CRC vs. AHD	0.0001	0.09
CRC vs. ALD	0.0001	0.007
CRC vs. UCL	1.0	0.04
CRC vs. UCS	0.0001	0.57
CRC vs. D	0.03	0.32
CRC vs. NCT	0.0001	0.001
CRC vs. HC	0.0001	0.0001
ALD vs. AHD	0.52	0.54
AHD vs. HC	1.0	0.02
ALD vs. HC	1.0	0.30
UCL vs. UCS	0.0008	0.01
UCL vs. HC	0.0001	0.0001
UCL vs. AHD	0.0001	0.001
UCL vs. ALD	0.0001	0.0001
HC vs. NCT	0.14	0.56

CRC, colorectal cancer; AHD, adenoma with high dysplasia; ALD, adenoma with low dysplasia; UCL, ulcerative colitis - duration of disease >10 years; UCS, ulcerative colitis - duration of disease <5 years; D, diverticulitis; NCT, normal colon tissues; HC, healthy controls.

vs. ALD (p=0.007); CRC vs. UCL (p=0.04); CRC vs. NCT (p=0.001); CRC vs. HC (p=0.0001); and AHD vs. HC (p=0.02); UCL vs. UCS (p=0.01); UCL vs. HC (p=0.0001); UCL vs. AHD (p=0.001); UCL vs. ALD (p=0.0001) (Table III).

The data regarding overall and pairwise inter-observer agreement in the stromal and epithelial compartment are reported in Table IV. In UC patients, full agreement (k=100%) was found for immunostaining evaluation of the stromal compartment and very good agreement for the epithelial compartment (k=94.6%). In the stromal compartment, pairwise agreement (A vs. B) was very good for CRC (k=89%), adenoma (k=93%), HC (k=84%), and NCT (k=83%), and good for D (k=75%), whereas in the epithelial compartment it was very good for D (k=81%) and moderate for the other diseases (Table IV). The differences in the level of agreement between the evaluation of epithelial and stromal immunostaining are essentially due to the histological characteristics of the colon epithelium, which is particularly rich in goblet cells. These cells contain mucin drops that occupy nearly the whole cell cytoplasm, making the assessment of cytoplasmic immunostaining more difficult in the epithelial compartment than in the stroma, where cells do not contain mucin drops.

Discussion

To the best of our knowledge, the present study is the first to examine HtrA1 expression in CRC, AHD, ALD, UCL, UCS, NCT and HC samples by immunohistochemistry. HtrA1 immunostaining was detected in the epithelium and stroma (lamina propria) of the colon mucosa in all the tissue samples analysed. Its expression patterns in the different HC and NCT colon segments examined showed no differences, suggesting that the various conditions depend on disease type rather than colon segment. Age was also unrelated to HtrA1 expression characteristics. The main finding of the present study was the significantly reduced HtrA1 expression found in CRC and UCL tissues compared with that in the NCT and HC samples and with the tissue specimens from the other patients. In particular, HtrA1 expression was similar in the stromal compartment of UCL and CRC sections whereas in the epithelial compartment it was weaker in UCL than CRC tissue. HtrA1 expression was also significantly lower in UCL than UCS samples, suggesting that it declines with disease duration, particularly in the stromal compartment.

HtrA1 has a functional role both in epithelium and stroma compartments. HtrA1 is a serine protease involved in important physiological processes, including maintenance of mitochondrial homeostasis, apoptosis and cell signalling (32,33). HtrA1 has also the capacity to degrade numerous extracellular matrix (ECM) proteins, produced by the stromal cells, as well as to decrease biological function of vascular endothelial cells, and thus, able to act even in the stromal compartment (34,35). The effects of HtrA1 on

Table IV. Agreement on immunostaining assessment.

Disease	Overall agreement (%)	Agreement among observers A, B and C Pairwise comparisons (Cohen's κ statistic)		
		A vs. B (95% CI)	A vs. C (95% CI)	B vs. C (95% CI)
Stromal compartment				
CRC	92.8	0.89 (0.69-1.0)	0.65 (0.35-0.95)	0.75 (0.49-1.0)
A	95.2	93.7 (93.1-94.3)	93.7 (93.1-94.3)	93.7 (93.1-94.3)
UC	100.0	1.0	1.0	1.0
D	74.0	0.75 (0.52-0.98)	0.57 (0.30-0.84)	0.69 (0.46-0.93)
NCT	79.3	0.83 (0.65-1.0)	0.77 (0.57-0.98)	0.82 (0.62-1.0)
HC	92.8	0.84 (0.67-1.0)	0.53 (0.28-0.78)	0.5 (0.25-0.75)
Epithelial compartment				
CRC	83.8	0.63 (0.40-0.86)	0.63 (0.40-0.86)	0.77 (0.54-0.98)
A	89.1	75.6 (0.56-0.96)	82.6 (0.66-0.98)	68.7 (0.48-0.89)
UC	94.6	0.66 (0.35-0.96)	0.51 (0.13-0.88)	0.73 (0.46-1.0)
D	87.4	0.81 (0.61-1.0)	0.69 (0.45-0.93)	0.62 (0.35-0.88)
NCT	87.4	0.65 (0.35-0.95)	0.65 (0.35-0.95)	0.56 (0.26-0.87)
HC	85.6	0.77 (0.47-1.0)	0.54 (0.14-0.93)	0.60 (0.25-0.95)

CRC, colorectal cancer; A, adenoma; UC, ulcerative colitis; D, diverticulitis; NCT, normal colon tissues; HC, healthy controls.

proliferation and apoptosis of epithelial cells and the effects on the ECM remodelling can intervene at different phases of colorectal carcinogenesis. In UCL, both the epithelial proliferation and apoptosis and ECM components in the mucosa and submucosa are altered (18,36,37), findings that may link the risk of CRC of UCL to the altered expression of HtrA1 observed in this subset of UC patients.

UCL is a well-known risk factor for CRC development, whereas UCS and D patients are not at great risk (16,18,38,39). The similar HtrA1 expression patterns found in UCS and D samples in the present study provide support for this concept. It may thus be hypothesized that different pathological inflammatory microenvironments induce a reduction in HtrA1 expression in CRC and UCL, and that such low HtrA1 levels underpin the risk of progression to CRC. Moreover, different inflammatory soluble factors are found in acute and chronic inflammation. In long-standing chronic inflammation some molecules foster epithelial hyperproliferation and the genetic and epigenetic alterations that promote CRC (17-19,38-40). It is clearly useful to establish which of the specific chronic inflammatory mediators of UCL correlate with HtrA1 expression, but this was outside the scope of the present study. However, the present data do suggest that HtrA1 may provide an important link between UCL and CRC, and that epithelium and stroma are differentially affected (41). Notably, it has been suggested that HtrA1 responds to different environments in different ways, probably due to its secretory characteristic (42), and that high HtrA1 levels are associated with chronic inflammatory conditions such as rheumatoid arthritis, osteoarthritis and macular degeneration (26,27,43-45). The latter data appear to contrast with the decreased HtrA1 expression found in UCL, where patterns were similar to those observed in CRC. However, it should be noted that different cellular and molecular mechanisms act in rheumatoid arthritis, osteoarthritis and macular degeneration, which are not associated with an increased risk of cancer (45). Furthermore, unlike the case of UC and CRC (17), the intestinal microbiota do not appear to play an important pathogenic role in these inflammatory diseases.

Concerning AHD and ALD tissues, HtrA1 levels were not significantly different compared with those measured in HC samples in the two mucosal compartments, except for a slight reduction found in the epithelial compartment. Since adenoma is considered as the main risk factor for sporadic CRC, the present data appear to exclude a direct involvement of HtrA1 in the progression from adenoma to CRC.

HtrA1 does not appear to be involved in the first stage of adenoma formation (which is mainly related to *APC* gene mutations) or in the first stage of development of dysplasia in adenoma, but rather in a later stage of carcinogenesis. This notion is partly supported by the distribution of percentage, found in the present study, of HtrA1 expression, histological grade and CRC stage. We did not apply a statistical test due to the small numbers (data not shown). It is therefore likely that different molecular mechanisms are responsible for the development of dysplasia in adenoma and chronic UC.

There are important clinical and biological differences between the adenoma-carcinoma sequence and the UCL-carcinoma sequence (37,46). The molecular basis of the adenoma-carcinoma sequence involves first *APC* gene mutations and secondarily p53 mutations (46), whereas in the UCL-carcinoma sequence the p53 mutations come first (37). The different sequence of events correlated with the different behaviour of HtrA1 in UCL and adenoma (AHD and ALD). In addition, the fact that adenomatous tissue does not contain

inflammatory components could explain the normal HtrA1 expression found in adenomas and its downregulation in UCL.

The present findings suggest that HtrA1 may be a promising marker to identify those UCL patients who are at high risk of developing CRC. Studies of larger patient samples are also required to demonstrate the ability of HtrA1 to screen patients at high risk of developing CRC, who may therefore require intensive endoscopic and histological monitoring.

References

1. Altobelli E, Lattanzi A, Paduano R, Varassi G and di Orio F: Colorectal cancer prevention in Europe: Burden of disease and status of screening programs. Prev Med 62: 132-141, 2014.
2. Altobelli E, D'Aloisio F and Angeletti PM: Colorectal cancer screening in countries of European Council outside of the EU-28. World J Gastroenterol 22: 4946-4957, 2016.
3. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
4. Pawa N, Arulampalam T and Norton JD: Screening for colorectal cancer: Established and emerging modalities. Nat Rev Gastroenterol Hepatol 8: 711-722, 2011.
5. Rubenstein JH, Enns R, Heidelbaugh J, Barkun A, Adams MA, Dorn SD, Dudley-Brown SL, Flamm SL, Gellad ZF, Gruss CB, *et al*; Clinical Guidelines Committee: American Gastroenterological Association Institute Guideline on the Diagnosis and Management of Lynch Syndrome. Gastroenterology 149: 777-782, quiz e16-e17, 2015.
6. Samadder NJ, Jasperson K and Burt RW: Hereditary and common familial colorectal cancer: Evidence for colorectal screening. Dig Dis Sci 60: 734-747, 2015.
7. Sebastian S, Hernández V, Myrelid P, Kariv R, Tsianos E, Toruner M, Marti-Gallostra M, Spinelli A, van der Meulen-de Jong AE, Yuksel ES, *et al*: Colorectal cancer in inflammatory bowel disease: Results of the 3rd ECCO pathogenesis scientific workshop (I). J Crohns Colitis 8: 5-18, 2014.
8. Parian A and Lazarev M: Who and how to screen for cancer in at-risk inflammatory bowel disease patients. Expert Rev Gastroenterol Hepatol 9: 731-746, 2015.
9. Breynaert C, Vermeire S, Rutgeerts P and Van Assche G: Dysplasia and colorectal cancer in inflammatory bowel disease: A result of inflammation or an intrinsic risk? Acta Gastroenterol Belg 71: 367-372, 2008.
10. Brenner H, Hoffmeister M, Stegmaier C, Brenner G, Altenhofen L and Haug U: Risk of progression of advanced adenomas to colorectal cancer by age and sex: Estimates based on 840,149 screening colonoscopies. Gut 56: 1585-1589, 2007.
11. Kuntz KM, Lansdorp-Vogelaar I, Rutter CM, Knudsen AB, van Ballegooijen M, Savarino JE, Feuer EJ and Zauber AG: A systematic comparison of microsimulation models of colorectal cancer: The role of assumptions about adenoma progression. Med Decis Making 31: 530-539, 2011.
12. Terzić J, Grivennikov S, Karin E and Karin M: Inflammation and colon cancer. Gastroenterology 138: 2101-2114.e5, 2010.
13. Sakellakis M, Makatsoris T, Gkermpesi M, Peroukidis S and Kalofonos H: Ulcerative colitis six years after colon cancer: Only a coincidence? Int Med Case Rep J 7: 85-88, 2014.
14. Dyson JK and Rutter MD: Colorectal cancer in inflammatory bowel disease: What is the real magnitude of the risk? World J Gastroenterol 18: 3839-3848, 2012.
15. Herszényi L, Barabás L, Miheller P and Tulassay Z: Colorectal cancer in patients with inflammatory bowel disease: The true impact of the risk. Dig Dis 33: 52-57, 2015.
16. O'Connor PM, Lapointe TK, Beck PL and Buret AG: Mechanisms by which inflammation may increase intestinal cancer risk in inflammatory bowel disease. Inflamm Bowel Dis 16: 1411-1420, 2010.
17. Azer SA: Overview of molecular pathways in inflammatory bowel disease associated with colorectal cancer development. Eur J Gastroenterol Hepatol 25: 271-281, 2013.
18. Foersch S and Neurath MF: Colitis-associated neoplasia: Molecular basis and clinical translation. Cell Mol Life Sci 71: 3523-3535, 2014.
19. Hartnett L and Egan LJ: Inflammation, DNA methylation and colitis-associated cancer. Carcinogenesis 33: 723-731, 2012.
20. Lipinska B, Zylicz M and Georgopoulos C: The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase. J Bacteriol 172: 1791-1797, 1990.
21. Spiess C, Beil A and Ehrmann M: A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell 97: 339-347, 1999.
22. Zumbrunn J and Trueb B: Primary structure of a putative serine protease specific for IGF-binding proteins. FEBS Lett 398: 187-192, 1996.
23. Zhao Z, Li H, Wang C, Xu W, Sun J and Zhao W: Serine protease HtrA1 as an inhibitor on proliferation invasion and migration of gastric cancer. Med Oncol 32: 112, 2015.
24. Baldi A, De Luca A, Morini M, Battista T, Felsani A, Baldi F, Catricalà C, Amantea A, Noonan DM, Albini A, *et al*: The HtrA1 serine protease is down-regulated during human melanoma progression and represses growth of metastatic melanoma cells. Oncogene 21: 6684-6688, 2002.
25. Grau S, Richards PJ, Kerr B, Hughes C, Caterson B, Williams AS, Junker U, Jones SA, Clausen T and Ehrmann M: The role of human HtrA1 in arthritic disease. J Biol Chem 281: 6124-6129, 2006.
26. Altobelli E, Marzioni D, Lattanzi A and Angeletti PM: HtrA1: Its future potential as a novel biomarker for cancer. Oncol Rep 34: 555-566, 2015.
27. Weger M, Renner W, Steinbrugger I, Köfer K, Wedrich A, Groselj-Strele A, El-Shabrawi Y, Schmut O and Haas A: Association of the HTRA1 -625G>A promoter gene polymorphism with exudative age-related macular degeneration in a Central European population. Mol Vis 13: 1274-1279, 2007.
28. Xia J, Wang F, Wang L and Fan Q: Elevated serine protease HtrA1 inhibits cell proliferation, reduces invasion, and induces apoptosis in esophageal squamous cell carcinoma by blocking the nuclear factor-κB signaling pathway. Tumour Biol 34: 317-328, 2013.
29. Lorenzi T, Lorenzi M, Altobelli E, Marzioni D, Mensà E, Quaranta A, Paolinelli F, Morroni M, Mazzucchelli R, De Luca A, *et al*: HtrA1 in human urothelial bladder cancer: A secreted protein and a potential novel biomarker. Int J Cancer 133: 2650-2661, 2013.
30. De Luca A, De Falco M, Severino A, Campioni M, Santini D, Baldi F, Paggi MG and Baldi A: Distribution of the serine protease HtrA1 in normal human tissues. J Histochem Cytochem 51: 1279-1284, 2003.
31. Marzioni D, Lorenzi T, Altobelli E, Giannubilo SR, Paolinelli F, Tersigni C, Crescimanno C, Monsurrò V, Tranquilli AL, Di Simone N, *et al*: Alterations of maternal plasma HTRA1 level in preeclampsia complicated by IUGR. Placenta 33: 1036-1038, 2012.
32. Zurawa-Janicka D, Skorko-Glonek J and Lipinska B: HtrA proteins as targets in therapy of cancer and other diseases. Expert Opin Ther Targets 14: 665-679, 2010.
33. Schmidt N, Irle I, Ripkens K, Lux V, Nelles J, Johannes C, Parry L, Greenow K, Amir S, Campioni M, *et al*: Epigenetic silencing of serine protease HTRA1 drives polyploidy. BMC Cancer 16: 399, 2016.
34. Tiaden AN and Richards PJ: The emerging roles of HTRA1 in musculoskeletal disease. Am J Pathol 182: 1482-1488, 2013.
35. Jiang J, Huang L, Yu W, Wu X, Zhou P and Li X: Overexpression of HTRA1 leads to down-regulation of fibronectin and functional changes in RF/6A cells and HUVECs. PLoS One 7: e46115, 2012.
36. Triantafillidis JK, Nasioulas G and Kosmidis PA: Colorectal cancer and inflammatory bowel disease: Epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res 29: 2727-2737, 2009.
37. Ullman TA and Itzkowitz SH: Intestinal inflammation and cancer. Gastroenterology 140: 1807-1816, 2011.
38. Antoniou E, Margonis GA, Angelou A, Zografos GC and Pikoulis E: Cytokine networks in animal models of colitis-associated cancer. Anticancer Res 35: 19-24, 2015.
39. Waldner MJ, Neurath MF and Markus M: Cytokines in colitis associated cancer: Potential drug targets? Inflamm Allergy Drug Targets 7: 187-194, 2008.
40. Goel GA, Kandiel A, Achkar JP and Lashner B: Molecular pathways underlying IBD-associated colorectal neoplasia: Therapeutic implications. Am J Gastroenterol 106: 719-730, 2011.

41. Ishiguro K, Yoshida T, Yagishita H, Numata Y and Okayasu T: Epithelial and stromal genetic instability contributes to genesis of colorectal adenomas. Gut 55: 695-702, 2006.
42. Clausen T, Southan C and Ehrmann M: The HtrA family of proteases: Implications for protein composition and cell fate. Mol Cell 10: 443-455, 2002.
43. Hou Y, Lin H, Zhu L, Liu Z, Hu F, Shi J, Yang T, Shi X, Guo H, Tan X, *et al*: The inhibitory effect of IFN-γ on protease HTRA1 expression in rheumatoid arthritis. J Immunol 193: 130-138, 2014.
44. Wu J, Liu W, Bemis A, Wang E, Qiu Y, Morris EA, Flannery CR and Yang Z: Comparative proteomic characterization of articular cartilage tissue from normal donors and patients with osteoarthritis. Arthritis Rheum 56: 3675-3684, 2007.
45. Tomasello G, Tralongo P, Damiani P, Sinagra E, Di Trapani B, Zeenny MN, Hussein IH, Jurjus A and Leone A: Dismicrobism in inflammatory bowel disease and colorectal cancer: Changes in response of colocytes. World J Gastroenterol 20: 18121-18130, 2014.
46. Hardy RG, Meltzer SJ and Jankowski JA: ABC of colorectal cancer. Molecular basis for risk factors. BMJ 321: 886-889, 2000.