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ABOUT COVER

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Retrospective Study

Expression of Notch pathway components (Numb, Itch, and Siah-1) in colorectal tumors: A clinicopathological study

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Abstract

BACKGROUND

The role of the Notch pathway in carcinogenesis and tumor progression has been demonstrated in many organs, including the colon. Accordingly, studies aimed at developing therapies targeting this pathway in various cancers require the identification of several factors that may play a role in regulating Notch-1 expression. Although Numb, Itch, and seven in absentia homolog-1 (Siah-1) have been shown to contribute to the regulation of Notch signaling, their role in colorectal carcinogenesis and tumor progression has not been fully elucidated to date.

AIM

To evaluate Numb, Itch, and Siah-1 expression in colorectal tumors to clarify their relationship with Notch-1 expression and their role in carcinogenesis and tumor behavior.

METHODS

Expression of Notch-1, Numb, Itch, and Siah-1 was investigated in 50 colorectal carcinomas, 30 adenomas, and 20 healthy colonic tissues by immunohistochemistry and quantitative real-time polymerase chain reaction (PCR) analyses.

RESULTS

In contrast to Notch-1, which is expressed at higher levels in tumor tissues and adenomas, expression of Numb, Itch, and Siah-1 was stronger and more frequent in normal mucosa ($P < 0.01$). There was a positive correlation between Notch-1

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Patients were not required to give informed consent to the study because the analysis used anonymous data that were obtained after each patient agreed to treatment by written consent.

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expression and high histological grade, the presence of lymph node metastasis, and advanced-stage tumors, whereas expression of Numb, Itch, and Siah-1 was absent or reduced in tumors with these clinicopathological parameters ($P < 0.05$). In survival analysis, expression of Notch was related to poor prognosis but that of Numb, Itch, and Siah-1 correlated with improved survival ($P < 0.05$). Multivariate analysis revealed Notch-1 expression and loss of Numb expression to be independent prognostic parameters together with lymph node metastasis ($P < 0.05$).

CONCLUSION

Our findings support the role of Notch-1 in colorectal carcinoma and indicate that loss of Numb, Itch, and Siah-1 expression is associated with carcinogenesis. Our data also suggest that these three proteins might be involved in the Notch-1 pathway during colorectal carcinoma (CRC) progression and might play an essential role in approaches targeting Notch as novel molecular therapies for CRC.

Key words: Colorectal adenomas; Colorectal cancer; Notch-1; Numb; Itch; Siah-1

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Core tip: This report describes a preliminary study to investigate Numb, Itch, and Siah-1 expression in colorectal tumors and to evaluate their relationship with Notch-1 expression and their roles in oncogenesis and prognosis. Our findings confirm the role of Notch-1 in colorectal carcinoma (CRC) and emphasize that loss of Numb, Itch, and Siah-1 expression is associated with carcinogenesis. Our data also indicate that Numb, Itch, and Siah-1 might be involved in the Notch-1 signaling pathway during CRC progression. Accordingly, these three proteins might be key in possible therapies targeting Notch-1 to treat colorectal carcinoma.

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common cancers worldwide and, despite significant advances in treatment, ranks third in cancer-related mortality and morbidity^[1,2]. Although curative surgery is possible in some cases, the fact that one-fifth of patients have metastatic disease at the time of diagnosis and sixty percent of patients undergoing surgical resection experience recurrence and metastasis emphasizes the need to identify new strategies for the determination of CRC tumor behavior and treatment^[3,4]. In this context, numerous studies have been conducted to elucidate signaling pathways that contribute to colorectal tumor development and progression and to use them as potential therapeutic targets, mainly to prevent aggressive tumor behavior and treatment resistance^[5-7].

Although Notch signaling has traditionally been known for its role in determining cell fate, its identification as one of the most active pathways in cancer cells renders it a potential therapeutic target in CRC^[8]. In recent years, the role of the Notch signaling pathway in gastrointestinal development and normal gut homeostasis has been thoroughly demonstrated^[9]. However, differences in the findings of studies on its use in CRC as a therapeutic target exist, which necessitates an understanding of the roles of other components in the Notch pathway^[10-12].

Numb is a conserved evolutionary protein that antagonizes Notch signaling activities in regulating the balance between proliferation and differentiation in development and homeostasis^[13]. Ubiquitination of the membrane-bound Notch receptor Notch-1 and subsequent attenuation of its activity are the basis of this molecular mechanism, which involves the binding of Numb to the E3 ubiquitin-ligase

Itch. Numb and Itch function in concert to promote ubiquitination of the Notch receptor before activation^[14]. Furthermore, the networking of Numb into E3 ubiquitin ligase-based circuitries in the regulation of Notch signaling involves other E3 ubiquitin ligases, including seven in Siah-1. Siah-1 has an inhibitory effect on Numb but a stimulatory effect on Notch-1^[15]. In addition, data obtained in recent years indicate that Siah-1 may also play a role in the degradation of β -catenin^[16]. Numb is expressed in many adult tissues, such as the breast, lung, testis, and salivary gland; it is detected in the villus crypt axis in the mouse intestine and participates in goblet cell transformation by inhibiting the Notch pathway in intestinal epithelial cells^[17]. Numb also increases mucin secretion in intestinal cells and helps to maintain the integrity of the epithelial barrier. In recent years, there has been evidence that aggressive tumor behavior in various solid tumors is associated with loss of Numb expression^[18].

Some experimental studies have suggested that loss of Numb expression is associated with tumor behavior in CRC^[19,20]. Regardless, the relationship between expression of Numb and clinicopathological parameters, survival, and Notch-1 expression in patients with colorectal carcinoma remains to be investigated. Similarly, the roles of Itch and Siah-1 expression in colorectal carcinogenesis and their relationship to tumor behavior, survival, Notch-1 expression, and Numb expression in CRC have not been evaluated.

Therefore, this preliminary study was undertaken to investigate expression of Numb, Itch, and Siah-1 in colorectal tumors to clarify their relationship with Notch-1 expression and their role in carcinogenesis and tumor behavior.

MATERIALS AND METHODS

Study design

Expression of Notch-1, Numb, Itch, and Siah-1 was evaluated in 50 samples of CRC diagnosed as adenocarcinoma, not otherwise specified (NOS), in 30 adenomas (10 tubular, 10 tubulovillous and 10 villous), and in 20 healthy colonic tissues diagnosed at the Department of Pathology. The ethics committee of Akdeniz University Medical School approved the study.

Immunohistochemistry

Tissue sections from surgical specimens fixed in 40 g/L formaldehyde and embedded in paraffin were reviewed, and representative tissue blocks were selected. Slides were immunostained with anti-Notch-1 (Dilution 1/25), anti-Numb (Dilution 1/25), anti-Itch (Dilution 1/100), and anti-Siah-1 (Dilution 1/100) monoclonal antibodies (Thermo Fisher Scientific, United States) using the avidin-biotin immunoperoxidase technique.

Each sample was scored on a scale of 0-4, as follows: 0, negative; 1, positive staining in 1%-25% of tumor cells; 2, positive staining in 26%-50% of tumor cells; 3, positive staining in 51%-75% of tumor cells; and 4, positive staining in 76%-100% of tumor cells. The intensity of immunostaining was determined as follows: 0, negative staining; 1, weakly positive staining; 2, moderately positive staining; and 3, strongly positive staining. The distribution of staining was also evaluated.

Quantitative real-time PCR analysis

PCR analysis was performed using Solaris qPCR Gene Expression Assay for Human Gene Notch-1, Numb, Itch, Siah-1, and GAPDH Kits (Thermo Fisher Scientific). In particular, reverse transcription of total RNA was performed using Maxima First Strand complementary DNA (cDNA) Synthesis Kit for qPCR (Thermo Fisher Scientific). Data were analyzed by the C_T method.

RNA isolation

RNA extraction was performed according to the RNeasy formalin-fixed, paraffin-embedded (FFPE) Kit protocol (Qiagen GmbH, Hilden, Germany). Briefly, 10 μ m thick sections were cut from formalin-fixed, paraffin-embedded blocks, and tumor areas were collected from the sections and transferred to a microcentrifuge tube. Deparaffinization was performed by adding 1 mL xylene and incubating for 10 min at room temperature. This step was repeated twice, after which 1 mL absolute ethanol was added. The deparaffinized tissue sections were incubated in lysis buffer containing proteinase K at 56 °C for 15 min and then 80 °C for 15 min. This step was followed by DNase treatment to eliminate all genomic DNA, including very small DNA fragments that are often present in FFPE samples. Next, ethanol was added to

provide binding conditions for RNA; lysate was applied to an RNeasy MinElute spin column, with the membrane binding total RNA. The RNA was eluted in a minimum V of 14 μ L of RNase-free water. The c of the isolated RNA and the ratio of absorbance at 260 nm to 280 nm (A260/A280 ratio) were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Montchanin, DE, United States).

First-strand cDNA synthesis

Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Cat # K1641) was used according to the manufacturer's instructions. Five micrograms of RNA was transcribed with random primers. Briefly, 5X reaction mix, maximum enzyme mix, nuclease-free water, and template RNA were mixed and incubated for 10 min at 25°C followed by 15 min at 50°C; the reaction was terminated by heating at 85°C for 5 min. The product of the first-strand cDNA synthesis was stored at -20°C until qPCR.

qPCR

Gene expression analysis was performed using Solaris qPCR Gene Expression Assay (Thermo Fisher Scientific) for Human Gene Notch-1 (Cat # AX-007771-00-0100), Numb (Cat # AX-015902-00-100), Itch (Cat # AX-007196-00-0100), Siah-1 (Cat # AX-012598-00-0100), and GAPDH (Cat # AX-004253-00-0100). The probes were labeled with FAM-MGM dyes. Briefly, the reaction mixture consisted of 12.5 μ L of the 2X Solaris qPCR Master Mix, 1.25 μ L of the 20X Solaris Primer/Probe set, and PCR grade water. Five microliters of cDNA was used as a template for PCR in a final V of 25 μ L. The master mix contains all components for real-time PCR: (1) Thermo-Start DNA polymerase, a hot-start version of Thermoprime DNA polymerase; (2) dNTPs, with dTTP replacing dUTP to maximize amplification efficiency; (3) Proprietary reaction buffer, optimized to work with Solaris primer/probe assays; and (4) ROX. The cycle conditions were set as follows: preincubation step for 15 min at 95°C for activation of the Thermo-Start DNA polymerase (1 cycle) followed by 40 cycles each of 15 s at 95°C for template denaturation and 60 s at 60°C for annealing/extension. A Rotor-Gene Q instrument was used. Expression data were normalized to GAPDH using the $\Delta\Delta C_q$ method.

Statistical analysis

Data were analyzed by SPSS 20.0. The χ^2 test was employed to examine categorical data. Relative gene expression levels between the groups and their relationship with clinicopathological parameters were investigated by *t* test. Univariate analysis, including survival analysis, was estimated with the Kaplan-Meier method. The log-rank test was employed for comparison of survival rates. A Cox proportional hazards regression model was applied for multivariate analysis. A *P* value < 0.05 was considered to be significant. Spearman's correlation test was used to determine relationships between Notch-1, Numb, Itch, and Siah-1 expression.

RESULTS

As shown in [Figure 1](#), both granular cytoplasmic and nuclear staining for Notch-1, Numb, Itch, and Siah-1 was observed; in some cases, Itch also displayed focal cytoplasmic staining. The intensity and distribution of staining did not differ between the groups. However, when considering the presence or absence of staining, Notch-1 staining occurred at a more frequent rate in CRC than in adenomas and controls, whereas the frequency of Numb, Itch, and Siah-1 staining was higher in controls and adenomas (*P* < 0.05) ([Table 1](#)). In terms of Notch-1, Numb, Itch, and Siah-1 expression, there was no significant difference between adenomas, healthy colon (controls), or tissues adjacent to carcinoma and adenomas (*P* > 0.05) ([Table 1](#)). Similarly, the distribution of Notch-1, Numb, Itch, and Siah-1 expression among adenoma subtypes did not differ (*P* > 0.05) ([Table 1](#)). [Table 2](#) summarizes the correlations between immunohistochemical expression of Notch-1, Numb, Itch, and Siah-1 and clinicopathological parameters. Expression of Notch-1 was more frequently observed in tumors with poor histological grade, lymph node metastasis, and an advanced stage (*P* < 0.05). In contrast, Numb, Itch, and Siah-1 expression was more frequent in tumors with well-differentiated morphology, without lymph node metastasis, and at an earlier stage (*P* < 0.05). The survival of patients with Notch-1 expression was shorter than that among those without Notch-1 expression. Conversely, Numb, Itch, and Siah-1 expression was related to better survival (*P* < 0.05) ([Table 2](#) and [Figure 2](#)).

Although the expression levels of Notch-1 were higher in colorectal carcinomas, those of Numb, Itch, and Siah-1 were higher in adenomas and healthy colonic mucosa,

Table 1 Distribution of immunohistochemical expression of Notch, Numb, Itch, and Siah-1 among the three groups, tissues adjacent to carcinoma, tissues adjacent to adenoma, and subtypes of adenomas

	Controls	Adenomas	Carcinomas	<i>P</i> value	Tissues adjacent to carcinoma ^a	Tissues adjacent to adenoma ^a	Subtypes of adenomas ^a		
							Tubular	Tubulovillous	Villous
Notch expression									
Negative (%)	15 (75)	12 (40)	18 (36)	0.02	25 (50)	16 (53)	4 (40)	5 (50)	3 (30)
Positive (%)	5 (25)	18 (60)	32 (64)		25 (50)	14 (47)	6 (60)	5 (50)	7 (70)
Numb expression									
Negative (%)	6 (30)	21 (70)	30 (60)	0.008	26 (52)	14 (47)	6 (60)	7 (70)	8 (80)
Positive (%)	14 (70)	9 (30)	20 (40)		24 (48)	16 (54)	4 (40)	3 (30)	2 (20)
Itch expression									
Negative (%)	8 (40)	24 (80)	30 (60)	0.01	27(54)	19 (63)	9 (90)	7 (70)	8 (80)
Positive (%)	12 (60)	6 (20)	20 (40)		23 (46)	11 (37)	1 (10)	3 (30)	2 (20)
Siah-1 expression									
Negative (%)	5 (25)	19 (63.3)	29 (58)	0.024	31(62)	21 (70)	7 (70)	5 (50)	7 (70)
Positive (%)	15 (75)	11 (36.7)	21 (42)		19 (38)	9 (30)	3 (30)	5 (50)	3 (30)

^a $P < 0.05$.

consistent with the immunohistochemical findings ($P < 0.05$) (Table 3, Figure 3 and 4). The relationships between the gene expression levels of Notch-1, Numb, Itch, and Siah-1 and clinicopathological parameters are presented in Table 4, and the significant findings are illustrated in Figure 5. The level of Notch-1 gene expression was significantly higher in poorly differentiated and moderately differentiated tumors than in well-differentiated tumors ($P < 0.05$). This level was also 7-fold higher in tumors with lymph node metastases and 3-fold higher in advanced-stage tumors than in tumors without lymph node metastasis and in tumors with earlier stages, respectively ($P < 0.05$). Moreover, the mean level of Notch-1 gene expression was 8-fold higher in patients with shorter survival times than in patients who survived for more than 5 years ($P < 0.05$). Overall, the expression levels of Numb, Itch, and Siah-1 were higher in well-differentiated tumors (6-fold increase), with lymph node metastasis (8-fold increase) and at advanced stages (5-fold increase) ($P < 0.05$). The survival of patients with higher Numb, Itch, and Siah-1 expression levels was better than that of patients with a low expression level of these genes ($P < 0.05$).

Similarly, when cases were grouped according to upregulation and downregulation of gene expression, upregulation of Notch-1 was more frequently observed in poorly differentiated tumors, in tumors with lymph node metastasis and in advanced stages. However, Numb, Itch, and Siah-1 upregulation correlated with better differentiation, absence of lymph node metastasis, and earlier stages ($P < 0.05$) (Table 5). Downregulation of Notch-1 and upregulation of both Numb and Itch were associated with better prognosis ($P < 0.05$) (Table 5 and Figure 6). Spearman's test revealed that the Notch-1 expression correlated inversely with the levels of Numb ($r: -0.4$, $P < 0.05$), Itch ($r: -0.3$, $P < 0.05$) and Siah-1 ($r: -0.8$, $P < 0.01$) expression.

In log-rank univariate analysis, the status of Notch-1, Numb, Itch, and Siah-1 expression and their expression levels, as well as lymph node metastasis and stage, were found to be significantly associated with survival among CRC patients (Figure 2 and Figure 6). According to Cox regression analysis, lymph node metastasis, upregulation of Notch, and downregulation of Numb were independent prognostic

Table 2 Correlations between immunohistochemical expression of Notch, Numb, Itch, and Siah-1 and clinicopathological parameters

Parameters	n	Notch		Numb		Itch		Siah-1	
		Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)
Age	18	32	30	20	30	20	29	21	
64 ± 12.35 ≥	24	8 (44.3)	16 (66.7)	13 (54.1)	11 (45.9)	14 (58.3)	10 (41.7)	16 (66.7)	8 (33.3)
64 ± 12.35 <	26	10 (38.5)	16 (61.5)	17 (65.3)	9 (34.7)	16 (61.5)	10 (38.5)	13 (50)	13 (50)
Gender									
Female	17	6 (35.3)	11 (66.7)	10 (58.8)	7 (41.2)	10 (58.8)	7 (42.2)	7 (41.2)	10 (58.8)
Male	33	12 (36.4)	21 (63.6)	20 (60.6)	13 (39.4)	20 (60.6)	13 (39.4)	22 (66.7)	11 (33.3)
Localization									
Caecum	8	3 (37.59)	5 (62.5)	6 (75)	2 (25)	5 (62.5)	3 (37.5)	4 (50)	4 (50)
Colon	16	5 (31.3)	11 (68.7)	9 (56.3)	7 (43.7)	8 (50)	8 (50)	10 (62.5)	6 (37.5)
Sigmoid	10	4 (40)	6 (60)	7 (70)	3 (30)	7 (70)	3 (30)	5 (50)	5 (50)
Rectum	16	6 (37.5)	10 (62.5)	8 (50)	8 (50)	10 (62.5)	6 (37.5)	10(50)	6 (50)
Differentiation ^a									
WD	18	16 (88.8)	2 (11.2)	3 (16.6)	15 (83.4)	5 (27.8)	13 (72.8)	8 (44.5)	10 (55.5)
MD	15	1 (6.6)	14 (93.4)	12 (80)	3 (20)	10 (66.7)	5 (33.3)	10 (66.6)	5 (33.4)
PD	17	1 (5.8)	16 (94.2)	15 (88.2)	2 (11.8)	15 (88.2)	2 (11.8)	11 (64.8)	6 (35.2)
Invasion									
T1	7	6 (85.7)	1 (14.3)	4 (57.1)	3 (42.9)	2 (28.5)	5 (71.5)	3 (48.3)	4 (51.7)
T2	10	6 (60)	4 (40)	7 (70)	3 (30)	7 (70)	3 (30)	6 (60)	4 (40)
T3	23	4 (17.4)	19 (82.6)	12 (52.1)	11 (47.9)	15 (65.2)	8 (34.8)	14 (60.9)	9 (39.1)
T4	10	2 (20)	8 (80)	7 (70)	3 (30)	6 (60)	4 (40)	6 (60)	4 (40)
LN ^a									
Absent	17	1 (5.3)	17 (94.7)	5 (29.4)	12 (70.6)	7 (41.1)	10 (58.9)	4 (23.6)	13 (76.4)
Present	33	17 (61.39)	21(38.7)	25 (75.8)	8 (24.2)	23 (69.6)	10 (30.4)	25 (75.8)	8 (24.2)
Lymphatic invasion									
Absent	37	17 (45.9)	20 (54.1)	22 (59.4)	15 (40.6)	23 (62.1)	14(37.9)	25 (67.6)	12 (32.4)
Present	13	1 (7.6)	12 (92.4)	8 (61.5)	5 (38.5)	7 (53.8)	6 (45.2)	4 (30.8)	9 (69.2)
Vascular invasion									
Absent	42	16 (38.1)	26 (61.9)	25 (61.9)	17(38.1)	27 (64.3)	15 (35.7)	26 (61.9)	16 (38.1)
Present	8	2 (25)	6 (75)	5 (62.5)	3 (37.5)	3 (62.5)	5 (37.5)	3 (37.5)	5 (62.5)
Perineural invasion									
Absent	31	11 (35.4)	20 (64.6)	21 (67.7)	10 (32.3)	20 (64.5)	11 (35.5)	18 (58.1)	13 (41.9)
Present	19	7 (36.8)	12 (63.2)	9 (47.4)	10 (52.6)	10 (52.6)	9 (47.4)	11 (57.9)	8 (42.1)
Stage ^a									
1 + 2	24	13 (54.1)	11 (45.9)	8 (33.4)	16 (66.6)	10 (41.6)	14 (58.4)	10 (41.7)	14 (58.3)
3 + 4	26	5 (19.2)	21 (80.8)	22 (84.6)	4 (15.4)	20 (76.9)	6 (23.1)	19 (73.1)	7 (26.9)
Distant metastasis									
Absent	26	8 (30.7)	18 (69.3)	15 (57.6)	11 (42.4)	13 (50)	13 (50)	15 (57.7)	11(42.3)
Present	24	10 (41.6)	14 (58.4)	15 (62.5)	9 (37.5)	17 (70.8)	7 (29.2)	14 (58.4)	10 (41.6)
Survival (mo) ^a									
60.62 ± 26.68 ≥	26	2 (7.6)	24 (92.4)	20 (76.9)	6 (23.1)	22 (84.6)	4 (15.4)	19 (73.1)	7 (26.9)

60.62 ± 26.68 <	24 16 (66.6)	8 (33.4)	10 (41.7)	14 (58.3)	8 (33.4)	16 (66.6)	10 (41.7)	14 (58.3)
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^aP < 0.05. WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LNM: Lymph node metastasis.

Table 3 Distribution of Notch-1, Numb, Itch, and Siah-1 expression levels assessed by qPCR analysis in adenoma and carcinoma groups

Gene expression levels	Adenomas	Carcinomas	P value
Notch			
mean ± SD	3.1016 ± 1.0452	10.2628 ± 5.8043	0.03
Median	2.6992	1.5305	
Normal (%)	0	12 (24)	0.0001
(↑) (%)	15 (50)	22 (44)	
(↓) (%)	15 (50)	16 (32)	
Numb			
mean ± SD ¹	8.1362 ± 1.6299	2.3153 ± 2.2267	0.03
Median	4.0287	-2.2616	
Normal (%)	4 (13.3)	0	0.0001
(↑) (%)	18 (60)	24 (58)	
(↓) (%)	8 (26.7)	26 (52)	
Itch			
mean ± SD	9.2899 ± 2.7369	2.1982 ± 1.0461	0.006
Median	2.0422	1.5933	
Normal (%)	11 (36.7)	12 (32)	0.0001
(↑) (%)	15 (50)	18 (38)	
(↓) (%)	4 (13.3)	20 (30)	
Siah-1			
mean ± SD	5.9024 ± 1.18671	0.8358 ± 1.3831	0.03
median	1.4031	-2.0322	
Normal (%)	6 (20)	10 (20)	0.001
(↑) (%)	11 (36.7)	13 (26)	
(↓) (%)	13 (43.3)	27 (54)	

¹Fold increase $2^{-\Delta\Delta Ct}$. (↑): Upregulation; (↓): Downregulation.

factors (Table 6).

DISCUSSION

In this study, Notch-1 gene and protein expression levels were higher in carcinoma and adenomas than in control tissues, and the expression differences among these three groups were significant. Our observation is not surprising, given the previous clinical and experimental data demonstrating the relationship of the Notch pathway with carcinogenesis in the colon^[21,22]. In CRC, expression of Notch-1, its ligand Jagged-1 and its target genes Hes-1 and Hey-1 is significantly higher than in normal mucosa^[23,24], and expression of Notch-1 and its ligands is also higher in precancerous conditions, such as inflammatory bowel diseases, than in the healthy colon^[25]. In adenomas of adenomatous polyposis coli gene (APC)-mutant mice, γ-secretase inhibitors that suppress the Notch pathway promote goblet cell differentiation while

Table 4 Mean and median values of relative gene expression levels of Notch-1, Numb, Itch and Siah-1 in tumors with different clinicopathological parameters (qPCR analysis)

Parameters	n	Notch-1		Numb		Itch		Siah-1	
		mean ± SD ¹	Median	mean ± SD	Median	mean ± SD	Median	mean ± SD	Median
Age									
64 ± 12.35 ≥	24	11.23 ± 5.23	-1.18	1.08 ± 1.03	-1.03	1.73 ± 2.34	-1.22	0.81 ± 0.32	-1.22
64 ± 12.35 <	26	9.20 ± 4.34	1.67	3.28 ± 4.32	-2.26	3.35 ± 1.64	1.99	0.85 ± 0.42	-2.99
Gender									
Female	17	10.21 ± 6.02	1.38	1.74 ± 1.34	-2.43	1.93 ± 1.34	1.35	0.23 ± 0.03	-3.19
Male	33	10.58 ± 5.03	1.67	3.42 ± 3.42	4.67	2.70 ± 2.10	-1.21	2.00 ± 0.93	-1.22
Localization									
Caecum	8	13.10 ± 5.23	12.39	1.17 ± 1.45	1.31	1.85 ± 1.62	2.43	2.40 ± 1.11	0.41
Colon	16	7.99 ± 7.32	-1.50	2.60 ± 2.34	1.20	3.33 ± 2.03	2.30	0.26 ± 0.23	-2.56
Sigmoid	10	15.03 ± 6.23	20.79	3.69 ± 2.45	3.03	-1.03 ± 0.3	-1.75	-0.77 ± 0.22	-1.49
Rectum	16	8.37 ± 3.41	1.16	1.72 ± 1.65	-2.96	2.63 ± 1.22	1.67	1.62 ± 0.51	-3.48
Differentiation ^a									
WD	18	3.92 ± 2.45	-3.08	6.64 ± 3.46	9.92	5.26 ± 2.42	7.05	3.67 ± 1.53	-1.22
MD	15	9.85 ± 4.46	21.67	1.26 ± 1.04	-2.26	-1.20 ± 0.84	-2.41	-1.66 ± 0.66	-3.24
PD	17	17.28 ± 9.03	22.54	-3.62 ± 1.81	-4.45	-0.03 ± 0.01	-1.86	-1.91 ± 0.65	-4.21
Invasion									
T1	7	5.39 ± 5.93	-7.70	9.40 ± 4.72	14.62	10.04 ± 5.80	12.06	2.75 ± 1.36	-3.89
T2	10	10.01 ± 6.82	4.45	3.63 ± 2.45	-3.06	1.41 ± 0.56	-2.26	1.43 ± 0.92	-3.86
T3	23	11.38 ± 7.02	1.67	1.63 ± 1.85	-2.26	1.39 ± 0.8	-1.91	1.87 ± 0.23	-1.16
T4	10	11.75 ± 6.88	9.59	-2.40 ± 2.03	-3.39	-1.65 ± 0.3	-1.51	-3.51 ± 1.48	-2.94
LNM ^a									
Absent	17	2.45 ± 2.67	-4.54	8.66 ± 4.82	13.40	5.91 ± 2.20	7.84	5.77 ± 2.81	-1.16
Present	33	15.17 ± 6.89	19.90	-1.57 ± 1.24	-2.35	-1.07 ± 0.51	-1.65	-2.19 ± 1.08	-3.54
Lymphatic invasion									
Absent	37	10.84 ± 6.36	1.67	1.54 ± 3.42	-2.26	1.68 ± 0.83	1.35	0.45 ± 0.23	-3.19
Present	13	8.92 ± 5.32	1.38	4.51 ± 3.83	3.79	3.67 ± 1.43	1.38	1.90 ± 0.92	-1.35
Vascular invasion									
Absent	42	11.77 ± 8.43	1.67	1.92 ± 1.65	-2.26	1.44 ± 0.96	1.07	-0.48 ± 0.28	-3.22
Present	8	12.96 ± 7.32	-2.98	9.63 ± 5.32	13.13	6.17 ± 3.02	5.66	7.77 ± 3.82	5.50
Perineural invasion									
Absent	31	8.92 ± 5.32	-1.01	2.58 ± 2.48	-2.26	2.86 ± 1.32	2.00	1.22 ± 0.32	-3.22
Present	19	12.65 ± 6.02	17.79	1.87 ± 1.82	2.27	1.17 ± 0.61	-1.21	0.20 ± 0.80	5.50
Stage ^a									
1 + 2	24	4.45 ± 4.02	-9.81	12.85 ± 5.63	7.53	5.24 ± 2.31	6.89	1.24 ± 0.51	-1.64
3 + 4	26	15.78 ± 7.94	-8.25	9.72 ± 4.63	-2.29	-1.61 ± 0.22	-1.88	0.21 ± 0.03	-2.42
Distant metastasis									
Absent	26	9.83 ± 7.34	-1.18	3.42 ± 1.86	1.99	3.17 ± 1.24	1.77	0.99 ± 0.23	-2.45
Present	24	10.89 ± 6.53	1.67	1.11 ± 1.02	-2.86	1.14 ± 0.82	-1.15	0.66 ± 0.23	-2.02
Survival (mo) ^a									

60.62 ± 26.68 ≥	26	17.16 ± 6.04	21.22	-5.47 ± 2.24	-4.90	-2.03 ± 1.06	-2.47	-4.63 ± 2.07	-4.05
60.62 ± 26.68 <	24	2.95 ± 2.02	-5.33	10.75 ± 5.21	13.13	6.78 ± 2.83	8.51	6.76 ±	3.15

^a*P* < 0.05.¹Fold increase (2^{-ΔΔC_t}). WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LNM: Lymph node metastasis.

reducing cell proliferation^[26]. In addition, in both familial adenomatous polyposis and adenomas, upregulation of genes that function in the Notch pathway, including Notch-1, as well as the relationship between Notch-1 to Wnt-β-catenin are notable findings demonstrating the role of Notch-1 in tumor development^[27,28]. Our findings, together with all these data, support the role of Notch signaling in CRC carcinogenesis.

To date, the relationship between tumor behavior and the Notch pathway in CRC has been investigated in many studies. Although Notch-1 has been shown to be associated with aggressive tumor characteristics, there are some inconsistencies among the results of these studies. In some studies, Notch-1 expression has been found to be associated with grade^[29], lymph node metastasis^[30], and advanced stage^[31], similar to our findings; other studies have reported that Notch-1 expression is related to the presence of distant metastasis^[32,33] and lymphatic and vascular invasion^[34]. These different results may be explained by differences in the number of cases and in the techniques and antibodies used to detect Notch-1. In our study group, Notch-1 expression was associated with a poor prognosis: 25% of the patients with high Notch expression (32 cases) survived 5 years, but this rate was 88.8% in the group without Notch expression (18 cases). Moreover, multivariate analysis revealed Notch-1 to be an independent prognostic factor. Overall, the number of studies demonstrating that Notch-1 expression is associated with poor prognosis (although not identified as an independent prognostic factor in all of these studies) is considerably higher than those showing that it is not^[35-39]. Despite the relatively small number of cases in the present study, our results support that Notch-1 expression plays a role in determining aggressive tumor behavior in CRC and a poor prognosis.

In contrast to Notch-1-related findings, Numb expression levels gradually decreased from normal tissue to carcinoma in our samples, and the difference between the groups was significant. Accumulated evidence indicates that Numb, which plays an essential role in stem cell compartment maintenance and cell fate determination, has the potential to function in the oncogenesis of many tumors^[40-42]. However, Numb may have promoter function in some cancers and a suppressor function in others. For example, Numb overexpression inhibits tumor growth and the epithelial-mesenchymal transition in prostate carcinoma, esophageal squamous cell carcinoma, adenocarcinoma of the lung, and breast cancer^[43,44], but it promotes cell proliferation in hepatocellular carcinoma and squamous cell carcinoma of the lung^[45]. Therefore, the influence of Numb in oncogenesis might be related to the type of malignancy or the histological subtype. Although Numb has been studied during carcinogenesis in various organs due to its effect on Notch signaling, few studies have investigated variations in Numb expression during human colorectal carcinogenesis, and a vast majority of the studies investigating changes in Numb expression in colorectal tissues are experimental studies, particularly those involving carcinoma cell cultures^[46,47]. In intestinal epithelial cells, Numb induces the goblet cell phenotype by inhibiting Notch^[31]. In cell culture, inhibition of Numb by several microRNAs (miR-142-3p, miR-34-a, Musashi-1, and 2) induces stemness and proliferation of cancer cells. Parallel to our study, Zhao *et al.*^[48] reported that expression of Numb in CRC tissues was significantly lower than that in the adjacent normal intestinal mucosa, suggesting that Numb may exert a suppressor function in the development of CRC. Interestingly, these researchers also observed that splice variants of Numb might have different effects on colorectal carcinogenesis. In a study by Meng *et al.*^[23], Notch-1 expression was detected in patients with colon cancer, but Numb levels were decreased. To the best of our knowledge, no study has evaluated Numb expression in adenoma tissues. In our study, expression levels of Numb decreased significantly from adenomas to CRC. When all these data are evaluated together, our findings support that loss of Numb expression contributes to colorectal carcinogenesis.

The present study revealed that Numb expression is higher in well-differentiated tumors, at earlier stages, and in patients without lymph node metastases, in contrast to the findings for Notch-1. Numerous experimental studies have shown that Numb protein expression is associated with cell differentiation in both normal tissues and tumors, concluding that it is a determinant of cell fate^[41-44]. Similar findings have been

Table 5 Correlations between expression levels of Notch, Numb, Itch, and Siah-1 and clinicopathological parameters in colorectal carcinoma

Levels		Notch			Numb		Itch			Siah-1		
Parameters	<i>n</i>	(-)	(↓)	(↑)	(↓)	(↑)	(-)	(↓)	(↑)	(-)	(↓)	(↑)
		12	16	22	26	24	16	19	15	10	27	13
Age												
64 ± 12.353 ≥	22	7 (31.8)	4 (18.2)	11 (50)	12 (54.5)	10 (45.5)	7 (31.8)	6 (27.3)	9 (40.9)	4 (18.2)	13 (59.1)	5 (22.7)
64 ± 12.353 <	28	5 (17.9)	12 (42.9)	11 (39.3)	14 (50)	14 (50)	5 (17.9)	12 (42.9)	11 (39.3)	6 (21.4)	14 (50)	8 (28.6)
Gender												
Female	17	3 (17.6)	5 (29.5)	9 (52.9)	17 (51.5)	16 (48.5)	6 (35.3)	7 (41.2)	4 (23.4)	3 (17.6)	10 (58.8)	4 (23.5)
Male	33	9 (27.3)	11 (33.3)	13 (39.4)	9 (52.9)	8 (47.1)	6 (18.2)	11 (33.3)	16 (48.5)	7 (21.2)	17 (51.5)	9 (27.3)
Localisation												
Caecum	8	1 (12.5)	3 (37.5)	4 (50)	4 (50)	4 (50)	0	3 (37.5)	5 (62.5)	2 (25)	4 (50)	2 (25)
Colon	16	3 (18.8)	3 (18.8)	10 (62.5)	9 (56.2)	7 (43.8)	5 (31.2)	5 (31.2)	6 (37.5)	3 (18.8)	9 (56.2)	4 (25)
Sigmoid	16	7 (43.8)	7 (43.8)	2 (12.5)	6 (60)	4 (40)	3 (30)	3 (30)	4 (40)	2 (12.5)	10 (62.5)	4 (25)
Rectum	10	1 (10)	3 (30)	6 (60)	7 (43.8)	9 (56.2)	4 (25)	7 (43.8)	5 (31.2)	3 (30)	4 (40)	3 (30)
Differentiation ^a												
WD MD	24 13	6 (25) 4 (30.7)	13 (54.1) 2 (15.3)	5 (20.9) 7 (54)	8 (33.3) 8 (61.5)	16 (66.7) 5 (38.5)	5 (20.8) 3 (23.1)	5 (20.8) 5 (38.5)	14 (58.3) 5 (38.5)	7 (29.2) 2 (15.49)	7 (29.2) 10 (76.9)	10 (41.7) 1 (7.7)
PD	13	2 (15.4)	1 (7.7)	10 (76.9)	10 (76.9)	3 (23.1)	4 (30.8)	8 (61.5)	1 (7.7)	1 (7.7)	10 (76.9)	2 (15.4)
Invasion												
T1	7	1 (14.3)	1 (14.3)	5 (71.4)	1 (14.3)	6 (85.7)	2 (20)	0	5 (71.4)	2 (28.6)	3 (42.9)	2 (28.6)
T2	10	3 (30)	3 (30)	4 (40)	6 (60)	4 (40)	2 (20)	(30)	5 (50)	1 (10)	5 (50)	4 (40)
T3	23	6 (26.1)	7 (30.4)	10 (43.5)	15 (65.2)	8 (34.8)	5 (21.7)	11 (47.8)	7 (30.4)	5 (21.7)	5 (50)	4 (17.4)
T4	10	2 (20)	5 (50)	3 (30)	4 (40)	6 (60)	24 (30)	18 (36)	20 (40)	2 (20)	5 (50)	3 (30)
LNM ^a												
Absent	19	3 (15.8)	13 (68.4)	3 (15.8)	6 (31.6)	13 (68.4)	5 (26.3)	2 (10.5)	12 (63.2)	6 (31.6)	5 (26.3)	8 (42.1)
Present	31	9 (29)	3 (9.7)	19 (61.3)	20 (64.5)	11 (35.5)	7 (22.6)	16 (88.9)	8 (25.8)	4 (12.9)	22 (71)	5 (16.1)
Lymphatic invasion												
Absent	37	11 (29.7)	13 (35.1)	13 (35.1)	20 (54.1)	17 (45.9)	7 (18.9)	13 (35.1)	17 (45.9)	7 (18.9)	24 (64.9)	6 (16.2)

Present	13	1 (7.7)	3 (23.1)	9 (69.2)	6 (46.2)	7 (53.8)	5 (38.5)	5 (38.5)	3 (23.1)	3 (23.1)	3 (23.1)	7 (53.8)
Vascular invasion												
Absent	42	12(28.6)	14(33.3)	16 (38.1)	23 (54.8)	19 (45.2)	8 (19)	16 (38.1)	18 (42.9)	8 (19)	25 (59.5)	9 (21.4)
Present	8	0	2 (25)	6 (75)	3 (37.5)	5 (62.5)	4 (50)	2 (25)	2 (25)	2 (25)	2 (25)	4 (50)
Perineural invasion												
Absent present	31	6 (19.4)	12 (38.7)	13 (41.9)	15 (48.4)	16 (51.6)	8 (25.8)	10 (32.3)	13 (41.9)	5 (16.1)	17 (54.8)	9 (29)
	19	6 (31.6)	4 (21.1)	9 (47.4)	11 (57.9)	8 (42.1)	4 (21.1)	8 (42.1)	7 (36.8)	5 (26.3)	10 (52.6)	4 (21.1)
Stage ^a												
1 + 2	24	8 (33.3)	15 (62.5)	9(4.2)	7 (29.2)	17 (70.8)	5 (20.8)	4 (16.7)	15 (62.5)	7 (29.2)	9 (37.5)	8 (33.3)
3 + 4	26	4 (15.4)	1(11.6)	19(73)	19 (73.1)	7 (26.9)	7 (26.9)	14 (53.8)	5 (19.1)	3 (11.5)	18 (69.2)	5 (19.2)
Distant metastasis												
Absent	26	7 (26.9)	8 (30.8)	11 (42.3)	11 (42.3)	15 (57.7)	7 (26.9)	7 (26.9)	12 (46.2)	4 (15.4)	16 (61.5)	6 (23.1)
Present	24	12(20.8)	8 (33.3)	11 (45.8)	15 (62.5)	9 (37.5)	5 (20.8)	11 (45.8)	8 (33.3)	6 (25)	11 (45.8)	7 (39.2)
Survival (mo) ^a												
60.62 ± 26.68 ≥	26	11 (42.3)	0	15 (57.7)	22 (84.6)	4 (15.4)	7 (26.9)	15 (57.6)	4 (42.4)	4 (15.5)	21 (80.7)	1 (3.8)
60.62 ± 26.68 <	24	1 (4.2)	16 (66.6)	7 (29.2)	4 (16.7)	20 (83.3)	5 (20.8)	3 (12.5)	16 (87.5)	6 (25)	6 (25)	12 (50)

^a*P* < 0.05. Downregulation of Notch and upregulation of Numb, Itch, and Siah-1 are associated with better prognosis. (↑): Upregulation, (↓): Downregulation; WD: Well-differentiated; MD: Moderately differentiated; PD: Poorly differentiated; LNM: Lymph node metastasis.

obtained in experimental studies involving CRC cell culture. Indeed, many microRNAs that induce stemness and aggressive behavior in CRC stem cells stabilize β-catenin through Numb repression^[46,47]. A recent study demonstrated that low Numb serum levels are associated with cetuximab resistance and worse prognosis in patients with CRC, suggesting that Numb is a contributor to the prevention of tumor aggression^[49]. In another, Numb expression was investigated in patients with CRC^[48], and similar to our data, Numb expression was found to be reduced in patients with lymph node metastasis, deeper invasive tumors, and at a more advanced stage, though the difference was not significant.

Nonetheless, our study revealed a significant inverse correlation between these parameters and Numb expression. Although our results need to be supported by further studies in larger patient series, the results show that Numb plays a role in preventing aggressive tumor behavior in CRC. To the best of our knowledge, the relationship between Numb expression and survival in CRC has not been investigated

Table 6 Independent prognostic factors according to Cox regression analysis

Parameter	Regression coefficient	P value	Relative risk
LN metastasis	2.406	0.001	11.088
Notch expression levels	1.579	0.03	3.677
Numb expression levels	-1.166	0.01	0.561

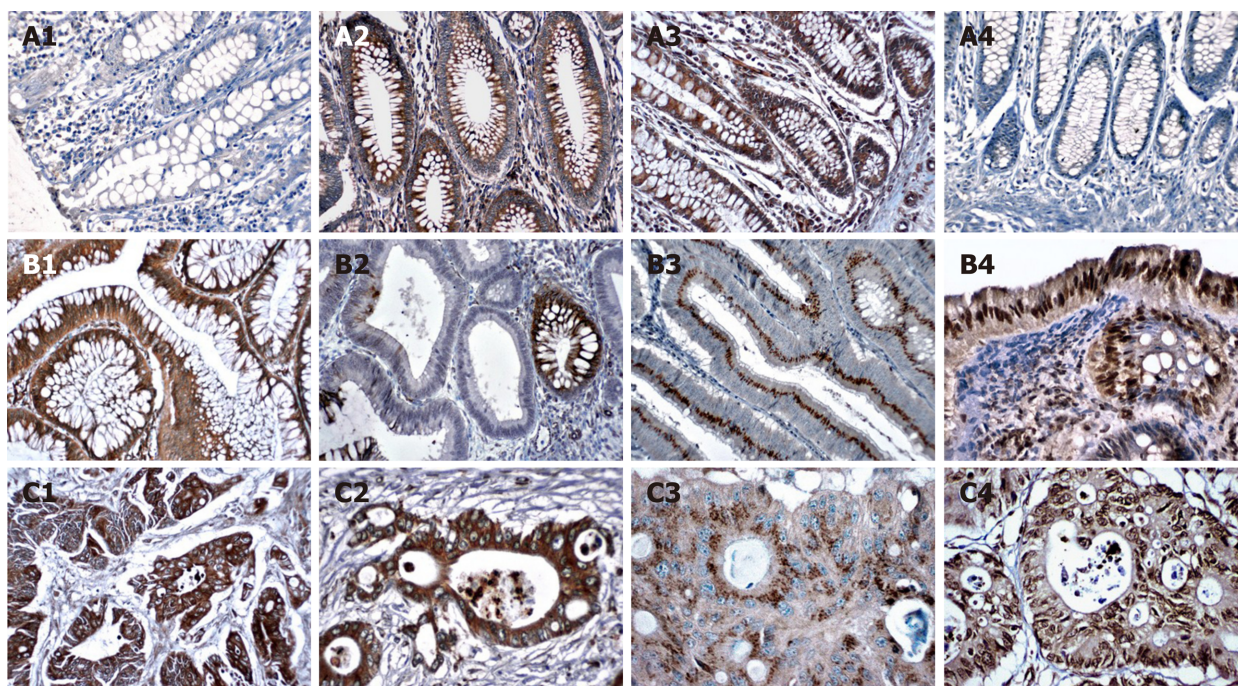


Figure 1 Notch (A1, B1 and C1), Numb (A2, B2 and C2), Itch (A3, B3 and C3), and Siah-1(A4, B4 and C4) immunohistochemical expression in the three groups. Expression of Notch is higher in CRCs (C1-4), and that of Numb Itch and Siah-1 is higher in adenomas (B1-4) and controls (A1-4).

to date. Our results indicate that loss of Numb expression is an independent prognostic factor and predictor of poor prognosis in CRC.

In our study, Itch gene expression was normal in all healthy colon mucosa samples. In adenomas, Itch levels were decreased, paralleling *Numb* gene expression, and we observed an inverse correlation between Itch and Notch-1 gene expression. The frequency of cases with low Itch gene expression was higher in the adenocarcinoma group, and our immunohistochemical evaluation revealed similar correlations. This finding is in line with the results of several studies in which Itch was shown to induce activity of the Numb protein, thereby acting as a suppressor of Notch-1 expression and leading to a reduction in its level^[50]. Although there are few studies investigating Itch expression in the colon, in an elegant study by Kathania *et al*^[51], Itch-depleted animals showed increased inflammation in the colon, increased proinflammatory cytokines, and a tendency to develop cancer associated with colitis. Interestingly, in the same study, it was noted that the effect of Itch on homeostasis in the small intestine differed, suggesting organ-specific effects in the gastrointestinal tract. In another study, Huang *et al*^[52] found less frequent expression of Itch in colorectal carcinoma than in normal tissue in 45 cases, and it was concluded that Itch can suppress carcinogenesis by suppressing the Wnt- β catenin pathway. These findings, together with our findings, suggest that loss of Itch expression participates in colorectal carcinogenesis *via* the Notch-1-Numb axis.

The study presented herein revealed that similar to Numb, increases in Itch expression and upregulation are associated with better characteristics of tumor behavior and better survival in CRC. Recent studies investigating the role of Itch in tumors of different organs have reported different results^[53-55]. For instance, some investigators have observed that Itch has a role in treatment resistance and poor prognosis, and the use of Itch inhibitors due to its interaction with p63 and p73,

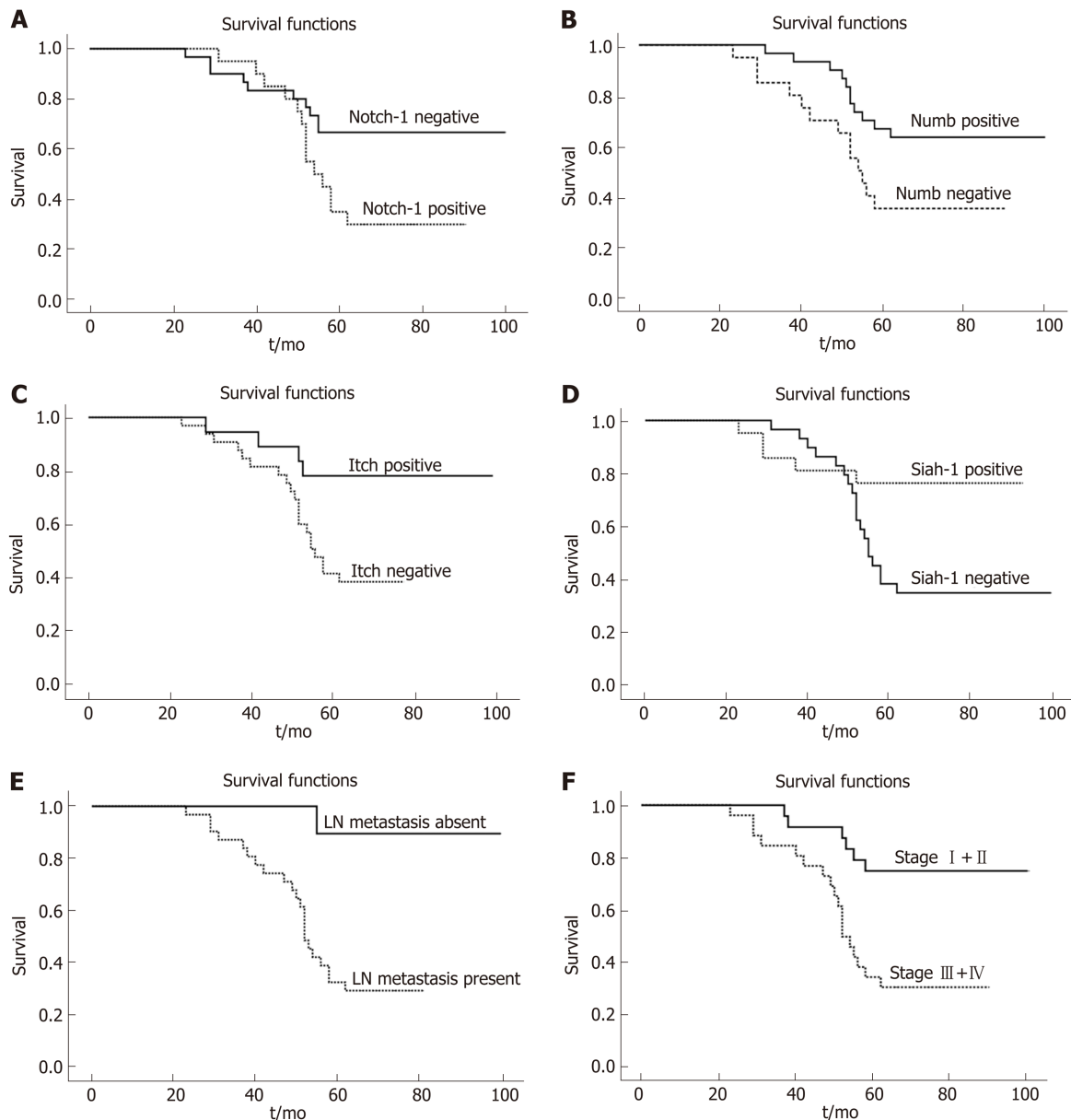


Figure 2 Survival of patients according to Notch (A), Numb (B), Itch (C), and Siah-1 (D) immunohistochemical expression, lymph node metastasis (E), and stage (F). Survival of patients with Notch expression is shorter than that of those without Notch expression. In contrast, expression of Numb, Itch, and Siah-1 is related to better survival.

especially in tumors without functional p53, has been proposed^[56,57]. Because Itch is associated with many pathways, including Notch-1 signaling, researchers have suggested that not only its inhibition but also its stimulation can be used in some cancers. Peng *et al.*^[58] demonstrated that lithium exerts its antitumor effect *via* Itch stimulation in pancreatic cancer. In general, neither the role of Itch as a potential treatment target nor its role in tumor behavior has been completely clarified. The effect of Itch in the colon has been investigated owing to its association with IL-17, which plays a role in colorectal inflammation and carcinogenesis^[51]. Unfortunately, we could not find any studies in the literature examining the relationship between Itch expression and CRC tumor behavior and survival for a comparison with our results. Only one recent study was found, demonstrating that a decrease in circ-Itch, a circulating antitumor-acting RNA that affects the function of Itch, is associated with a poor prognosis in patients with CRC^[52]. The current data suggest that loss of Itch expression is associated with a poor prognosis and aggressive tumor behavior in CRC, warranting further large-scale studies.

In our study group, Siah-1 was expressed more frequently in healthy mucosa, and similar to Numb and Itch, its gene expression was normal. However, Siah-1 expression, as detected by immunohistochemistry, and its gene expression level, as

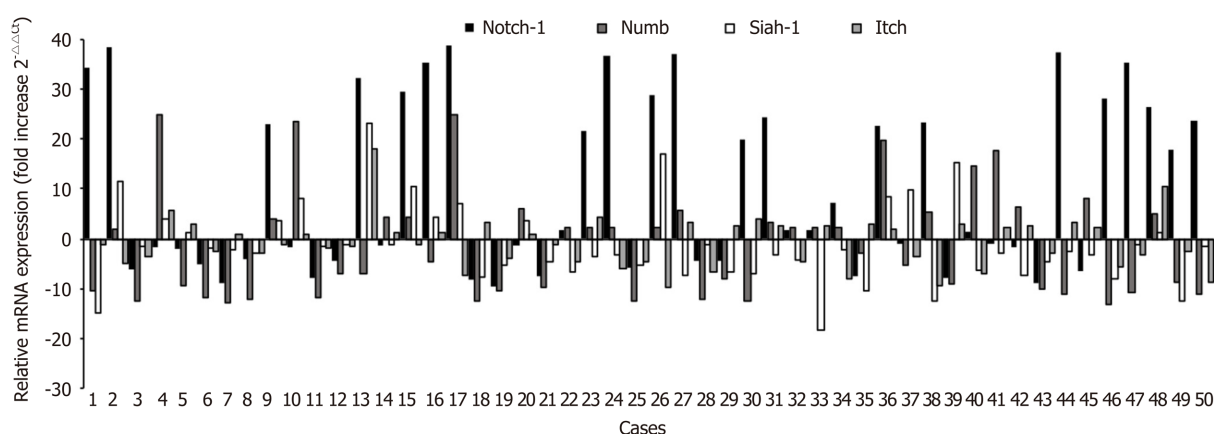


Figure 3 Relative gene expression levels of Notch, Numb, Itch, and Siah-1 in 50 patients diagnosed with colorectal carcinoma (qPCR analysis).

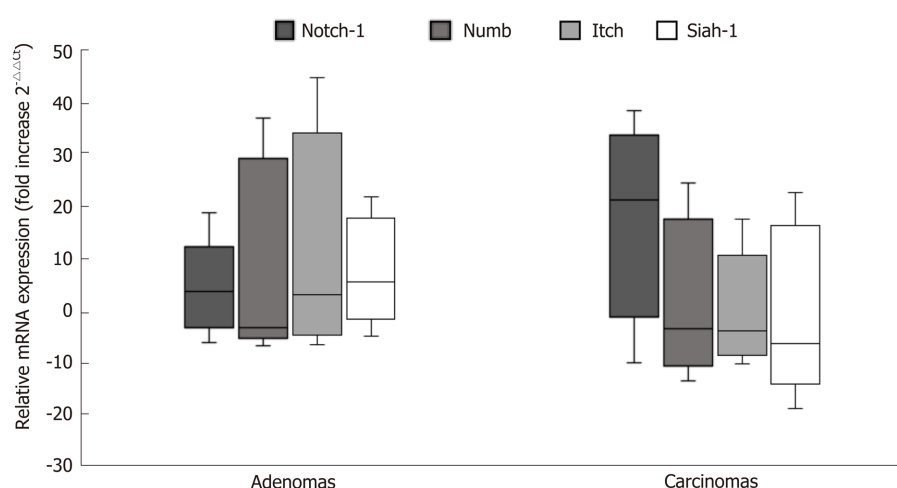


Figure 4 Levels of Notch, Numb, Itch, and Siah-1 expression in adenomas and carcinomas. Expression levels of Notch are higher in colorectal carcinoma; in contrast, expression levels of Numb, Itch, and Siah-1 are higher in adenomas (qPCR analysis).

determined by molecular methods, were decreased in adenomas compared to the control group, and this decrease was more pronounced in the adenocarcinoma group. Unfortunately, the relationship between Siah-1 and colorectal carcinogenesis and tumor behavior has not been addressed in any study to date. In a few early studies, data suggesting that Siah-1 may act as a stimulator of Notch-1 due to its ability to target Numb for degradation^[19]. However, the results of subsequent studies have shown that the effect of Siah-1 is not limited to Numb and Notch and that it also plays a role in Ras and hypoxia pathways. More notably, Siah-1 plays an active role in the degradation of β -catenin, which is constitutively activated in the development of most CRC (90%)^[59]. Siah-1 interacts with the carboxyl terminus of APC, recruiting the ubiquitination complex and promoting the degradation of β -catenin in the E3 ubiquitin ligase complex. Although further studies should be carried out to test this hypothesis, in light of all these data, our results suggest that Siah-1 is involved in Notch-1 suppression, rather than its stimulation, in colorectal carcinogenesis.

Among our cases, loss of and downregulation of Siah-1 expression were associated with aggressive tumor behavior and poor prognosis, similar to Numb and Itch and in contrast to Notch-1 expression. At present, data on the potential use of Siah-1 as a prognostic marker and as a therapeutic target vary depending on the organ and even according to different histological types of tumors of the same organ^[60,61]. However, in addition to its relationship with Notch-1, numerous studies have shown that Siah-1 is involved in β -catenin degradation, which supports evidence that Siah-1 has a tumor-suppressor role. Indeed, an experimental study in CRC demonstrated that Siah-1 interacts with the APC gene and degrades β -catenin^[62]. Regardless, to the best of our

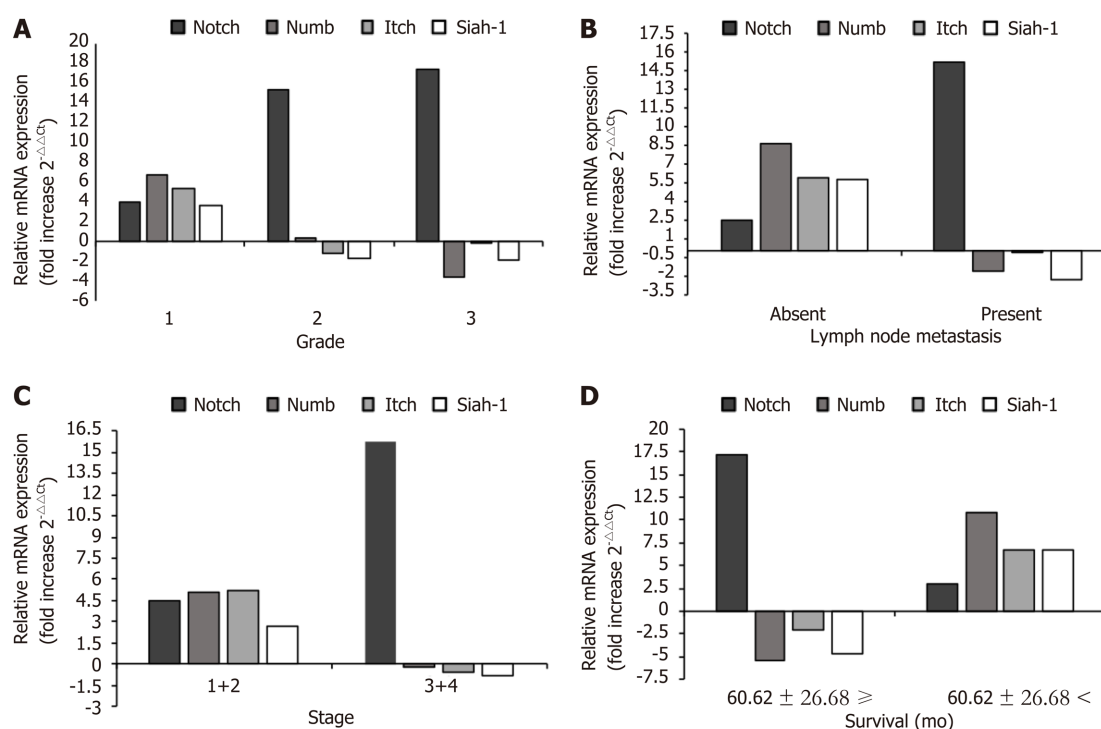


Figure 5 Distribution of relative gene expression levels of Notch, Numb, Itch, and Siah-1 according to grade (A), lymph node metastasis (B), stage (C) and survival (D) in colorectal carcinoma (qPCR analysis).

knowledge, no study has investigated the relationship between Siah-1 expression and the clinicopathological parameters of and survival in CRC patients. Our results suggest that Siah-1 expression plays an important role in the negative regulation of CRC progression, and our data are not consistent with the findings that Siah-1 induces Notch-1 by inhibiting Numb. In contrast to the negative correlation of Siah-1 with Notch-1, its positive association with Numb and Itch indicates that in CRC, its effect is not limited to Notch-1 induction and also involves other pathways.

In conclusion, this preliminary study indicates that overexpression of Notch-1, as well as loss of Numb, Itch, and Siah-1 expression, contributes to colorectal carcinogenesis. In patients with CRC, Notch-1 expression is associated with aggressive tumor behavior and is an independent adverse prognostic marker. In our study group, decreased Numb expression supports the findings that loss of Numb expression in CRC is a determinant of aggressive tumor behavior, indicating that it is an independent prognostic parameter. Similar to Numb, reductions in Itch and Siah-1 expression are associated with aggressive tumor behavior and prognosis in CRC. Positive relationships between Numb, Itch, and Siah-1 and their inverse correlation with Notch-1 expression indicate that these proteins are involved in the Notch pathway during CRC progression. Finally, Numb, Itch, and Siah-1 may be useful in determining the progression of CRC and are also potential new therapeutic targets. However, our results should be supported by further studies involving large numbers of cases. Another essential factor to be considered is that this study was conducted only using samples from patients diagnosed with adenocarcinoma, NOS. Therefore, the relationship between Notch-1, Numb, Itch, and Siah-1 expression and their impacts on carcinogenesis and tumor behavior should be investigated in other histological subtypes of CRC as well as their precursors, particularly in subtypes with a more aggressive course, such as serrated carcinoma.

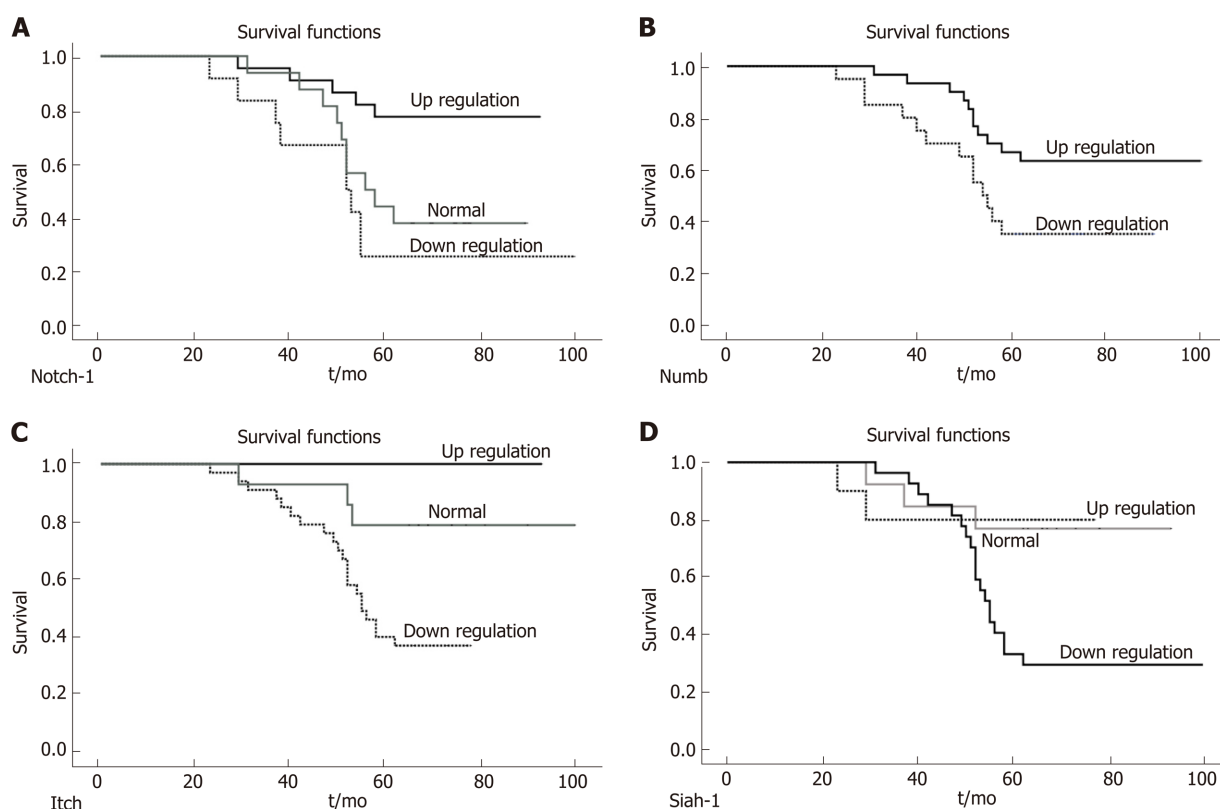


Figure 6 Survival of patients according to expression levels of Notch (A), Numb (B), Itch (C), and Siah-1 (D). Survival of patients with Notch upregulation is shorter than that of patients without Notch upregulation. Conversely, upregulation of Numb, Itch, and Siah-1 is related to better survival.

ARTICLE HIGHLIGHTS

Research background

The advances in immunohistochemical and molecular techniques in recent years, many studies have been carried out, aiming to determine new targets that can be useful in the treatment as well as predicting colorectal carcinoma (CRC) behavior. In this context, the role of Notch-1 in oncogenesis, tumor behavior, and survival have been investigated in recent studies with different results.

Research motivation

The contribution of Numb, Itch and Siah-1 in the Notch pathway is documented. On the other hand, the roles of Numb, Itch and Siah-1 expression in colorectal carcinogenesis and their relationship to tumor behavior, survival, Notch-1 expression have not been evaluated.

Research objectives

In this preliminary study, we aimed to evaluate the expression of Numb, Itch, and Siah-1 in colorectal tumors to clarify their relationship with Notch-1 expression and their role in carcinogenesis and tumor behavior.

Research methods

We retrospectively investigated the expression of Notch, Numb, Itch and Siah-1 in 50 colorectal adenocarcinomas, 30 adenomas, and 10 healthy colon tissue by immunohistochemistry and quantitative real-time PCR analysis. Data were analyzed by the χ^2 test, *t*-test and Spearman's correlation test. Survival probability curves were calculated using the Kaplan-Meier method. A Cox proportional hazards regression model was applied for multivariate analysis. A *P* value < 0.05 was considered to be significant.

Research results

Notch-1 staining was more frequent in CRC than in adenomas and controls. However, the frequency of Numb, Itch, and Siah-1 staining was higher in controls and adenomas. While the expression of Notch-1 was more frequently observed in tumors with poor histological grade, lymph node metastasis, and an advanced stage, the expression of Numb, Itch, and Siah-1 was more frequent in tumors with well-differentiated morphology, without lymph node metastasis, and at an earlier stage. Although the survival of patients with Notch-1 expression was shorter than that among those without Notch-1 expression, Numb, Itch, and Siah-1 expression was related to better survival. Similarly, it was noted that the gene expression levels of Notch, Numb, Itch and Siah-1 were in line with the results of the immunohistochemical evaluation. The status of Notch-1, Numb, Itch, and Siah-1 expression and their expression levels, as well as lymph node metastasis and stage, were found to be significantly associated with survival. Cox regression analysis revealed that lymph node metastasis, upregulation of Notch, and downregulation of Numb were independent prognostic factors. The Notch-1 expression correlated inversely with the levels of Numb, Itch and Siah-1 expression.

Research conclusions

Our findings support the role of Notch-1 in CRC and indicate that loss of Numb, Itch, and Siah-1 expression is associated with carcinogenesis. Our data also highlight that Numb, Itch, and Siah-1 might contribute to the Notch-1 signaling pathway during CRC progression. Therefore, these three proteins might be key in possible therapies targeting Notch-1 in the treatment of CRC.

Research perspectives

In CRC, Numb, Itch and Siah-1 contribute to carcinogenesis and tumor behavior. In contrast to Notch-1, the relationship of Numb, Itch and Siah-1 expression with better prognostic parameters and improved survival can make them a potential therapeutic target. In this regard, further studies in large patient series and cell cultures can provide comprehensive information on the efficacy of the Notch pathway in therapy.

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