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Independent induction of caspase-8 and cFLIP expression during colorectal carcinogenesis in sporadic and HNPCC adenomas and carcinomas

D.M. Heijink [a], J.H. Kleibeuker [b], M. Jalving [a,b], W. Boersma-van Ek [b], J.J. Koornstra [b], J. Wesseling [c] and S. de Jong [a,*]

[a] *Department of Medical Oncology, University Medical Center Groningen, University of Groningen, The Netherlands*
[b] *Department of Gastroenterology and Hepatology, University Medical Center Groningen, University of Groningen, The Netherlands*
[c] *Department of Pathology, Netherlands Cancer Institute/Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands*

Abstract. *Background*: TNF-Related Apoptosis Inducing Ligand (TRAIL) is a promising agent for the induction of apoptosis in neoplastic tissues. Important determinants of TRAIL sensitivity are two intracellular proteins of the TRAIL pathway, caspase-8 and its anti-apoptotic competitor cellular Flice-Like Inhibitory Protein (cFLIP). *Methods*: The aim of this study was to investigate basic expression of caspase-8 and cFLIP in normal colorectal epithelium ($n = 20$), colorectal adenomas ($n = 66$) and colorectal carcinomas ($n = 44$) using immunohistochemistry performed on both sporadic and Hereditary Non-Polyposis Colorectal Cancer (HNPCC or Lynch syndrome)-associated adenomas and carcinomas. *Results*: Expression of both caspase-8 and cFLIP was similar in cases with sporadic and hereditary origin. Expression of caspase-8 in colorectal adenomas and carcinomas was increased when compared to normal colon tissue ($P = 0.02$). Nuclear, paranuclear as well as cytoplasmic localizations of caspase-8 were detected. Immunohistochemistry revealed an upregulation of cFLIP in colorectal carcinomas in comparison to normal epithelium and colorectal adenomas ($P < 0.001$). A large variation in the caspase-8/cFLIP ratio was observed between the individual adenomas and carcinomas. *Conclusion*: Caspase-8 and cFLIP are upregulated during colorectal carcinogenesis. Upregulation of caspase-8 and/or downregulation of cFLIP may be interesting approaches to maximize TRAIL sensitivity in colorectal neoplasms.

Keywords: Caspase-8, cFLIP, colorectal cancer, HNPCC, apoptosis, TRAIL

1. Introduction

Colorectal cancer is one of the leading causes of cancer-related deaths in the western world [44]. The development of colorectal cancer is a multistep process in which normal epithelium progresses through adenoma to carcinoma [42]. Sporadic neoplasms arise due to accumulating somatic mutations. In hereditary syndromes these somatic mutations occur on top of a germ line mutation in a specific gene such as a mismatch repair (MMR) gene in hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome [32].

The mainstay for the treatment of sporadic and hereditary colorectal cancer is surgery. Additionally, chemotherapy and radiotherapy are frequently used [2]. Resistance to chemotherapy often occurs, so there is a need for targeted therapy bypassing this resistance. The use of biological agents, especially apoptosis inducers such as Tumor Necrosis Factor (TNF) – Related Apoptosis Inducing Ligand (TRAIL) or other TRAIL-receptor agonists, can be of great value in this respect [10,43].

Recombinant human (rh)TRAIL can induce apoptosis in malignant cells without harming normal cells

*Corresponding author: S. de Jong, PhD, Department of Medical Oncology, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands. Tel.: 31 50 3612964; Fax: 31 50 3614862; E-mail: s.de.jong@int.umcg.nl.

through binding to its death receptors DR4 and DR5 on the cell membrane [3,4]. Despite the generally extensive expression of the death receptors in both sporadic and hereditary colorectal adenomas and carcinomas [23], several studies show a range of sensitivities for rhTRAIL induced apoptosis in different colon carcinoma cell lines [16,40]. In ex-vivo experiments, colorectal adenomas with high-grade dysplasia were sensitive to rhTRAIL while adenomas with low-grade dysplasia were not [17]. Little is known about the mechanisms behind the varying sensitivity for rhTRAIL [9].

Binding of TRAIL to its death receptors causes trimerization of the receptors and recruitment of the adaptor protein FADD and of the initiator caspases, procaspase-8 and procaspase-10, which results in the formation of the death-inducing signalling complex (DISC) [7,21]. Caspase-8 is currently regarded as the most important initiator caspase in the extrinsic pathway. Procaspase-8 binding to the DISC then results in autocleavage and activation of caspase-8. This caspase-8 activation leads directly to the activation of caspase-3 and subsequently apoptosis [3]. In addition, active caspase-8 thereby triggers the mitochondrial pathway [11]. An important modulator of caspase - 8 activation is the cellular Flice Like Inhibitory Protein (cFLIP). cFLIP shows great homology to pro-caspase-8, but lacks the enzymatic activity of its C-terminal caspase-like domain. There are several splicing variants of cFLIP, $cFLIP_L$ and $cFLIP_S$ being the most predominant [35]. cFLIP is upregulated in many cancer types when compared to normal tissue and is thought to be involved in resistance to death receptor induced apoptosis and also in resistance to chemotherapy [12,27,29]. From previous experiments, we and others have shown that differences in rhTRAIL sensitivity cannot solely be attributed to differences in TRAIL receptor expression [40]. Several groups suggested the ratio of caspase-8 and cFLIP to be important in rhTRAIL sensitivity [15,28,40]. In colorectal adenomas and carcinomas TRAIL receptor expression has been studied, however, little is known about the expression of cFLIP and caspase-8.

The aim of the present study was to investigate the expression of caspase-8 and cFLIP in normal colon epithelium, sporadic adenomas and carcinomas, and HNPCC adenomas and carcinomas. Finally, caspase-8 and cFLIP expression are related to parameters previously determined in these tissues such as apoptosis and TRAIL receptor expression [23].

2. Methods

2.1. Patient and tissue selection

HNPCC patients had a germ line mutation in one of the MMR genes and/or fulfilled the Amsterdam II criteria. All colorectal adenomas and carcinomas removed from HNPCC patients at the University Medical Center Groningen between 1979 and 2002, with sufficient material available, were selected. In total, 33 adenomas and 17 carcinomas (20 from mutation carriers and 30 from patients fulfilling the Amsterdam II criteria) were examined.

Sporadic colorectal adenomas ($n = 33$) and carcinomas ($n = 27$), were selected from previously studied material, of which sufficient material was available to allow serial sectioning for immunohistochemical staining [25]. Sporadic adenomas were selected after matching to HNPCC adenomas for degree of dysplasia.

Twenty samples of normal colonic mucosa were selected from archival materials, which had been obtained in 1999. These are samples from patients with normal macroscopic findings at colonoscopy. Randomly taken biopsies which did not show any abnormalities at histological examination were included. Biopsies from normal mucosa from patients with a history of adenomas or carcinomas were excluded.

2.2. Histopathological classification

For histopathological evaluation haematoxylin and eosin stained slides were used. Classification was performed according to the WHO criteria for gastrointestinal tumours [19]. For adenomas, circumferential size was measured and degree of dysplasia was determined. Adenomas were classified as tubular, tubulovillous, or villous. For statistical purposes, adenomas with tubulovillous or villous histology were combined. For carcinomas, stage and differentiation were determined.

2.3. Immunohistochemistry for cFLIP and caspase-8

For immunohistochemical staining, serial 3 μm thick sections were cut from paraffin blocks. After deparaffinization in xylene and rehydration in alcohol, antigen retrieval was performed by high pressure cooking. Slides were immersed in 200 μl blocking reagent (Boehringer Mannheim, Germany) and underwent 3 sessions of 5 minutes at 115 degrees Celsius. Endoge-

nous peroxidase was blocked with 3% hydrogen peroxide for 30 minutes. For cFLIP staining, a mouse monoclonal primary antibody, detecting both $FLIP_L$ and $FLIP_S$, (1:25; clone NF6; Alexis, Lausen, Switzerland) was applied for 24 hours at 4 degrees Celsius. Then, a highly sensitive horseradish peroxidase system, EnVision, was used according to the instructions of the manufacturer (DAKO, Glostrup, Denmark). Caspase-8 (both full length and cleaved) was detected with a mouse monoclonal antibody (1:50; clone 1C12, Cell Signaling Technology, Beverly, MA, USA) and incubated for 1 hour at room temperature. Slides were incubated with rabbit anti-mouse peroxidase antibody (1:50; DAKO, Glostrup, Denmark), followed by addition of goat anti-rabbit peroxidase antibody (1:50; DAKO, Glostrup, Denmark). Peroxidase activity was visualized with diaminobenzidine for all slides. Slides were counterstained with haematoxylin.

Staining was evaluated by light microscopy by two independent investigators, with re-evaluation under a multi-headed microscope if results did not agree. Results from both stainings were assessed as negative (−) when there was no significant or very weak cytoplasmic immunostaining. Positivity was scored as +/− (moderate staining) or + (strong staining). If staining intensity in a slide was heterogeneous, the area with the highest staining intensity was chosen for scoring if present in more than 10% of the slide. Additionally, for caspase-8 the presence or absence of nuclear staining and of paranuclear staining were assessed and scored as positive (more than 10% positive cells) or negative (less than 10% positive cells).

2.4. Immunohistochemistry for DR4, DR5, apoptosis and proliferation in adenomas and carcinomas

Staining procedures and controls for DR4, DR5 and apoptosis staining were described previously [24,25]. For DR4, a goat polyclonal IgG (1:100; clone C-20; Santa Cruz, CA, USA) was used and no antigen retrieval was required. For DR5 antigen retrieval was carried out by microwave treatment and a rabbit polyclonal IgG (1:100; Oncogene Research, Cambridge, MA, USA) was used. The percentage of staining cells was estimated semi-quantitatively. Samples with DR4 or DR5 staining in more than 10% of cells were considered positive. Apoptotic cells were determined by using the monoclonal antibody M30 (Boehringer Mannheim, Mannheim, Germany) after antigen retrieval by microwave treatment. Apoptosis was assessed in at least 1000 epithelial cells and expressed as a percentage of the total number of cells counted (apoptotic index).

For proliferation, antigen retrieval was performed by high pressure cooking. The primary antibody MIB-1 was applied (against Ki67; 1:400; Immunotech, Marseilles, France). MIB-1 positive cells were assessed in at least 1000 cells in full length crypts and expressed as a percentage of the total number of epithelial cells counted.

2.5. Controls

To verify the specificity of the primary antibodies, several experiments were conducted. First, a Ramos B-cell cell line, stably transfected with a $cFLIP_L$ construct ($cFLIP_L$ cell line), as well as a mock transfected cell line (LZRS cell line) (a generous gift from Dr. B.J. Kroesen, Department of Tumor Immunology, UMCG, the Netherlands), and the untransfected Ramos B-cell cell line expressing endogenous cFLIP were used. Western blots yielded protein expression of expected size, with low expression of $cFLIP_L$ in Ramos and LZRS cell lines and high expression of $cFLIP_L$ in the overexpression cell line. After culturing, the cells were embedded in paraffin, and 3 μm thick sections were cut from paraffin blocks to be used as controls representing high and low expression in immunohistochemistry. Immunostaining of the cell line controls revealed a clearly higher cFLIP expression level in the $cFLIP_L$ overexpressing cell line as compared to the non-transfected Ramos cell line with endogenous cFLIP level, indicating a specific and sensitive staining (Fig. 1).

For caspase-8, two ovarian cancer cell lines were used with low (CP70 cell line) and high (A2780 cell line) expression of caspase-8. Western blotting confirmed the expected levels of caspase-8 in these cell lines. As with the FLIP cell line model, the cell line with high levels of caspase-8 showed higher immunostaining intensity than the cell line with low levels of caspase-8.

Secondly, a number of slides were immunostained using non-immunized mouse IgG1 antibody (Alexis, Lausen, Switzerland) at the same IgG concentration as for the primary antibody. In these cases, no immunostaining was detected.

Thirdly, several slides were treated with phosphate-buffered saline (PBS) in the absence of the primary antibody. In these slides, no immunostaining was seen.

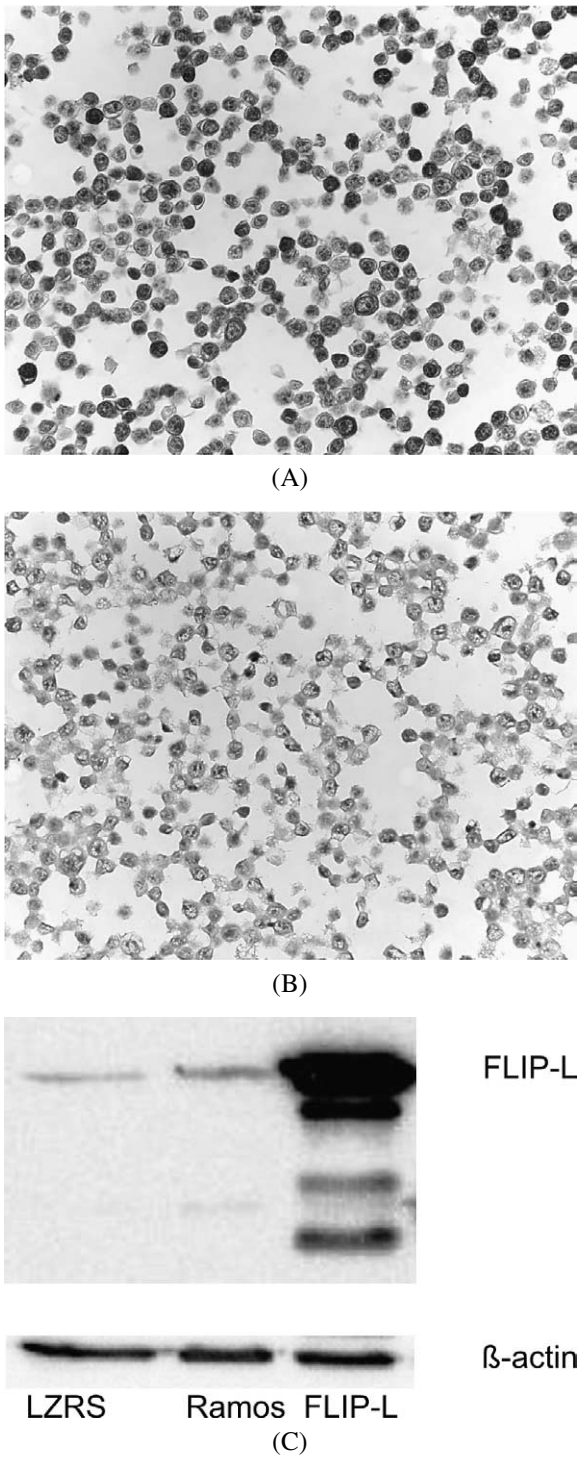


Fig. 1. Control model for cFLIP staining. (A) Strong cFLIP staining in $cFLIP_L$ overexpression cell line. (B) Weak FLIP staining in Ramos cell line. (C) Western-blot analyses for cFLIP confirming differences in cFLIP expression between $cFLIP_L$ overexpression cell line and Ramos cell line; the extra bands probably represent splice variants.

2.6. Statistical analysis

Appropriate tests were used to assess differences in patient and tumour characteristics (χ^2 test for discontinuous variables, Mann–Whitney test for continuous variables) and immunohistochemical findings (χ^2 test for discontinuous variables, Kruskal–Wallis test for continuous variables). Relationships between stainings of DR4, DR5, degrees of apoptosis, degree of proliferation and stainings of cFLIP and caspase-8 were tested by using Kruskal–Wallis testing. SPSS (version 12.0) for Windows software (SPSS Inc., Chicago, IL, USA) was used in all statistical analyses. P values < 0.05 were considered statistically significant.

3. Results

3.1. Patient and tumour characteristics

Patient and tumour characteristics are summarized in Table 1.

Several differences in patient and tumour characteristics were observed between the two patient groups. HNPCC patients were more often women ($P = 0.001$). Mean age at time of diagnosis of patients with sporadic tumours was higher than in HNPCC patients ($P = 0.007$). Mean adenoma size was smaller in patients with HNPCC ($P = 0.01$) and tumour stage was lower in HNPCC-associated cancer cases than in sporadic cancer cases ($P < 0.001$).

3.2. Expression of caspase-8 in normal colonic epithelium, colorectal adenomas and carcinomas

There were no differences in the expression patterns of caspase-8 between sporadic and HNPCC adenomas and between sporadic and HNPCC carcinomas. Therefore HNPCC and sporadic patients were further analyzed as one group.

Caspase-8 cytoplasmic staining intensity was higher in adenomas and carcinomas compared to normal colonic epithelium ($P = 0.02$) (Figs 2 and 3). In addition to the cytoplasmic staining, caspase-8 was also detected at other subcellular localizations (Fig. 4). It was present in the nucleus in 75% of the cases, in normal epithelium as well as in adenomas and carcinomas. Paranuclear staining was detected in normal colon, adenomas and carcinomas in 15, 33 and 27 percent of the cases respectively, and was heterogeneously distributed. Cytoplasmic, nuclear and paranuclear stain-

Table 1
Patient and tumour characteristics

		Sporadic		HNPCC	
		Ad	Ca	Ad	Ca
n		33	27	33	17
Male (%)		67	59	52	12
Age (years) mean, range		66 (50–82)	66 (40–88)	48 (34–67)	55 (31–77)
Size (mm) mean, range		12 (3–45)	–	6.5 (2–18)	–
Tubular (%)		61	–	67	–
HGD (%)		42	–	42	–
Tumour stage (%)	I/II		33		94
	III/IV		67		6
Differentiation (%)	Good/moderate		81		76
	Poor		19		24

Ad, adenoma; Ca, carcinoma; HGD, high-grade dysplasia; HNPCC, hereditary non-polyposis colorectal cancer.

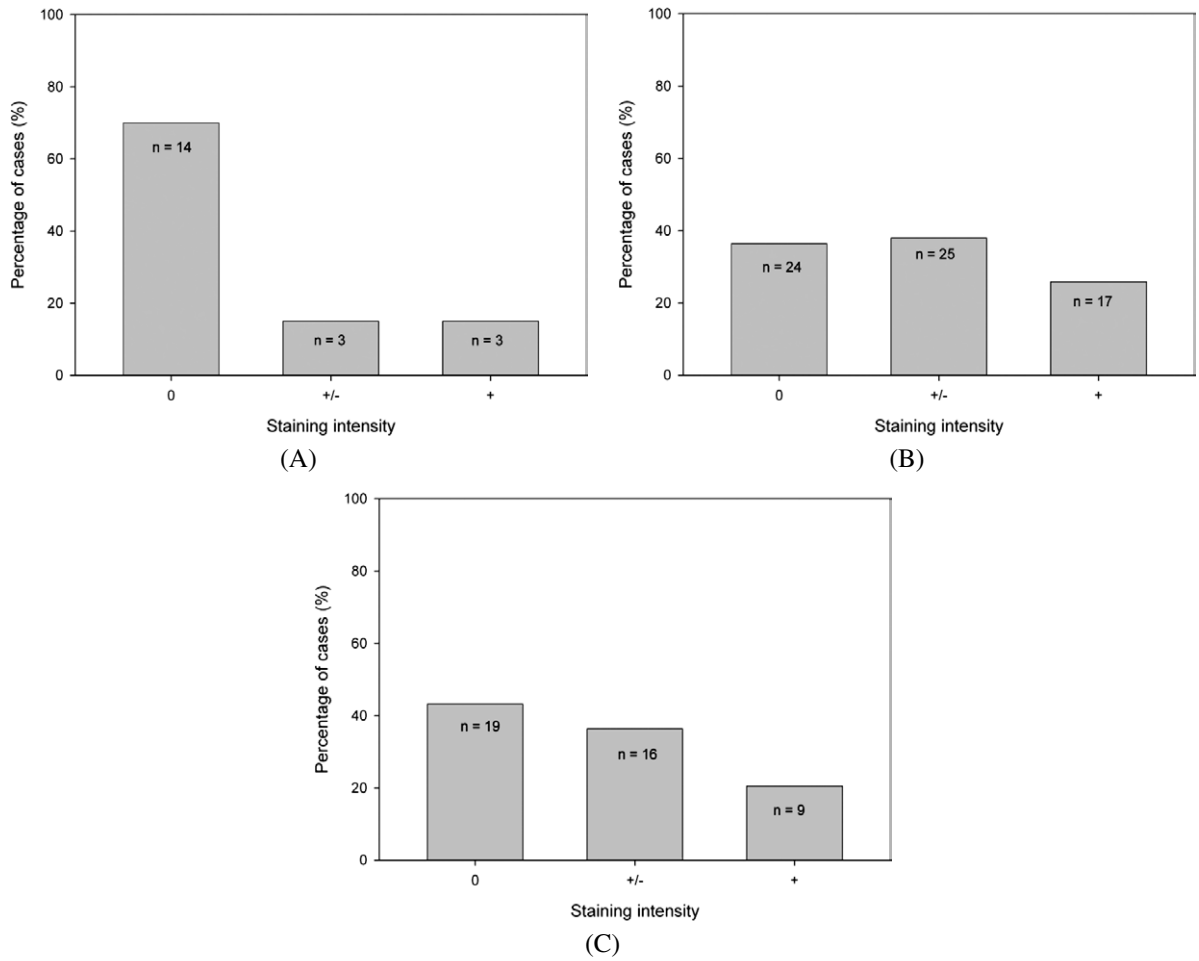
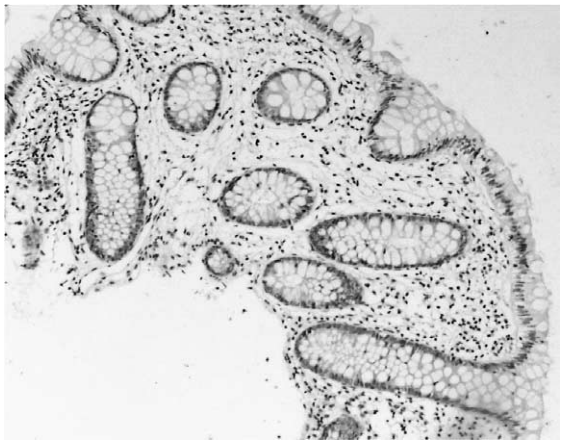
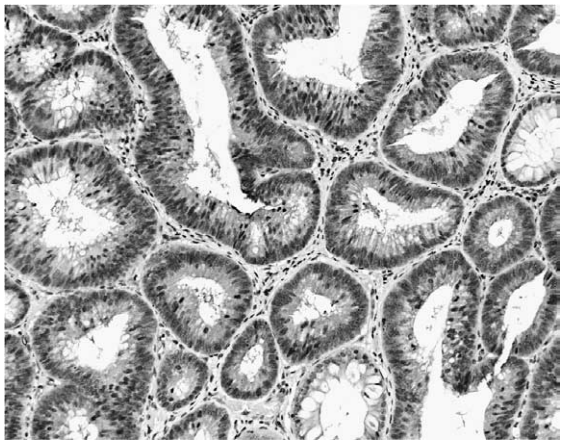


Fig. 2. Cytoplasmic expression of caspase-8 in normal colon tissue (A), colorectal adenomas (B) and colorectal carcinomas (C).



(A)



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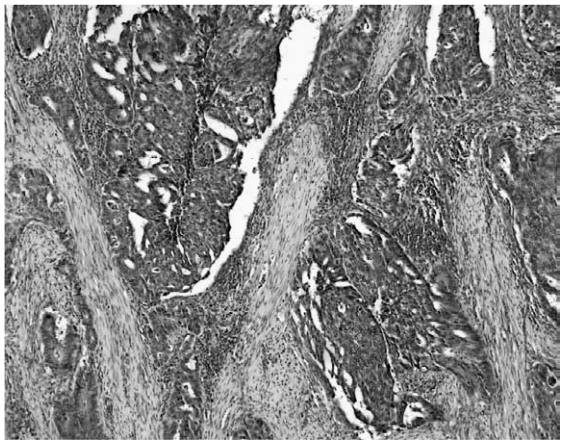
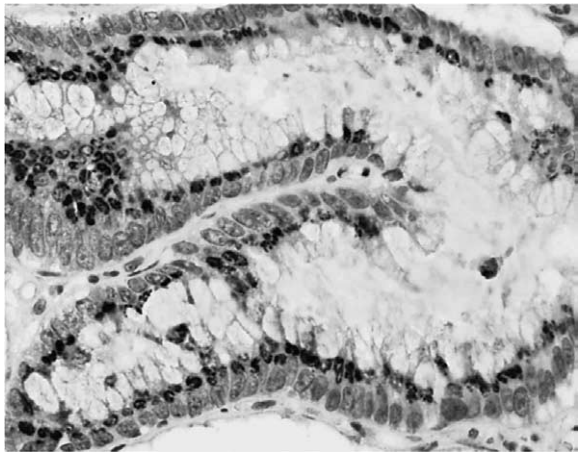
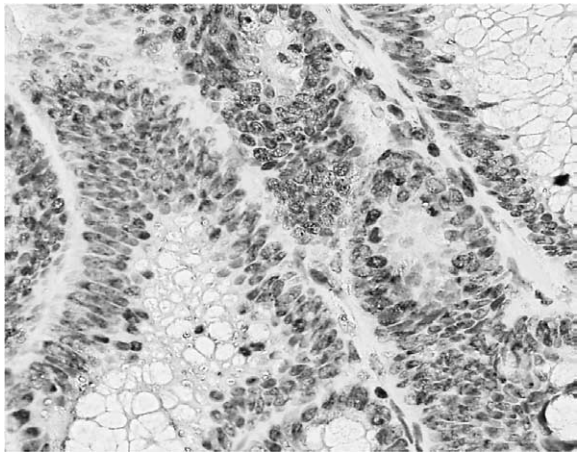


(C)

Fig. 3. Representative examples of expression of caspase-8 in normal colon tissue, colorectal adenomas and carcinomas. Normal tissue showing negative cytoplasmic staining (−). (B) Adenoma showing strongly positive staining (+). (C) Carcinoma showing strong positive staining (+).



(A)



(B)

Fig. 4. Different subcellular localization of caspase-8. (A) Paranuclear localization of caspase-8 in an adenoma. (B) Nuclear localization of caspase-8 in an adenoma.

ing intensity were independent of the histopathological characteristics of the adenomas (size, growth type and degree of dysplasia) and carcinomas (stage and differentiation).

3.3. Expression of cFLIP in normal colonic epithelium, colorectal adenomas and carcinomas

Since analysis did not reveal differences in the expression patterns of cFLIP between sporadic and HNPCC adenomas and between sporadic and HNPCC carcinomas, HNPCC and sporadic patients were further analyzed as one group.

cFLIP staining was moderately positive in one and strongly positive in one of the 20 normal colonic epithelium samples (10%). Staining was moderately and

strongly positive in respectively 14 and 1 out of the 66 colorectal adenomas (23%), which is not significantly different from the results in the controls. In colorectal carcinomas, 75% of the cases showed positive staining, being either moderately positive (19 out of 44 cases) or strongly positive (14 out of 44 cases) ($P < 0.001$ compared to both normal tissue and adenomas, Figs 5 and 6). Staining was heterogeneous and detected in the cytoplasm of the cells. Staining intensity was independent of the histopathological characteristics of the adenomas (size, growth type and degree of dysplasia) and carcinomas (stage and differentiation).

3.4. Relation between cFLIP- and caspase-8 expression and DR4 and DR5 expression, apoptosis and proliferation

Although both caspase-8 and cFLIP expression were elevated in colon carcinomas, there was no correlation between expression of caspase-8 and cFLIP. In contrast, all possible ratios were observed, varying from high to low caspase-8/cFLIP ratios. In a previous study, we determined TRAIL receptor DR4 and DR5 expression, apoptosis and proliferation in these adenomas and carcinomas allowing us to relate caspase-8 or cFLIP with these parameters (Table 2). There was no association between expression of caspase-8 and any one of the four parameters. In carcinomas, the expression of DR4 increased with increasing expression of cFLIP ($P = 0.03$). The caspase-8/cFLIP ratio did not show any relationship with one of the parameters studied.

4. Discussion

The present study shows that caspase-8 expression is increased in colorectal neoplasms when compared to normal colonic epithelium. We detected cytoplasmic as well as nuclear and paranuclear localization of caspase-8. cFLIP was upregulated in carcinomas compared to normal colonic epithelium and adenomas. There was a large variation in the ratio of caspase-8 and cFLIP in the different tumours. Despite the different pathways involved in carcinogenesis, levels of cFLIP and caspase-8 were similar in tumours occurring sporadically and HNPCC-associated tumours.

The upregulation of caspase-8 in colorectal adenomas and carcinomas in comparison to normal colonic epithelium is not entirely in line with findings in a previous study from which it was concluded that cytoplasmic caspase-8 expression was unchanged or only

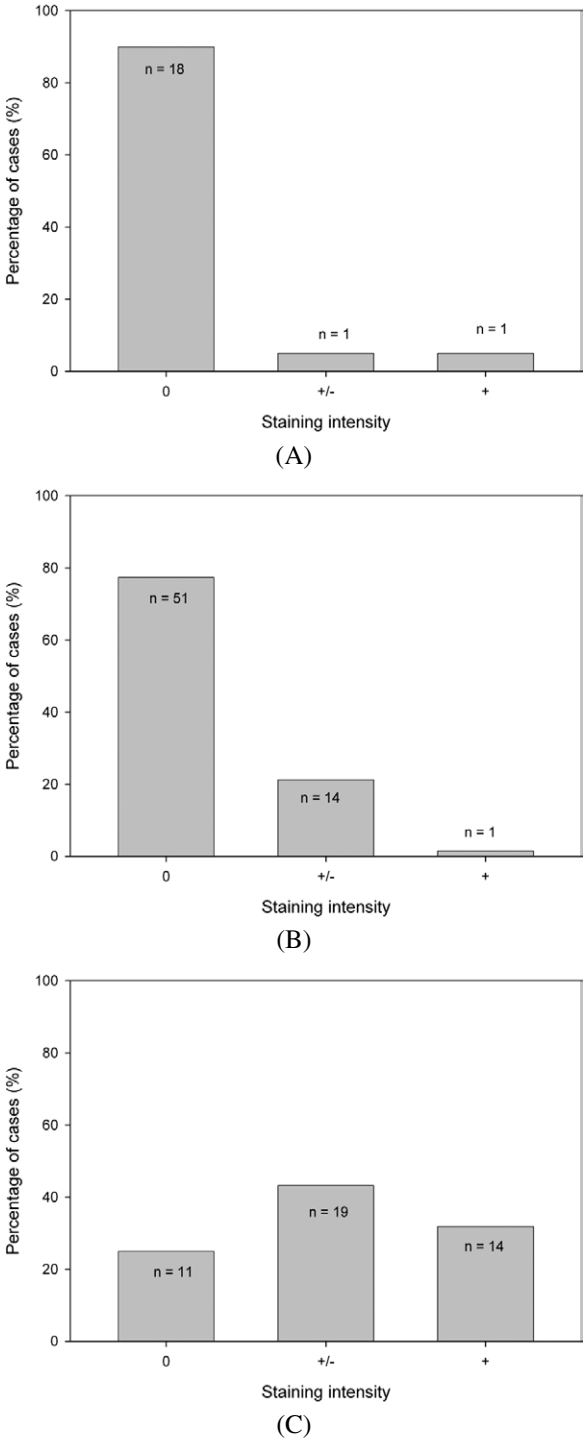


Fig. 5. Cytoplasmic expression of cFLIP in normal colon tissue (A), colorectal adenomas (B) and colorectal carcinomas (C).

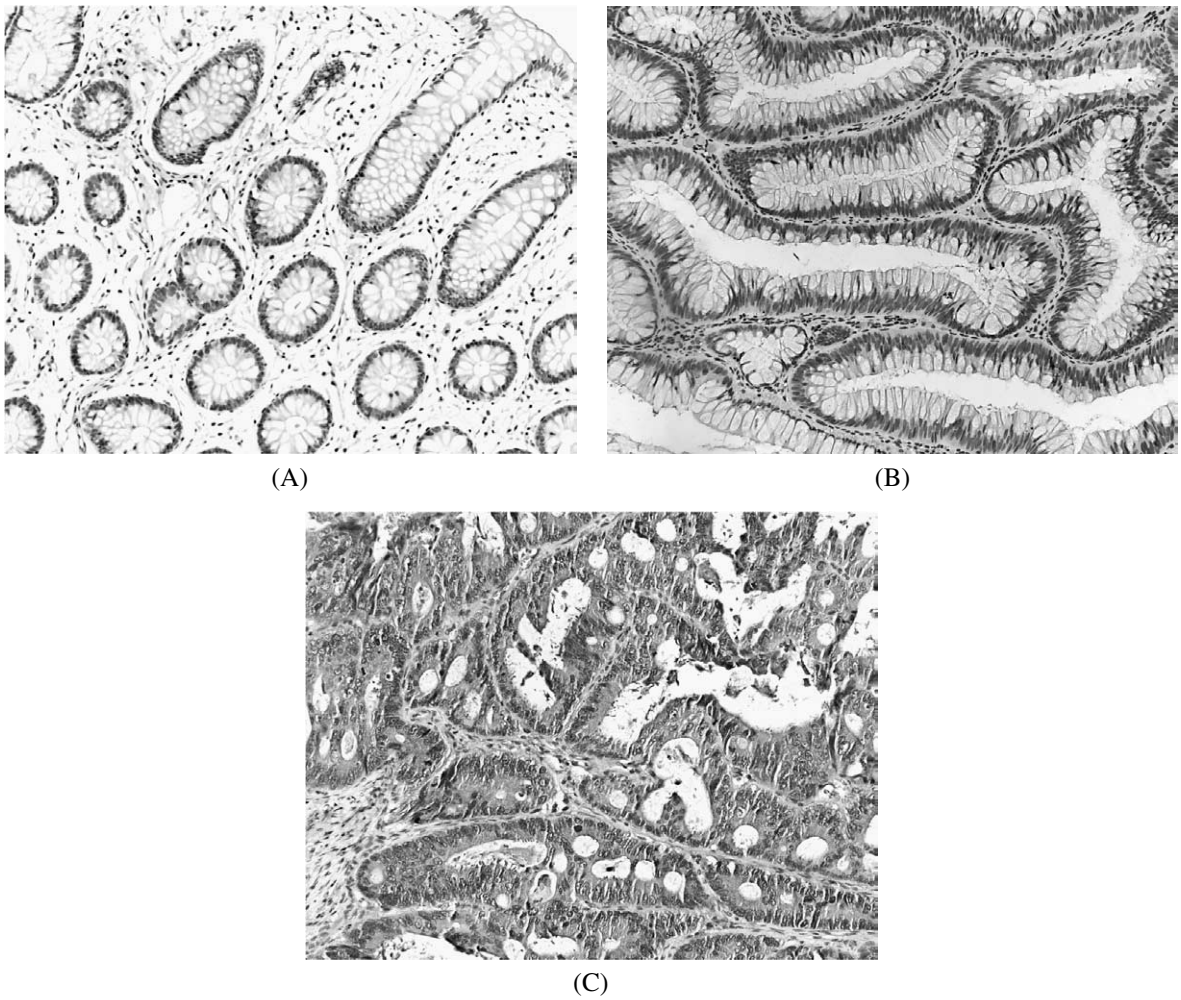


Fig. 6. Representative examples of expression of cFLIP in normal colon tissue, colorectal adenomas and carcinomas. (A) Normal colonic epithelium showing negative staining. (B) Colon adenoma showing negative staining. (C) Colon carcinoma representing strong cytoplasmic staining (+).

Table 2

Immunohistochemical staining for caspase-8, cFLIP, DR4, DR5, apoptosis and proliferation in sporadic (spor) and HNPCC adenomas (ad) and carcinomas (ca). Results are expressed as percentage of moderately or strongly positive cytoplasmic staining (caspase-8 and cFLIP) or median percentage of positive cells (DR4, DR5, apoptosis, proliferation)

	Spor Ad ($n = 33$)	HNPCC Ad ($n = 33$)	Spor Ca ($n = 27$)	HNPCC Ca ($n = 17$)
Caspase-8 (% positive cases)	70	58	59	53
cFLIP (% positive cases)	24	21	78	71
DR4 (%)	40	50	100	100
DR5 (%)	100	100	100	100
Apoptosis (%)	0.4	0.4	1.2	0.8
Proliferation (%)	36	45	70	60

slightly upregulated in carcinomas compared to the adjacent normal tissue [33]. This discrepancy may be due to the fact that we stained normal tissues from patients without any macroscopic or microscopic abnormalities in their colon and not normal looking mucosa adjacent to carcinomas of which we among others have demonstrated that it can not be regarded as truly normal mucosa [25].

The frequent nuclear localization of caspase-8 in both normal and neoplastic colonic epithelium is remarkable, because caspase-8 is thought to be a cytoplasmic protein. This might be explained by post-translational modifications of caspase-8 such as sumoylation that

co-localization of caspase-8 with a known adaptor molecule that can be expressed in the nucleus such as FADD [1,6,37]. Additionally, paranuclear localization of caspase-8 was observed in a substantial number of colorectal tissues. This subcellular localization of caspase-8 has not been described before in colorectal tissue and has never been demonstrated by immunohistochemistry. There are several possible explanations for paranuclear localization of caspase-8. Paranuclear localization could reflect caspase-8 in the mitochondria, which is released upon apoptotic stimulation [34]. More speculative, overexpressed procaspase-8 colocalizes with overexpressed FADD in the Golgi complex [38]. Although caspase-8 expression was higher in colorectal adenomas and carcinomas compared to normal colonic epithelium, further upregulation and/or a shift in cellular localization may be an effective way to potentiate rhTRAIL and agonistic TRAIL receptor antibody induced apoptosis. Caspase-8 expression has been shown to be induced by interferon alpha, interferon gamma and several chemotherapeutic drugs in colon cancer cell lines [14,26,30]. Further research, however is necessary to obtain more insight in the mechanisms and consequences of subcellular localization of caspase-8 since it may influence the availability of caspase-8 for the assembling of the DISC, necessary for apoptosis induction via rhTRAIL or agonistic TRAIL receptor antibodies.

Immunohistochemical studies have indicated an upregulation of cFLIP in cancer tissue in comparison to normal tissue in several tumour types including sporadic colon cancer [12,27,29,36,45]. In the present study, both sporadic adenomas and carcinomas as well as HNPCC adenomas and carcinomas were included to investigate cFLIP expression in relation to colorectal carcinogenesis. We conclude that cFLIP is upregulated in both sporadic and HNPCC colorectal carcinomas in comparison to normal colonic epithelium and adenomas. Previous studies in cell line models have shown that cFLIP mediates resistance to chemotherapy and death receptor induced apoptosis [12,27,29]. In concordance, high cFLIP levels are associated with a poor prognosis [39]. In the present study, we found an association of high cFLIP expression with higher expression of DR4. High DR4 expression has been found to be associated with shorter overall and disease-free survival in colon cancer [41]. Whether the concomitant expression of c-FLIP and DR4 has biological relevance with respect to survival in colon cancer needs to be established.

Since we observed a strong upregulation of cFLIP in cancer tissue, cFLIP may be a potential target for anticancer therapy in combination with rhTRAIL or agonistic TRAIL receptor antibody, especially in those colorectal cancer patients who have high cFLIP levels. NSAIDs can influence the expression of inhibitors of apoptosis such as cFLIP either directly or indirectly by inhibiting NF-κB [18]. Furthermore, PPARγ modulators [22] and a variety of other substances [35] have shown to reduce levels of cFLIP.

This study shows a large variation in expression of caspase-8 and cFLIP in individual adenomas and carcinomas. Several studies imply that the caspase-8/cFLIP ratio is important in determining rhTRAIL sensitivity [13,15,20,28]. Optimizing this ratio by upregulating caspase-8 and/or downregulating cFLIP seems a reasonable approach for optimal apoptosis induction via rhTRAIL. Contradictory to many studies mentioned before suggesting an anti-apoptotic function of cFLIP, some studies state that cFLIP is able to function as an activator of caspase-8 [8,31]. In these cases, downregulation of cFLIP would be unwanted. Future studies are needed to determine the functional relevance of the caspase-8/cFLIP ratio and the caspase-8 regulating functions of cFLIP.

In conclusion, our study demonstrates that both cFLIP and caspase-8 are upregulated during colorectal carcinogenesis. In individual cases there is large variation in expression of caspase-8 and cFLIP. If future studies reveal that the caspase-8/cFLIP ratio is functionally important, upregulation of caspase-8 and drug-induced downregulation of cFLIP may be important approaches in optimizing TRAIL or agonistic TRAIL receptor antibody sensitivity in colorectal cancer.

Acknowledgements

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