

# Fas Ligand Expression in Lynch Syndrome-Associated Colorectal Tumours

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**Abstract** Fas Ligand (FasL) expression by cancer cells may contribute to tumour immune escape via the Fas counterattack against tumour-infiltrating lymphocytes (TILs). Whether this plays a role in colorectal carcinogenesis in Lynch syndrome was examined studying FasL expression, tumour cell apoptosis and number of TILs in colorectal neoplasms from Lynch syndrome patients (50 adenomas, 20 carcinomas) compared with sporadic cases (69 adenomas, 52 carcinomas). FasL expression was observed in 94% of Lynch syndrome adenomas and in all carcinomas. FasL expression patterns and apoptotic indices were similar in Lynch syndrome-associated neoplasms and sporadic cases. The number of TILs was higher in Lynch syndrome neoplasms than in sporadic cases. There were no correlations between FasL expression and tumour cell apoptosis or number of TILs in Lynch syndrome-associated neoplasms. So, FasL expression is an early event in Lynch syndrome and sporadic colorectal carcinogenesis, but not related to TIL number. Taken together, our data do not support a role for the Fas counterattack in colorectal carcinogenesis in Lynch syndrome.

**Keywords** Apoptosis · Colorectal cancer · Counterattack · Fas ligand · HNPCC · Lynch syndrome

## Abbreviations

FasL	Fas Ligand
TIL	tumour-infiltrating lymphocytes
LS	Lynch syndrome
HNPCC	hereditary nonpolyposis colorectal cancer
MSI	microsatellite instability
MSS	microsatellite stable
TNF	tumour necrosis factor
WHO	World Health Organization

## Introduction

Lynch syndrome (LS), or hereditary nonpolyposis colorectal cancer (HNPCC), is characterised by an autosomal dominant inheritance of early-onset colorectal cancer and an increased risk of other cancers, in particular cancer of the endometrium [1]. LS is caused by germline mutations in a mismatch repair gene, mostly hMLH1, hMSH2 and hMSH6 [1]. These genes encode proteins involved in DNA repair. Failure of the DNA mismatch repair machinery to repair errors occurring during DNA replication leads to increased length variation of simple, repetitive sequences distributed throughout the genome, so-called microsatellite instability (MSI). An international set of markers is developed to test for MSI. Tumours are scored MSI-high if at least 30% of the markers show instability, MSI-low if less than 30% show instability, or MS-stable (MSS) if none of the markers shows instability [2]. The MSI-H phenotype is found in up to 92% of colorectal cancers associated with LS and in 10–15% of sporadic colorectal cancers [1].

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Dysfunction of one of the mismatch repair genes results in a rapid accumulation of genetic alterations in susceptible genes, including genes involved in growth suppression, apoptosis and signal transduction [1]. These alterations contribute to an accelerated adenoma–carcinoma sequence in LS compared to sporadic cancer. This is illustrated by the relative frequent occurrence of interval cancers between regular surveillance colonoscopies [3, 4]. In addition, LS adenomas frequently harbour a villous component and high-grade dysplasia, both markers of increased cancer risk [5, 6]. Among the genes that are targeted in the setting of MSI are *BAX* and *caspase 5*, suggesting that disturbances in apoptosis regulation may be important as a mechanism behind the accelerated colorectal carcinogenesis in LS [7, 8].

Apoptosis can be either initiated by the cell itself or by certain external stimuli. These external stimuli may induce apoptosis by targeting one of two pathways [7]. The ‘extrinsic’ pathway is initiated by triggering cell death receptors on the cell surface, leading to activation of the intracellular apoptotic machinery. The ‘intrinsic’ pathway of apoptosis is initiated via the mitochondria by cellular stress, such as chemotherapeutic drugs and radiation. Fas is a protein of the tumour necrosis factor (TNF) receptor superfamily, known to induce apoptosis through the extrinsic pathway in sensitive cells by binding to its natural ligand, Fas Ligand (FasL). In the normal human colon, all epithelial cells express Fas, but not FasL [9]. In contrast, many colorectal carcinomas and some adenomas show FasL expression [10–13]. It has been hypothesized that FasL expression in tumour cells might enable these cells to evade immune destruction by inducing apoptosis in tumour-infiltrating lymphocytes (TILs), a concept which has become known as the Fas counter-attack [14]. In a previous study, FasL expression was reported to be higher in MSI-H colorectal cancers compared to MSI-L and MSS cancers [15]. To date, FasL expression has not been examined in colorectal tumours from LS patients. We hypothesized that a diminished Fas counterattack between LS and sporadic colorectal tumours play a role as a mechanism to explain the difference in carcinogenesis. Therefore, FasL expression, tumour cell apoptosis and number of TILs in colorectal neoplasms from LS patients in comparison with sporadic cases were studied.

## Materials and Methods

### Patients

**Lynch Syndrome** At the University Medical Centre Groningen, patients with a germline mismatch repair gene mutation and/or fulfilling the Amsterdam II criteria undergo colonoscopy every 1–2years starting at the age of 25years, according to the current

surveillance guidelines [1]. All colorectal adenomas and carcinomas removed in these patients over a period of 23years with sufficient material available to allow serial sectioning for immunohistochemistry were selected for this study. In total, 20 carcinomas were examined, 17 from proven mutation carriers (8 *hMLH1*, 7 *hMSH2*, 2 *hMSH6*), as determined by sequencing of genomic DNA, and three from patients with a family history fulfilling the Amsterdam II criteria. Fifty adenomas, 23 from proven mutation carriers, 27 from patients with a family history fulfilling the Amsterdam II criteria, were analyzed.

**Sporadic cases** The control group consisted of sporadic carcinomas from a previously reported cohort of 500 patients with stage III colon cancer [16]. From this cohort, in which MSI analysis has been performed, we selected all MSI-H tumours ( $n = 26$ ) and randomly 26 MSS tumours. MSI-H tumours were included to determine possible differences between sporadic MSI-H tumours and LS-associated tumours which also are characterized by the MSI-H phenotype. None of the 26 patients with a MSI-H tumour was known with a MMR gene mutation or had a family history fulfilling the Amsterdam II criteria, so these were considered truly sporadic cases. Sporadic adenomas of which sufficient material was available to allow serial sectioning for immunohistochemical staining were selected from a previously described series [17] as control adenomas ( $n = 69$ ).

Histologic classifications were carried out on haematoxylin and eosin stained slides. The morphological classification of the carcinomas and adenomas was conducted according to the World Health Organization (WHO) criteria [18]. Adenocarcinomas were graded into well, moderately and poorly differentiated. For statistical purposes, tumours with good and moderate differentiation were compared with tumours with poor differentiation. From adenomas, the circumferential size was measured, and the severity of dysplasia was expressed as low- or high-grade. Adenomas were classified as tubular, tubulovillous or villous [18]. For statistical purposes, tubulovillous and villous adenomas were taken together.

### Immunohistochemistry

For immunohistochemical staining for FasL, sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol and finally in phosphate-buffered saline. For antigen retrieval, slides were subjected to steam-heat for 30min, using a commercially available steamer. After washing with PBS, sections were incubated with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA), followed by incubation for 1h at room temperature with a monoclonal anti-FasL antibody (MAb33, Transduction Laboratories, Lexington, KY, USA)

in a 1:300 dilution. After washing with PBS, slides were incubated with a 1:300 dilution of a biotinylated rabbit-anti-goat antibody (DAKO, Glostrup, Denmark), followed by addition of streptavidin-conjugated peroxidase (DAKO). Peroxidase activity was visualized with diaminobenzidine. Counterstaining was performed with haematoxylin. As negative controls, slides were immunostained in the absence of the primary antibody, and in these cases no immunostaining was detected. Evaluation of staining was performed by two independent investigators, without knowledge of the histopathological data. The percentage of positive cells was estimated and scored on a semiquantitative scale as (1) 0–25%, (2) 26–50%, (3) 51–75%, (4) 76–100%, in accordance with others [14, 15].

Apoptosis in epithelial cells was determined by immunohistochemistry with the murine monoclonal antibody M30 (Boehringer Mannheim, Mannheim, Germany), which reacts with a cleavage product of cytokeratin 18, released by activated caspase [19]. The immunoreactivity of the M30 antibodies is confined to the cytoplasm of apoptotic cells and is present during early apoptosis [20]. M30 staining has been shown to correlate well with the gold standard of morphological criteria [17]. Staining procedures for M30 were performed as described before [17]. Apoptosis was assessed in at least 1,000 tumour cells and expressed as a percentage of the total number of cells counted (apoptotic index). M30 positivity was identified as brown cytoplasmic staining.

The presence of TILs was assessed using a monoclonal antibody against CD8. Antigen retrieval was performed by immersing slides in 1mM EDTA buffer (pH 8.0) and heating in a microwave oven for 8min. The primary antibody (DAKO) was applied in a 1:50 solution for 1h followed by a secondary rabbit-anti-mouse antibody conjugated with peroxidase (DAKO) and a tertiary goat-anti-rabbit peroxidase-conjugated antibody.

For each tumour, the number of intraepithelial CD8+ cells was determined in ten high power fields (400 ×

magnification), in accordance with other studies [15, 21, 22]. TILs were defined as intraepithelial rather than peritumoural or stromal lymphocytes.

### Statistical Analysis

Statistical significance of differences between LS and sporadic cases was tested using the Mann-Whitney test for continuous variables and chi-square tests for discontinuous variables. To test the relationship between increasing FasL expression and continuous variables as apoptotic index and CD8 counts, the Wilcoxon rank sum test was used. P-values <0.05 were considered significant. SPSS for Windows software (SPSS Inc., Chicago, IL, USA) was used.

## Results

### Tumour Characteristics

Patient and tumour characteristics are summarized in Table 1. Inherent to the patient groups, several differences were observed between groups. Mean age of LS patients at the time of adenoma removal was lower than for those with sporadic disease ( $p < 0.0001$ ). LS-associated adenomas were smaller ( $p = 0.002$ ), more often containing high-grade dysplasia ( $p < 0.01$ ) and more often proximally localized ( $p < 0.05$ ), i.e. proximal to the splenic flexure, compared with their sporadic counterparts. LS-associated carcinomas and MSI-H sporadic carcinomas were more often localized in the proximal colon compared to sporadic MSS cases, ( $p < 0.001$ ).

### FasL Expression in LS and Sporadic Colorectal Neoplasms

Staining results are summarized in Table 2. FasL expression in epithelial cells was seen in 47/50 LS-associated adenomas and 64/69 sporadic adenomas (not significantly different). FasL expression was cytoplasmic and generally

**Table 1** Patient and tumour characteristics

	Adenomas		Carcinomas		
	LS	Sporadic	LS	Sporadic MSS	Sporadic MSI-H
n	50	69	20	26	26
Male (%)	48	30	85	73	69
Age in yrs (mean, range)	49 (30–67)	66 (28–89)	54 (31–77)	59 (40–75)	60 (26–76)
Size in mm (mean, range)	6.9 (2–30)	10.2 (3–45)			
Proximal localisation (%)	42	26	65	39	81
Pathology					
Tubular (%)	54	54			
High-grade dysplasia (%)	46	26			
Poor differentiation (%)			25	12	15

**Table 2** FasL expression, epithelial apoptosis (expressed as apoptotic index AI) and TILs in LS and sporadic colorectal adenomas and carcinomas

n	Adenomas		Carcinomas		
	LS	Sporadic	LS	Sporadic MSS	Sporadic MSI-H
	50	69	20	26	26
n FasL + (%)	47 (94)	64 (93)	20 (100)	26 (100)	26 (100)
0–25%	17 (34)	35 (51)	3 (15)	2 (8)	4 (15)
26–50%	4 (8)	9 (13)	1 (5)	1 (4)	2 (8)
51–75%	9 (18)	10 (14)	6 (30)	1 (4)	3 (12)
76–100%	20 (40)	15 (22)	10 (50)	22 (84) <sup>#</sup>	17 (65) <sup>#</sup>
AI (mean ± SEM)	1.1±0.22	0.4±0.05	1.5±0.38 <sup>¶</sup>	1.0±0.19*	1.1±0.28*
TILs (mean ± SEM)	6.6±0.96	3.9±0.38 <sup>###</sup>	18.6±4.84 <sup>¶¶</sup>	6.6±1.27	23.9±6.60 <sup>**</sup>

<sup>#</sup> $P < 0.001$  for sporadic carcinomas vs sporadic adenomas; <sup>¶</sup> $p < 0.05$  for LS carcinomas vs LS adenomas; \*  $p < 0.001$  for sporadic carcinomas vs sporadic adenomas; <sup>###</sup> $p < 0.05$  for LS adenomas vs sporadic adenomas; <sup>¶¶</sup> $p = 0.01$  for LS carcinomas versus sporadic MSS carcinomas and  $< 0.001$  for LS carcinomas vs LS adenomas; \*\*  $p = 0.02$  for sporadic MSI-H vs sporadic MSS carcinomas.

heterogeneously distributed within adenomatous tissue. In both groups, approximately half of the samples displayed expression in more than 50% of tumour cells. The extent of FasL positivity was not related to age, gender, adenoma size, location, degree of dysplasia or villous architecture, neither in LS-associated, nor in sporadic cases.

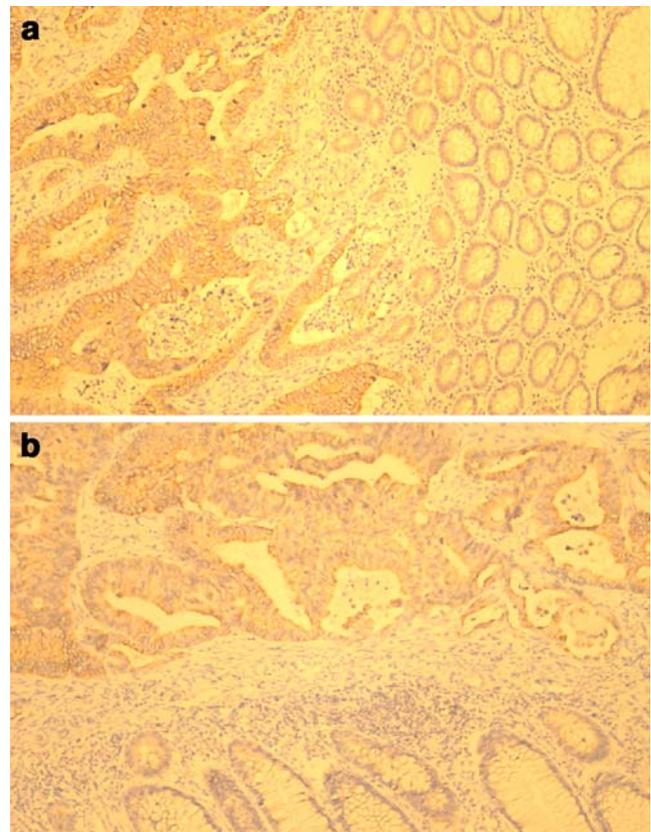
In carcinomas, FasL expression was present in all samples investigated. FasL expression was generally heterogeneously distributed. Percentages of positively staining cells were similar in LS, MSS and MSI-H sporadic tumours. In all three groups, approximately 80% of cases demonstrated FasL expression in more than 50% of cells. FasL expression was not related to age, gender, tumour location or degree of differentiation. When comparing FasL expression in adenomas and carcinomas, it was similar in LS-associated neoplasms (Fig. 1). In sporadic cases, percentages of positively staining cells were higher in carcinomas than in adenomas ( $p < 0.001$ ). This was true for MSS carcinomas as well as for MSI-H carcinomas.

#### Tumour Cell Apoptosis and Number of TILs in LS and Sporadic Colorectal Neoplasms

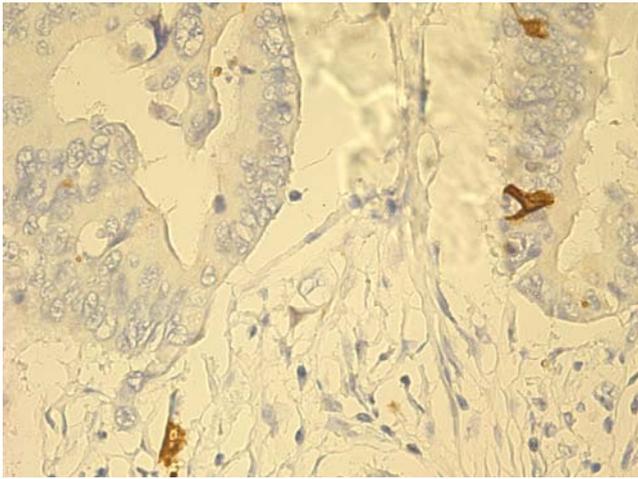
The level of tumour cell apoptosis, assessed by M30 immunoreactivity (Fig. 2), was similar between LS and sporadic adenomas, although there was a non-significant trend towards higher levels in LS adenomas ( $p = 0.08$ ). The same trend was observed in LS carcinomas, with apoptotic indices being slightly higher than in sporadic cancers ( $p = 0.07$ ). When comparing carcinomas with adenomas, mean apoptotic indices were higher in carcinomas, both in LS ( $p < 0.05$ ) and in sporadic cases ( $p < 0.001$ ).

The number of TILs, assessed by CD8 positivity, was higher in LS adenomas compared with sporadic adenomas ( $p < 0.05$ , Fig. 3). Within the group of LS adenomas, the mean number of TILs was higher in adenomas with high-

grade dysplasia compared with those with low-grade dysplasia ( $8.8 \pm 1.8$  vs  $4.7 \pm 0.8$ ,  $p < 0.001$ ). The mean number of TILs was also higher in adenomas with proximal location compared with those with distal location ( $9.4 \pm 1.9$  vs  $4.7 \pm 0.8$ ,  $p < 0.001$ ). The mean number of TILs was not correlated with adenoma size or the presence of villous



**Fig. 1** FasL expression (brown) in colorectal carcinomas with adjacent normal mucosa. A, Cancer associated with Lynch syndrome; B, Sporadic cancer. In both cases, stronger expression of FasL in tumour cells was seen compared to normal cells (magnification  $\times 100$ )



**Fig. 2** Example of M30 immunoreactivity in colon cancer, showing three cells with brown cytoplasmic staining (magnification  $\times 100$ )

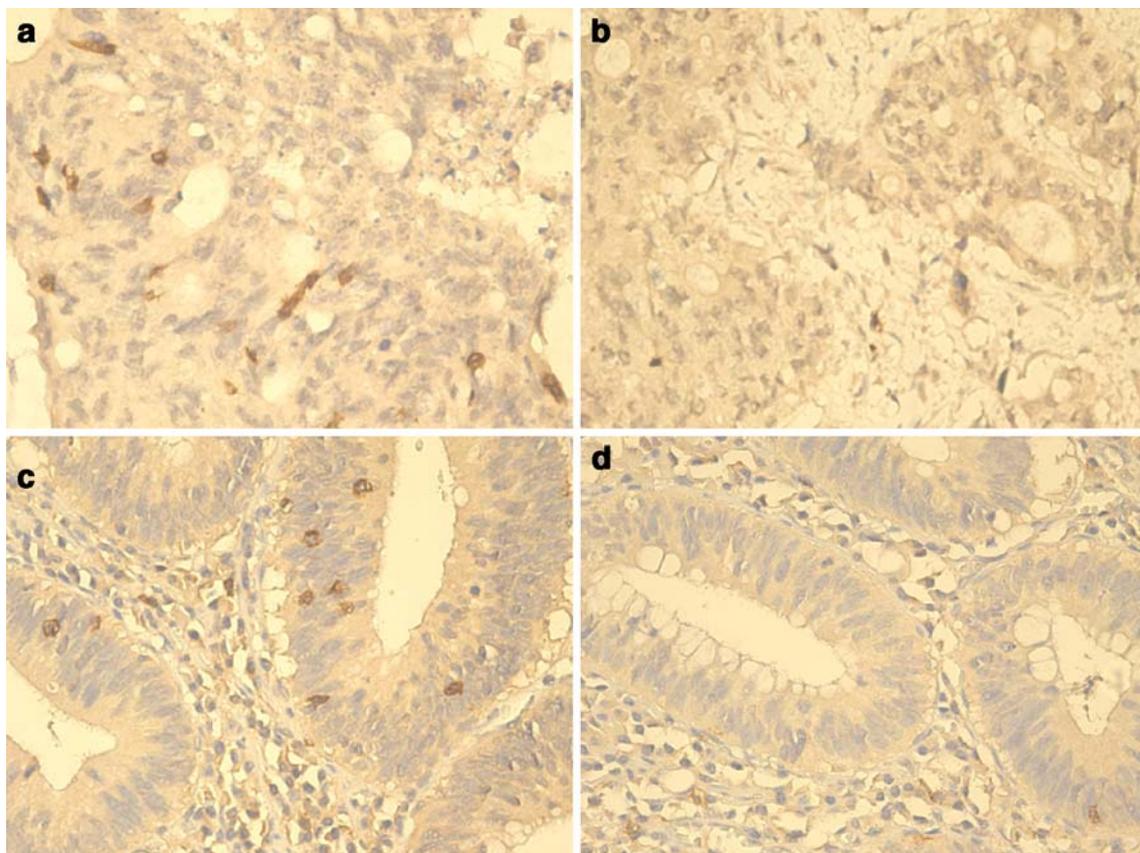
architecture in LS adenomas. In sporadic adenomas, the number of TILs was not correlated with adenoma size, degree of dysplasia, architecture or location.

In carcinomas, the number of TILs was similar in LS-associated carcinomas and MSI-H sporadic cancers but

higher in both these groups than in MSS sporadic tumours ( $p = 0.01$  and  $0.02$  respectively, Fig. 3). In LS-associated tumours, TIL density was higher in carcinomas than in adenomas ( $p < 0.001$ ). There was no correlation between the number of TILs and tumour location or degree of differentiation. This was the case in LS-associated cancers as well as in sporadic cancers.

#### Relationship Between FasL Expression, Tumour Cell Apoptosis and TILs

To search support for the FasL counterattack hypothesis, we determined whether higher FasL expression in neoplasms was associated with lower levels of tumour cell apoptosis or reduced TIL densities (Table 3). For this analysis, adenomas and carcinomas from LS patients and were taken together as well as adenomas and MSS carcinomas from sporadic origin. In LS-associated cases, there were no positive or negative correlations between the percentage of FasL positivity on the one hand and levels of tumour cell apoptosis or number of TILs on the other. There was also no correlation between the degree of tumour cell apoptosis and the number of TILs. In contrast, in sporadic cases, there



**Fig. 3** Staining of tumour-infiltrating lymphocytes (TILs) by CD8 immunoreactivity (brown). A, Lynch syndrome associated cancer; B, Sporadic MSS cancer; C, Lynch syndrome associated adenoma; D,

Sporadic adenoma. The number of TILs is higher in cases associated with Lynch syndrome compared to sporadic ones (magnification  $\times 400$ )

**Table 3** Relationship between FasL expression, epithelial apoptosis (expressed as apoptotic index AI, mean  $\pm$  SEM) and TILs (mean  $\pm$  SEM) in LS and sporadic colorectal neoplasms

	LS adenomas and carcinomas			Sporadic adenomas and MSS carcinomas		
	n	AI	TILs	n	AI	TILs
FasL expression						
0–25%	20	0.99 $\pm$ 0.25	6.7 $\pm$ 1.5	39	0.43 $\pm$ 0.07	3.4 $\pm$ 0.4
26–50%	5	1.84 $\pm$ 1.28	10.7 $\pm$ 4.7	11	0.68 $\pm$ 0.19	5.3 $\pm$ 1.9
51–75%	17	1.45 $\pm$ 0.48	11.9 $\pm$ 4.8	13	0.47 $\pm$ 0.09	3.9 $\pm$ 0.8
76–100%	28	1.13 $\pm$ 0.27	11.1 $\pm$ 2.7	32	0.82 $\pm$ 0.15	6.3 $\pm$ 1.0
Total	70	1.22 $\pm$ 0.19	10.0 $\pm$ 1.65	95	0.59 $\pm$ 0.07	4.7 $\pm$ 0.5

was a positive correlation between higher FasL expression and degree of tumour cell apoptosis (Spearman's rho correlation coefficient 0.27,  $p=0.008$ ). There was no correlation between FasL expression and the number of TILs, nor between the degree of tumour cell apoptosis and the number of TILs in sporadic neoplasms.

## Discussion

In this study, we sought to determine whether the Fas counterattack plays a role in the accelerated colorectal carcinogenesis in LS patients compared to sporadic disease. We found no support for such a mechanism, as FasL expression was similar between LS and sporadic tumours. In addition, there were no correlations between the percentages of FasL positivity and the number of TILs. A novel finding from our study was that LS-associated adenomas, in addition to LS-associated carcinomas, are also characterized by higher numbers of TILs compared to sporadic cases.

Colorectal carcinogenesis in LS is believed to be accelerated when compared with sporadic cases. This is illustrated by the relative frequent occurrence of interval cancers between regular surveillance colonoscopies. In addition, LS-associated adenomas frequently harbour villous components and high-grade dysplasia, which both are markers of increased cancer risk. Differences between LS and sporadic colorectal neoplasms have been extensively studied, demonstrating differences in molecular genetic alterations [23–26] and histopathological characteristics [6, 15, 27, 28]. The accelerated carcinogenesis in LS does not seem to be reflected by a difference in degree of apoptosis with sporadic carcinogenesis [29, 30], although several differences in expression of apoptosis-regulating proteins have been found [30, 31, 32]. To our knowledge, FasL expression has not been studied before in LS-associated neoplasms. Our results indicate similar expression patterns of FasL in LS and sporadic neoplasms. So, FasL expression seems to occur early in colorectal carcinogenesis, irrespective of the sporadic or hereditary origin of the lesions.

We found that FasL expression was more prevalent in carcinomas than in adenomas, consistent with other reports [10–13]. It was recently demonstrated that MSI-H tumours were associated with a reduced frequency of FasL expression relative to non-MSI-H tumours [33]. In our study, a similar trend was seen although this did not reach statistical significance.

Infiltration of lymphocytes is a prominent feature of MSI-H tumours, including LS-associated colorectal cancer [21, 22, 27, 34]. This was confirmed in the present report. In this study, we show for the first time that LS-associated adenomas display the same feature. Moreover, we found that the mean number of TILs was higher in LS adenomas with high-grade dysplasia compared with those with low-grade dysplasia. Only few studies are available that have evaluated the degree of infiltration with lymphocytes in adenomas [35–37]. From these studies, it appears that the number of TILs increases in the course of the adenoma-carcinoma sequence, which is corresponding to our results. It has also been shown that the number of TILs is an independent prognostic factor in colorectal cancer [38–40]. As LS-associated colorectal cancer and sporadic MSI-H colorectal cancer are associated with better survival rates than sporadic cases [1], it has been suggested that the presence of a cytotoxic anti-tumour immune response may at least in part contribute to the survival advantage described in these patients [34, 38]. Whether TILs in LS-associated and MSI-H sporadic tumours are really activated cytotoxic T-cells remains to be demonstrated.

The aim of our study was to determine whether higher FasL expression in neoplasms was associated with lower levels of tumour cell apoptosis or increased levels of apoptotic TILs, reflected by reduced TIL density. We found no such correlations in LS-associated neoplasms. In sporadic cases, a positive rather than a negative correlation between FasL expression and tumour cell apoptosis was found. This finding may be a consequence of elevated FasL levels triggering tumour cell apoptosis in a manner similar to autocrine suicide of activated T-cells [41]. Our findings are in contrast with those from Houston et al, who did not find a correlation between FasL expression and degree of

tumour cell apoptosis [42]. It must be noted that their series consisted of only ten colorectal tumours, which may explain the absence of a correlation in their study. The same research group recently found a non-significant negative association between intensity of FasL staining and number of TILs in a series of 91 colon carcinomas [43]. Two studies reported a positive correlation between FasL expression and apoptosis in TILs [43, 44], whereas we did not find an association between FasL expression and TIL density. The background for the discrepancy between these data and ours is unclear. A possible explanation may lie in the fact that in the other studies TUNEL and ISNT were used to assess TIL apoptosis. Both these techniques have poor specificity for apoptosis and their use is currently discouraged [7, 45].

The question whether the Fas counterattack plays a role at all in colorectal carcinogenesis is surrounded with controversy [46, 47]. The idea behind the concept is that FasL expression by tumour cells may enable those cells to kill anti-tumour immune effector T-cells that infiltrate the tumour. Many tumours and tumour cell lines of varying histologic origin express FasL [48]. Several functional studies have shown that FasL positive colon tumour cells can induce apoptosis of Fas-sensitive lymphocytes *in vitro* [49, 50]. In addition, inhibition of FasL expression reduced tumour development and growth in a xenograft experiment [51]. There are however also experimental data which seriously question the concept of the Fas counterattack. In a replication study, FasL-expressing colon cancer cells did not induce apoptosis in Fas-sensitive target cells [52]. In a gene transfer experiment, Fas resistant colon cancer cells, subcutaneously injected into mice, rapidly regressed upon transfection with FasL [53]. In another experiment, cytotoxic T-cells were not sensitive to FasL displayed on the surface of antigen-presenting cells [54]. Taken together, the role of the Fas counterattack in colorectal carcinogenesis is at least questionable [48].

In conclusion, we observed upregulation of FasL during colorectal carcinogenesis, both in LS-associated and in sporadic cases, without a correlation with the number of TILs. Our data do not support an important role for the Fas counterattack as a mechanism behind the difference between colorectal carcinogenesis in LS-associated and sporadic cases.

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