Clinicopathological significance of intelectin-1 in colorectal cancer: Intelectin-1 participates in tumor suppression and favorable progress

Running title: Intelectin-1 affects tumor suppression

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Abbreviations:

CAHG, conventional adenoma high grade; CALG, conventional adenoma low grade; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; ITLN1, intelectin-1; MMP7, matrix metalloproteinase 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SSA/P, sessile serrated adenoma/polyp; TSA, traditional serrated adenoma

Abstract

Intelectin-1 (ITLN1) is an adipokine with an anti-inflammatory function that is involved in neoplastic diseases such as pleural mesothelioma and gastric and prostate cancers. However, the expression and function of ITLN1 in colorectal cancer (CRC) remain unknown. To identify novel prognostic markers or therapeutic targets for CRC, we focused on ITLN1 protein. By immunohistochemistry, 87 (59%) of 148 CRC cases showed reduced expression of ITLN1. ITLN1-reduced CRC cases were associated with higher M grades (P=0.0017) than ITLN1-retained CRC cases. Furthermore, the cases with ITLN1 retained expression tended toward a more favorable prognosis than those with reduced expression. Cell growth of the CRC cell lines transfected with ITLN1 siRNA were greater than those of the negative control cell lines transfected with siRNA. Levels of phosphorylated epidermal growth factor receptor, Erk and Akt were higher in the CRC cells transfected with ITLN1 siRNA than in control cells. Immunohistochemical analysis of human colorectal polyp specimens also revealed a sequential decrease in the expression of ITLN1 through both the conventional adenoma-carcinoma pathway and the serrated pathway. These results indicated that ITLN1 might play an important role in regulating colorectal tumorigenesis.

KEYWORDS

colorectal cancer, EGFR, Erk, Akt, intelectin-1

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of cancer-related death worldwide.[1] Several molecules associated with carcinogenesis and tumor progression have been identified,[2–4] but the mechanisms remain unclear. It would be valuable to identify new therapeutic markers and to supplement standard clinicopathological staging using molecular markers to more precisely define the subset of patients at highest or lowest risk of recurrence following CRC surgery. In the search for new therapeutic or diagnostic markers, it is generally accepted that genes expressed at high levels in tumors and at very low levels in normal tissues are ideal diagnostic or therapeutic molecules.[4,5] We previously reported Reg IV, olfactomedin 4, claudin-18 and SPC18 to be prognostic markers for CRC.[6-10]

Intelectin-1 (ITLN1) is a secreted lectin that binds to galactofuranose. It is secreted mainly from goblet cells in the intestinal tract and is involved in inflammation and parasitic infection. Recently, ITLN1 was reported to be one of the adipokines with an anti-inflammatory function.[11] It decreases the expression of C-reactive protein and tumor necrosis factor but increases insulin-induced glucose uptake in yellow adipose tissue and nitric oxide synthesis, thereby helping to prevent the development of diabetes and ischemic heart disease. The concentrations of some adipokines such as leptin and PAI-I are also elevated in neoplastic diseases such as pleural mesothelioma, lung cancer, breast cancer, CRC and prostate cancer.[12–14] However the clinicopathological significance of ITLN1 in CRC remains unknown.

The conventional adenoma-carcinoma pathway and the serrated pathway are thought to be the major colorectal carcinogenesis pathways.[15,16] Activation of Wnt/β-catenin signaling has been reported to occur by different mechanisms in both pathways.[17] In addition, there are several immunohistochemical markers that can support traditional morphological diagnosis in tumors that arise through the colorectal carcinogenesis pathway, including p53, β-catenin,

claudin-18, SPC18 and MSH2.[9,10,18] Therefore, we investigated both the clinicopathological significance and biological function of ITLN1 in CRC and analyzed the expression of ITLN1 in precancerous lesions arising via the colorectal carcinogenesis pathways.

MATERIALS AND METHODS

Tissue samples and cell lines

We collected primary tumors from 148 patients diagnosed as having CRC who underwent curative resection surgery between 1997 and 2009 at Hiroshima University Hospital. Only patients untreated with preoperative radiotherapy or chemotherapy were enrolled in this study. The study population included 70 men and 78 women. Postoperative follow-up was scheduled every 1, 2 or 3 months during the first 2 years after surgery and every 6 months thereafter, unless more frequent follow-up was deemed necessary. The histological classifications were determined based on the World Health Organization system. Tumor staging was performed according to the TNM stage grouping system. Because written informed consent was not obtained, for strict privacy protection, all of the identifying information associated with the samples was removed before the analysis. This study was approved by the Ethical Committee for Human Genome Research of Hiroshima University, Hiroshima, Japan (no. IRINHI66).

Human colon cancer-derived cell lines WiDr, DLD-1, LoVo, COLO-201 and COLO-320 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All five cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS, Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Quantitative RT-PCR analysis and western blotting

Total RNA was extracted with a RNeasy Mini kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA using a First Strand cDNA Synthesis kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitative RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously.[19] The sequences were as follows: ITLN1, product length: 148 bp; Forward, 5′-ATGAACCAACTCAGCTTCCTGCTGTTTCTCATA-3′, Reverse: 5′-TCAACGATAGAATAGAAGCACAGCTGCCTCAGT-3′. β-Actin (*ACTB* gene) was used as an internal housekeeping control. Western blotting was performed as described previously.[20]

Antibodies

Anti-ITLN1 antibody was purchased from Immuno-Biological Laboratories (#10383, 1:300, Gunma, Japan). We also used 10 antibodies for analysis of the CRC: anti-β-catenin (#sc-7963, 1:100, Santa Cruz Biotechnology, Dallas, TX, USA), anti-matrix metalloproteinase 7 (MMP7) (#MAB3315, 1:100, Daiichi Fine Chemical, Takaoka, Japan), anti-p53 (#NCL-L-p53-DO7, 1:50, Novocastra, Newcastle, UK), anti-CDX2 (#GTX113160, 1:50, BioGenex, San Ramon, CA, USA), anti-MUC5AC (#760-4389, 1:50, Novocastra), anti-MUC2 (#760-4388, 1:50, Novocastra), anti-claudin-18 (#388000, 1:50, Invitrogen/Zymed Laboratories, San Francisco, CA, USA), anti-CD44 (#DF1485, 1:50, Santa Cruz Biotechnology) and anti-MLH1 and anti-MSH2 (#PC56-100UGCN and #NA27-100UGCN, both, 1:100; Merck, Darmstadt, Germany).

Immunohistochemistry

We used archival formalin-fixed paraffin-embedded tissues from the 148 study patients. The immunohistochemical analysis was performed with a Dako Envision+ Mouse Peroxidase

Detection System (Dako Cytomation, Carpinteria, CA, USA). Antigen retrieval was performed by microwave heating in citrate buffer (pH 6.0) for 30 min. Peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, and the sections were incubated with normal goat serum (Dako Cytomation) for 20 min to block nonspecific antibody binding sites. Sections were then incubated with a mouse monoclonal anti-ITLN1 antibody (dilution 1:100) for 1 h at room temperature, followed by incubation with Envision+ anti-mouse peroxidase for 1 h, and then by incubation with DAB Substrate-Chromogen Solution (Dako Cytomation) for 10 min for the color reaction. The sections were counterstained with 0.1% hematoxylin. Negative controls were created as above but with omission of the primary antibody.

The expression of ITLN1 in CRC was evaluated in all tumors as either preserved or reduced. When less than 5% of tumor cells were stained, the immunostaining was considered to indicate reduced expression of ITLN1 (according to the median cut-off values rounded off to the nearest 5%). The expression of MMP7, β-catenin, p53, CDX2, MUC2, MUC5AC, claudin-18, CD44, MLH1 and MSH2 was scored in all tumors as positive or negative. When more than 5% of tumor cells were stained, the immunostaining was considered to be positive for each molecule.

The expression of ITLN1 in pre-cancerous lesions was scored in all tumors as negative (score 0), mild (score 1), moderate (score 2) or strong (score 3) according to their immunostaining intensity. Using these definitions, two surgical pathologists (NK and KS), who had no knowledge of the clinical or pathological parameters or of the patients' outcomes, independently reviewed the immunoreactivity of each specimen. Interobserver differences were resolved by consensus review at a double-headed microscope after independent review.

RNA interference

To knock down endogenous ITLN1, RNAi was carried out as described previously.[19] siRNA oligonucleotides for ITLN1 and a negative control were purchased from Invitrogen. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. The cells were analyzed at 48 h after transfection in all experiments.

Cell growth assays

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to examine cell growth. The cells were seeded at a density of 4000 cells/well in 96-well plates. Cell growth was monitored after 1, 2 and 4 days. We performed three different experiments and calculated the mean and standard deviation in the MTT assay.

Statistical analysis

Correlations between the clinicopathological parameters and the expression of ITLN1 were analyzed using the $\chi 2$ test. Kaplan-Meier survival curves were constructed for patients with preserved ITLN1 and reduced ITLN1, and survival rates of the two groups were compared. Differences between the survival curves were tested for statistical significance by log-rank test. Univariate and multivariate Cox regression analyses were used to evaluate the associations between clinical covariates and survival. A *P*-value of < 0.05 was considered to indicate statistical significance. The hazard ratio (HR) and 95% confidence interval (CI) were estimated from Cox proportional hazard models. Statistical differences were evaluated using the two-tailed Student *t*-test. Age was treated as a categorical variable (≤ 65 years vs. ≥ 66

years). All variables found to be moderately associated ($P < 0.10$) with survival by a univariate analysis were included in the final multivariate Cox regression models. P values of < 0.05 were considered to indicate statistical significance. The SPSS software program (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses.

RESULTS

Messenger RNA expression of ITLN1 in systemic normal organs, CRC cell lines and CRC tissue

To confirm whether the ITLN1 gene is cancer specific, quantitative RT-PCR was performed in five CRC cell lines and in 14 types of normal tissue. ITLN1 expression was detected at higher levels in colon than in various normal organs. However, high ITLN1 expression was also observed in all five CRC cell lines (Supporting Figure. 1a), but these expression levels were all lower than that in normal colon. Moreover, we analysed ITLN1 expression in 20 CRC tissue samples and 20 corresponding non-neoplastic mucosa samples by quantitative RT-PCR. We calculated the ratio of mRNA expression levels between CRC tissue (T) and corresponding non-neoplastic mucosa (N). A T/N ratio < 1 was considered to represent reduced expression. ITLN1 mRNA was reduced in 16 (80%) of the 20 cases (Supporting Figure. 1b).

Expression and distribution of ITLN1 in CRC and its relationship with clinicopathological parameters

We used immunohistochemistry to investigate the expression of ITLN1 in the 148 human CRC samples. Reduced ITLN1 expression was observed in the tumor region compared to the non-neoplastic region (Fig. 1a). ITLN1 staining was reduced in 87 (59%) of the 148 CRC cases. In the non-neoplastic colonic mucosa, ITLN1 expression was limited to the cytoplasm

of the goblet cells (Fig. 1a). Staining for ITLN1 was detected in the cytoplasm of the tumor cells (Fig. 1b). Localization of ITLN1-preserved cases was irrespective of tumor center or tumor invasive region. Next, we analyzed the relationship between the reduced expression of ITLN1 and various clinicopathological characteristics. CRC cases with reduced ITLN1 expression showed a more advanced M classification ($P = 0.0017$), tumor stage ($P = 0.005$) and budding grade ($P = 0.001$) than those with preserved ITLN1 expression (Table 1). The reduced expression of ITLN1 was not associated with age, sex or histologic classification.

Relationship between reduced expression of ITLN1 in CRC and prognosis

We performed a Kaplan-Meier analysis to investigate the association between the expression of ITLN1 and prognosis to further elucidate the clinical impact of ITLN1 on CRC in our 148 patients. The reduced expression of ITLN1 was significantly associated with a poorer prognosis ($P = 0.0155$, Fig. 1c). In the univariate analysis, the reduced expression of ITLN1 ($P = 0.0126$), tumor stage ($P < 0.0001$) and budding grade ($P = 0.0006$) were associated with survival. In the multivariate analysis, the reduced expression of ITLN1, M grade and tumor stage were the independent prognostic predictors for survival in the 148 CRC patients (Supporting Table 1). According to OncoLnc, which was derived from The Cancer Genome Atlas database, the reduced expression of ITLN1 was significantly associated with a worse prognosis in CRC (Fig. 1d).[21]

Effect of ITLN1 inhibition on cell growth activity of CRC cells

We performed a biological study of ITLN1 using the CRC cell lines. Western blotting revealed that all three CRC cell lines expressed ITLN1 at various levels (Fig. 2a). The highest expression of ITLN1 was detected in DLD-1 followed by WiDr, whereas the remaining cell line expressed ITLN1 at a low level. Next, we examined the transition of ITLN1 expression

by Western blotting using DLD-1 and WiDr cell lines that had been transfected with three *ITLN1*-specific siRNAs (siRNA1, 2, and 3) because the highest expression of ITLN1 was detected in both of these cell lines (Fig. 2b). The expression of the ITLN1 protein in DLD-1 and WiDr was strongly suppressed by treatment with siRNA1 and 2. Thus, we used these two siRNAs in the following experiments to knock down the endogenous ITLN1. To investigate the possible anti-proliferative effects of ITLN1 knockdown, we performed an MTT assay at 4 days after the transfection of siRNA. The viability of the DLD-1 cells transfected with ITLN1 siRNA1 and 2 was significantly increased in comparison to the negative control DLD-1 cells transfected with siRNA (Fig. 2c). We performed the same assay in WiDr cells and obtained similar results (Fig. 2d).

Effect of ITLN1 inhibition on the phosphorylation of EGFR, AKT and ERK in CRC cells

It is well-known that TGF-α can phosphorylate epidermal growth factor receptor (EGFR), which subsequently stimulates the multiple signaling pathways involved in cellular proliferation, anti-apoptosis and other processes, including the Ras-Mek-Erk and Akt-PI3K pathways.[22,23] To confirm the ITLN1-induced activation of EGFR signaling in CRC, we analyzed the phosphorylation of EGFR, Akt and Erk in CRC cells with ITLN1 inhibition. The levels of phosphorylated EGFR, Erk, and Akt in the DLD-1 and WiDr cells transfected with ITLN1 siRNA1 or 2 were high in comparison to those in two CRC cell lines that had been transfected with negative control siRNA (Fig. 3a, b). These data suggested that ITLN1 could contribute to tumor progression in CRC.

Analysis of the correlation between ITLN1 expression and CRC-related molecules

We next investigated the relationship between the underexpression of ITLN1 and CRC-related major molecules, including β-catenin, MMP7, p53, CDX2, MUC2, MUC5AC, claudin-18, CD44, MLH1 and MSH2 (Fig. 4a–g). We revealed that the underexpression of ITLN1 was colocalized with β-catenin nuclear localization (P=0.012) and the expression of MMP7 (P=0.026), CDX2 (P=0.026), MUC5AC (P=0.02), claudin-18 (P=0.042) and CD44 (P=0.027) (Table 2). These data indicated the possibility of a correlation between ITLN1 and Wnt/β-catenin signaling.

Analysis of the expression of ITLN1 in pre-cancerous lesions

Finally, we performed an immunohistochemical analysis of ITLN1 in 96 human colorectal polyp specimens, including conventional adenoma low grade (CALG), conventional adenoma high grade (CAHG), traditional serrated adenoma (TSA) and sessile serrated adenoma/polyp (SSA/P) (Fig. 5a–d). Staining of weak or moderate intensity was sequentially increased in normal tissue, CALG and CAHG (P=0.012), and CALG showed higher ITLN1 expression than CAHG (Fig. 5e). The same tendency was also shown in normal tissue, TSA and SSA/P (Fig. 5f).

DISCUSSION

Quantitative RT-PCR and immunohistochemical analysis showed that ITLN1 expression was reduced in more than half of the CRC cases investigated. The expression of ITLN1 was associated with M classification, tumor stage and budding grade. The reduced expression of ITLN1 was also found to be an independent prognostic classifier of patients with CRC.

The histological features of CRC differ widely from area to area within the same tumor due to tumor heterogeneity. The most useful clinicopathological features and molecular signatures, including budding grade, can be deduced from the invasive front of the tumor,

where the most transformed and presumably most aggressive cells reside.[24] Although ITLN1 expression was not always observed at the invasive front, its expression correlated significantly with the budding grade. Thus, ITLN1 is likely to suppress tumor progression through several cancer-related molecules, especially in the invasive region in cases with preserved ITLN1. In fact, the reduced expression of ITLN1 correlated with β-catenin nuclear localization and the expression of MMP7, MUC5AC, claudin-18 and CD44. We previously revealed that MUC5AC and claudin-18 are also associated with poor prognosis in CRC.[25]

Downregulation of CDX2 in CRC was reported to increase throughout tumor progression,[26,27] and the expression of CDX2 was reported to reduce tumorigenesis in CRC cell lines.[28] Moreover, the prognosis of patients with negative CDX2 expression was significantly poorer than that of patients with positive expression. In the present study, the reduced expression of ITLN1 correlated with the negative expression of CDX2.

Although some reports suggested a positive influence of ITLN1 on tumor progression via the Akt signaling pathway,[29,30] we showed that ITLN1 could inhibit the activity of pAkt/Akt, and the underlying mechanism of inhibiting colon cancer stem cells and promoting apoptosis might be related to the inhibition of Akt activity, as reported in gastric cancer and neuroblastoma.[31] To our knowledge, this is the first study to show that the levels of phosphorylated EGFR and its downstream molecules, including Erk and Akt, were higher in CRC cells transfected with ITLN1 siRNA than in control cells. The phosphorylation of Erk and Akt was reported to result in the inhibition of apoptosis and to contribute to tumor progression, including metastasis.[32–34] Thus, these results suggested that ITLN1 reduces malignant behavior, including the cell growth, metastasis and invasion of CRC cells.

The present study also showed the sequentially reduced expression of ITLN1 in precancerous lesions. The expression of ITLN1 was significantly reduced in tumors related both to the conventional adenoma-carcinoma pathway (CALG and CAHG) and to the

serrated pathway (TSA and SSA/P). The conventional adenoma-carcinoma pathway is well known as a multistep carcinogenesis mechanism associated with the activation of Wnt/β-catenin signaling,[16,23] which promotes tumor malignancy and induces the expression of MMP7.[32] Meanwhile, claudin-18 is associated with the serrated pathway, which is overexpressed in hyperplastic polyps, SSA/Ps, and TSAs, compared with conventional adenomas.[9] In the present study, the reduced expression of ITLN1 correlated with the positive expression of claudin-18. These results suggested that ITLN1 may be involved in both pathways.

In summary, our results implied that the underexpression of ITLN1 is correlated with the progression of CRCs, and we revealed that CRC with the reduced expression of ITLN1 was independently associated with a poor prognosis. ITLN1 reduces the phosphorylation of EGFR and modulates downstream targets. Thus, ITLN1 may have potential as a predictive biomarker for the survival of patients with CRC.

DISCLOSURE STATEMENT

None declared.

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AUTHOR CONTRIBUTIONS

NK (first author) performed all data collection and analyses. NO, VA and YT assisted with the clinical data collection. YS, YY, GK and TB provided assistance with the molecular technologies used. WY provided input regarding the preparation of the manuscript. KS (corresponding author) contributed to the preparation of the manuscript.

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Figure legends

Figure 1 Immunohistochemical analysis of ITLN1 in colorectal cancer (CRC) tissue specimens. (a) ITLN1 immunostaining in the non-neoplastic colonic mucosa and CRC. ITLN1 staining was observed in the cytoplasm of goblet cells in non-neoplastic colonic mucosa, whereas ITLN1 staining was reduced in CRC cells (original magnification × 100). (b) ITLN1 immunostaining in CRC (original magnification × 100). Some ITLN1 staining was observed in the cytoplasm of CRC cells. (c) Kaplan-Meier plot of the survival of CRC patients. (d) Prognostic value of ITLN1 in CRC patients and the survival curve as plotted using the OncoLnc database.

Figure 2 Effects of the inhibition of ITLN1 on cell growth in colorectal cancer (CRC) cells. (a) Western blotting of ITLN1 in the cell lysates from three CRC cell lines. (b) Western blotting of ITLN1 in cell lysates from DLD-1 and WiDr transfected with ITLN1 siRNA or negative control siRNA. β-Actin was included as a loading control. (c, d) Effect of ITLN1 knockdown on the cell growth of DLD-1 (c) and WiDr (d). Cell growth was assessed by an MTT assay at 1, 2 and 4 days after seeding on 96-well plates. The mean (bars) and standard deviation (SD; error bars) of three independent experiments are shown. OD, optical density. $*P < 0.05$.

Figure 3 Effect of the downregulation of ITLN1 on the epidermal growth factor receptor (EGFR) signaling pathway. (a, b) Western blotting of ITLN1, EGFR, phospho-EGFR (pEGFR), Erk1/2, phospho-Erk1/2 (pErk1/2), Akt and phospho-Akt (pAkt) in cell lysates from DLD-1 (c) and WiDr (d) transfected with *ITLN1* siRNA or negative control siRNA.

β-actin was included as a loading control. We used three filters in each cell line. We confirmed that total proteins are almost the same amounts in all lanes as monitored by β-actin. In this figure, representative western blots for β-actin are shown.

Figure 4 Analysis of the correlation between the expression of ITLN1 and colorectal cancer (CRC)-related molecules in the invasive front. (a) Expression levels of ITLN1 (b–g) CRC-related molecules, including β-catenin, matrix metalloproteinase 7 (MMP7), CDX2, claudin-18, MUC5AC and CD44 were examined. A serial section showed that β-catenin, MMP7, CDX2, claudin-18, MUC5AC and CD44 at the invasive front were partially adjacent to the area in which ITLN1 was reduced.

Figure 5 Analysis of the expression of ITLN1 in pre-cancerous lesions of each colorectal carcinogenesis pathway. (a–d) Immunostaining of adenoma in colorectal polyps (CRPs). (a) Conventional adenoma low grade, n=32; (b) conventional adenoma high grade (CAHG), n=64; (c) traditional serrated adenoma (TSA), n=27 and (d) sessile serrated adenoma/polyp (SSA/P), n=20. (e, f) ITLN1 immunostaining scores in CRP. The graph indicates the percentage of sections with different scores (negative, weak, moderate and strong).

Supporting Information

Supporting Figure 1 Quantitative reverse transcriptase PCR analysis of intelectin-1 (ITLN1). (a) ITLN1 mRNA expression level in 13 normal tissues and 5 colorectal cancer (CRC) cell lines. (b) T/N ratio of ITLN1 mRNA level between CRC tissue (T) and corresponding nonneoplastic mucosa (N) in 20 CRC cases. Underexpression was defined as a T/N ratio < 1.0. Underexpression of the ITLN1 gene was observed in 16 (80%) of the 20 GC cases.

Supporting Table 1 Univariate and multivariate analysis of factors influencing survival in 148 patients with colorectal cancer

Table 1. Relationship between ITLN1 expression and clinicopathologic characteristics in the 148 CRC cases

	ITLN1 expression		*P*-value
	Reduced (%)	Preserved	
Age			
≦ 65 (*n* = 70)	41 (59%)	29	NS
≧ 66 (*n* = 78)	46 (59%)	32	
Sex			
Female (*n* = 89)	55 (62%)	34	NS
Male (*n* = 59)	32 (54%)	27	
T grade			
T1/T2 (*n* = 25)	11 (44%)	14	NS
T3/T4 (*n* = 123)	76 (62%)	47	
N grade			
N0 (*n* = 81)	46 (57%)	35	NS
N1/2/3 (*n* =67)	41 (61%)	26	
M grade			
M0 (*n* = 129)	70 (54%)	59	0.0017
M1 (*n* = 19)	17 (89%)	2	
Stage			
Stage I / II (*n* = 40)	16 (40%)	24	0.005
Stage III/IV (*n* = 108)	71 (66%)	37	
Budding Grade			
Low(Grade1) (*n* = 98)	48 (49%)	50	0.001
High(Grade2/3) (*n* = 50)	39 (78%)	11	
Histlogic classification			
Well/moderate (*n* = 144)	84 (58%)	60	NS
Poor/mucinous (*n* = 4)	3 (75%)	1	

P values were calculated with Fisher's exact test.

NS, not significant.

Table 2. Relationship between ITLN1 expression and cancer related molecules in 126 patients with colorectal cancer

	ITLN1 expression		*P*-value
	Reduced (%)	Reserved	
β-catenin (nuclear localization)			
Positive (*n* = 87)	55 (63%)	32	0.012
Negative (*n* = 39)	15 (38%)	24	
MMP7			
Positive (*n* = 40)	28 (70%)	12	0.026
Negative (*n* = 86)	42 (49%)	44	
P53 expression			
Positive (n = 69)	39 (57%)	29	NS
Negative (*n* = 58)	31 (53%)	27	
CDX2			
Positive (n = 73)	26 (36%)	47	<0.001
Negative (*n* = 53)	44 (83%)	9	
MUC2			
Positive (n = 79)	41 (52%)	38	NS
Negative (n = 47)	29 (61%)	18	
MUC5AC			
Positive (n = 67)	44 (65%)	23	0.02
Negative (n = 59)	26 (38%)	33	
Claudin-18			
Positive (n = 15)	12 (80%)	3	0.042
Negative (n = 111)	58 (53%)	53	
CD44			
Positive (n = 79)	50 (63%)	29	0.027
Negative (n = 47)	20 (42%)	27	
MLH1			
Positive (n = 70)	39 (56%)	31	NS
Negative (n = 56)	31 (55%)	25	
MSH2			
Positive (*n* = 78)	45 (57%)	33	NS
Negative (*n* = 48)	25 (52%)	23	

P values were calculated with Fisher's exact test.

NS, Not significant.

Supporting Table 1. Univariate and multivariate analysis of factors influencing survival in 148 patients with colorectal cancer

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age				
≦65	1 (Reference)	NS		
≧66	1.49 (0.79-2.92)			
Sex				
Female	1 (Reference)	NS		
Male	0.87 (0.45-1.65)			
Classification				
T grade	5.18 (0.97-5.03)	＜0.0001	2.47 (0.47-2.12)	NS
N grade	4.89 (2.45-10.59)	＜0.0001	2.05 (1.45-8.39)	NS
M grade	5.52 (2.78-10.51)	＜0.0001	2.81 (1.39-5.67)	0.003
Tumor stage				
Stage I/II	1 (Reference)	＜0.0001	1 (Reference)	0.0041
Stage III/IV	5.08 (0.10-12.04)		3.34（1.44-8.36）	
Budding Grade				
Low (Grade 1)	1 (Reference)	0.0006	1 (Reference)	NS
High (Grade 2/3)	3.31 (1.47-7.31)		1.56（077-4.47）	
ITLN1 expression				
Reduced	1 (Reference)	0.0126	1 (Reference)	0.0376
Preserved	2.93 (1.09-4.81)		2.57（1.16-5.34）	

P values were calculated with Fisher's exact test.

NS, not significant

Figure 1

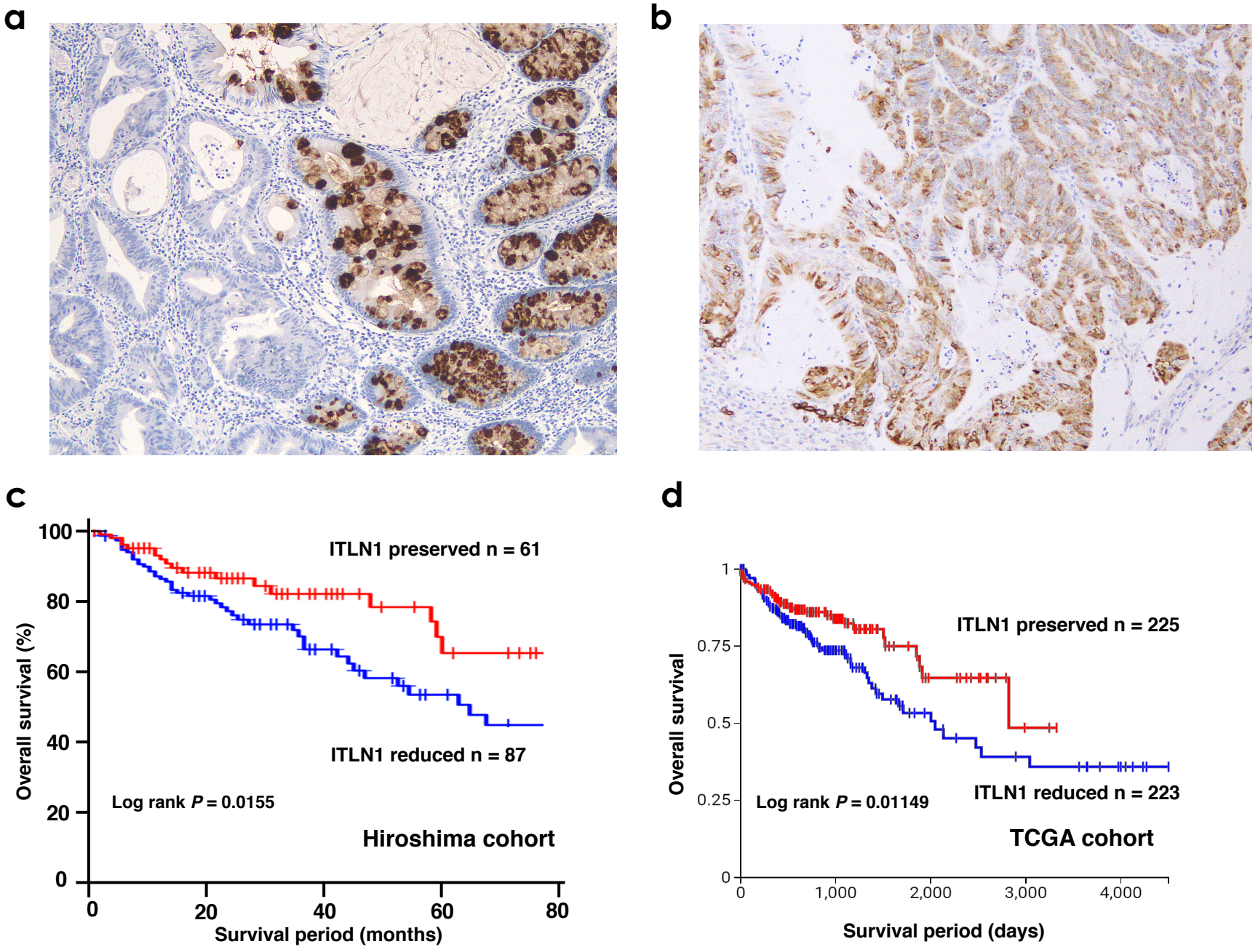

a
b
c
100
80
60
40
20
0
Overall survival (%)
ITLN1 preserved n = 61
ITLN1 reduced n = 87
Log rank P = 0.0155
Hiroshima cohort
0
20
40
60
80
Survival period (months)
d
1
0.75
0.5
0.25
0
Overall survival
ITLN1 preserved n = 225
ITLN1 reduced n = 223
Log rank P = 0.01149
TCGA cohort
0
1,000
2,000
3,000
4,000
Survival period (days)

Figure 2

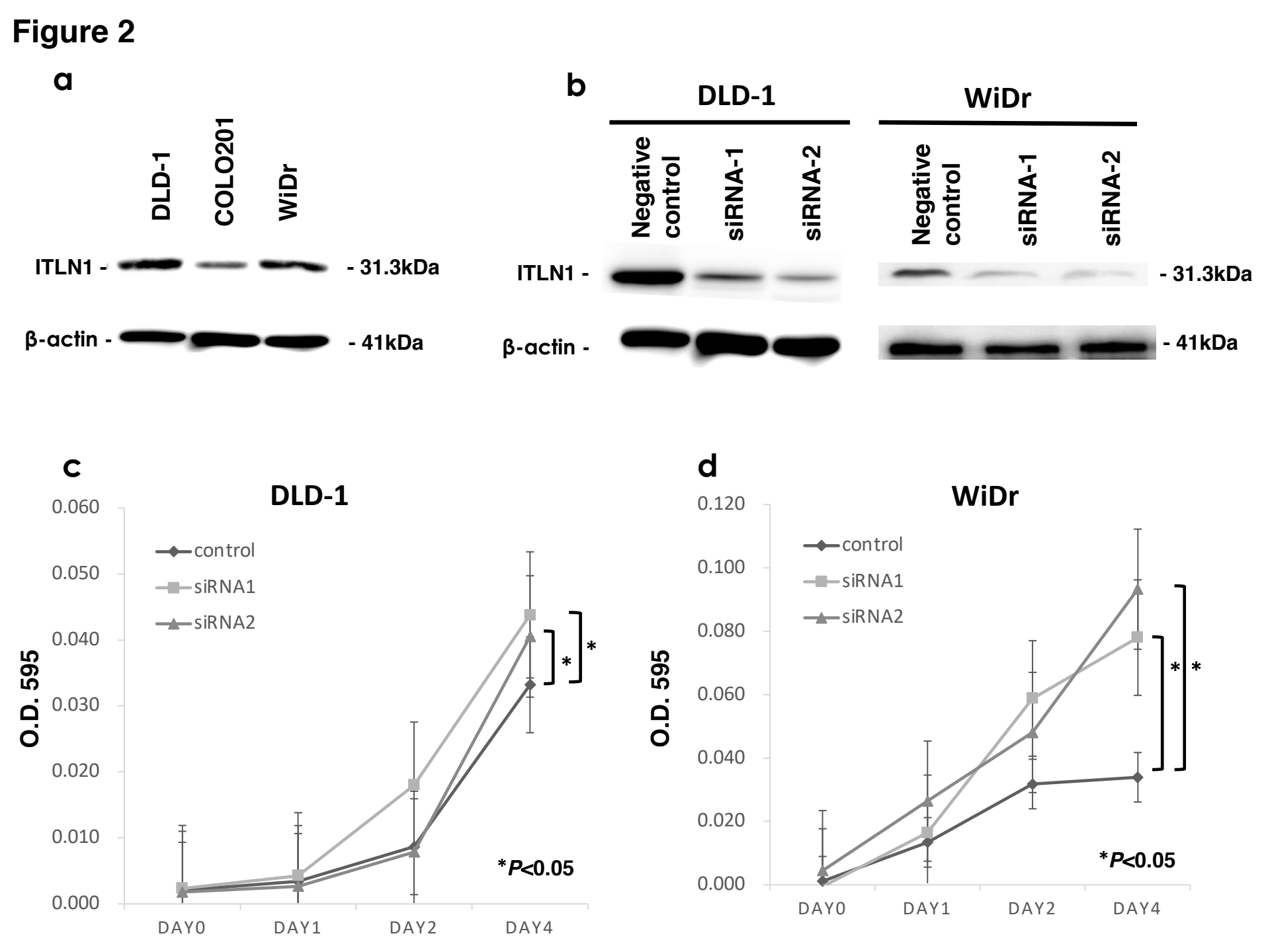

a
DLD-1
COLO201
WiDr
ITLN1 -
- 31.3kDa
β-actin -
- 41kDa
b
DLD-1
WiDr
Negative control
siRNA-1
siRNA-2
ITLN1 -
- 31.3kDa
β-actin -
- 41kDa
c
DLD-1
control
siRNA1
siRNA2
O.D. 595
0.060
0.050
0.040
0.030
0.020
0.010
0.000
DAY0
DAY1
DAY2
DAY4
*
*
*P<0.05
d
WiDr
control
siRNA1
siRNA2
O.D. 595
0.120
0.100
0.080
0.060
0.040
0.020
0.000
DAY0
DAY1
DAY2
DAY4
*
*
*P<0.05

Figure 3

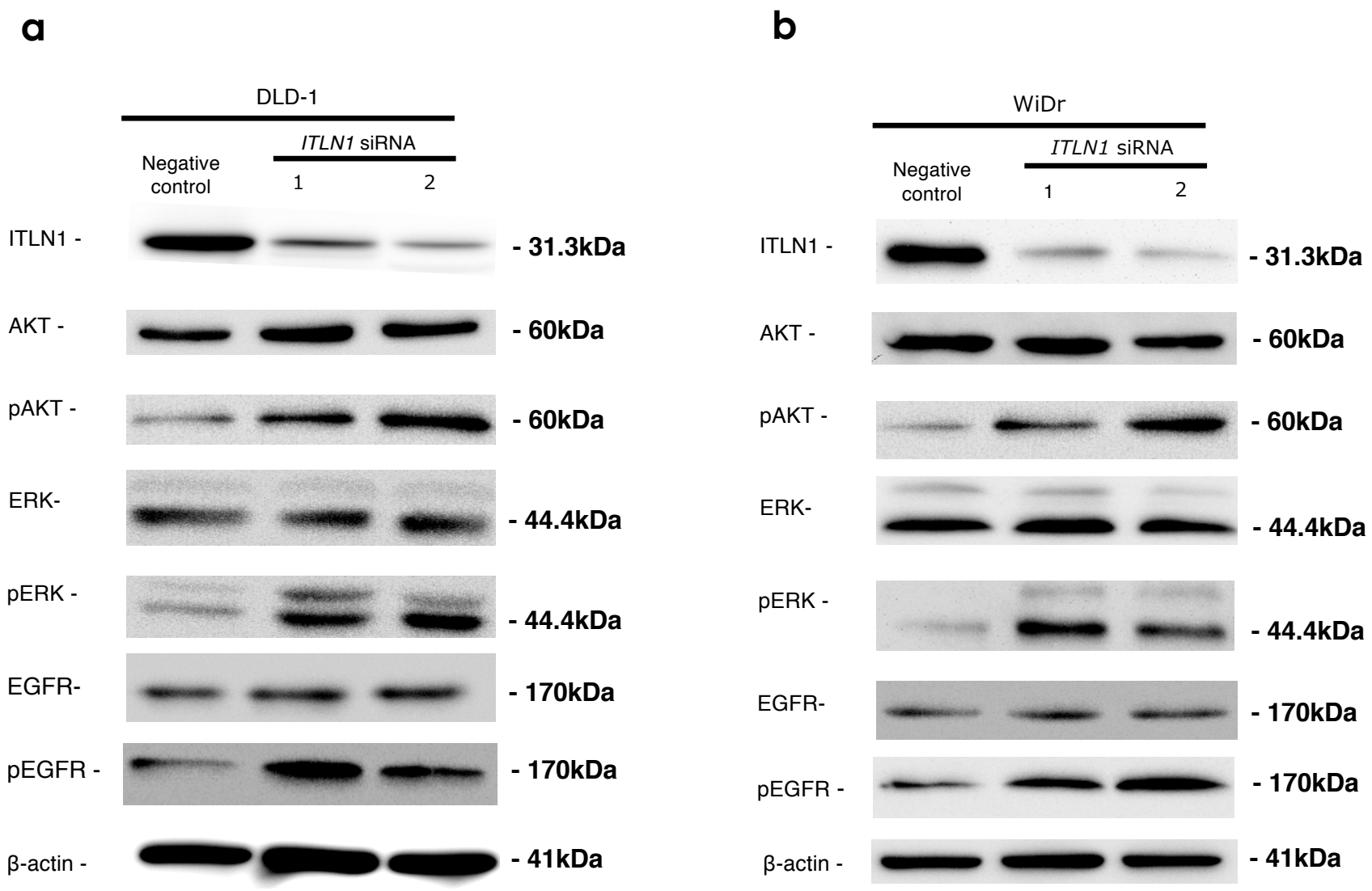

a
DLD-1
Negative control
ITLN1 siRNA
1
2
ITLN1 -
- 31.3kDa
AKT -
- 60kDa
pAKT -
- 60kDa
ERK-
- 44.4kDa
pERK -
- 44.4kDa
EGFR-
- 170kDa
pEGFR -
- 170kDa
β-actin -
- 41kDa
b
WiDr
Negative control
ITLN1 siRNA
1
2
ITLN1 -
- 31.3kDa
AKT -
- 60kDa
pAKT -
- 60kDa
ERK-
- 44.4kDa
pERK -
- 44.4kDa
EGFR-
- 170kDa
pEGFR -
- 170kDa
β-actin -
- 41kDa

Figure 4

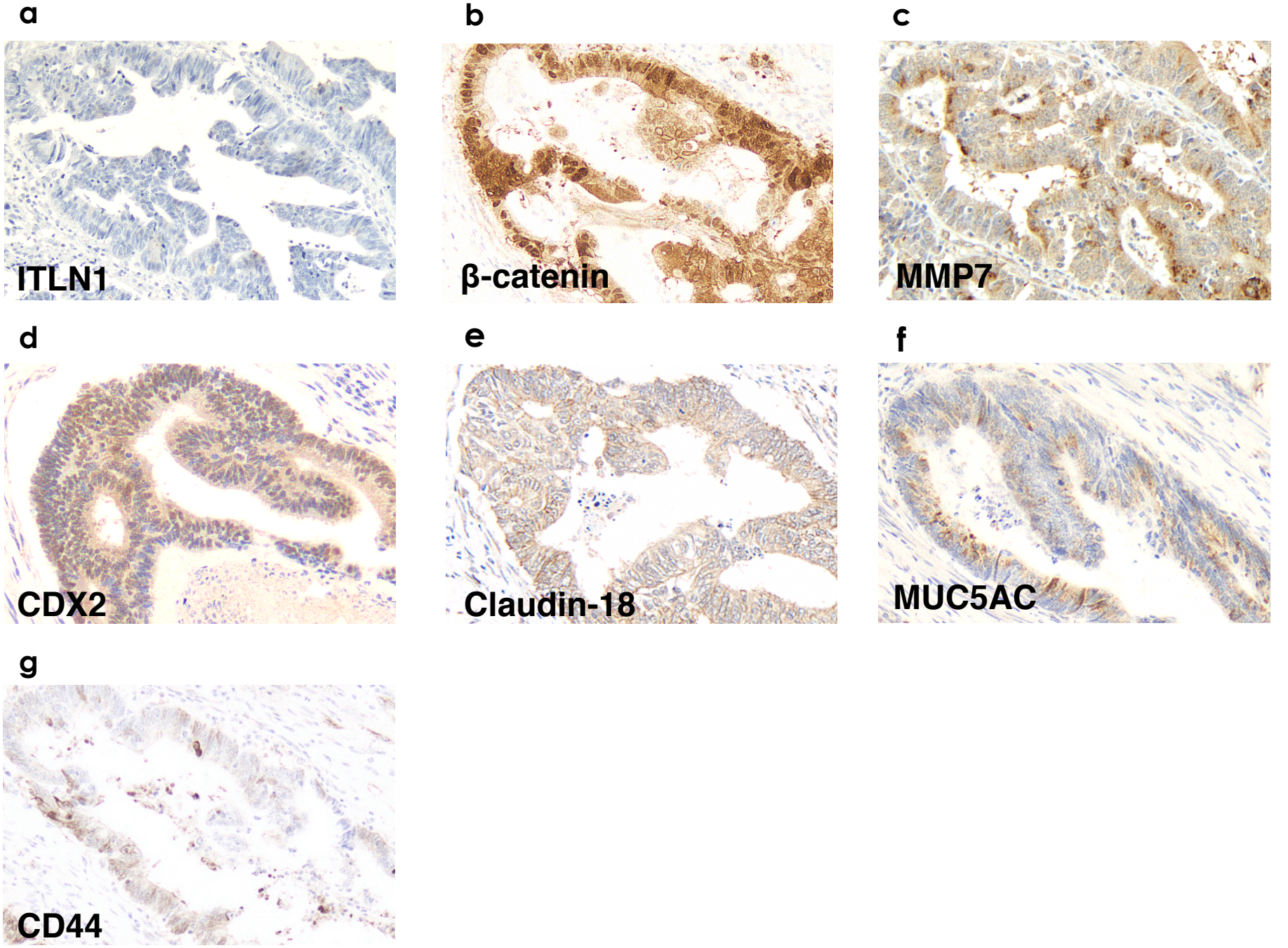
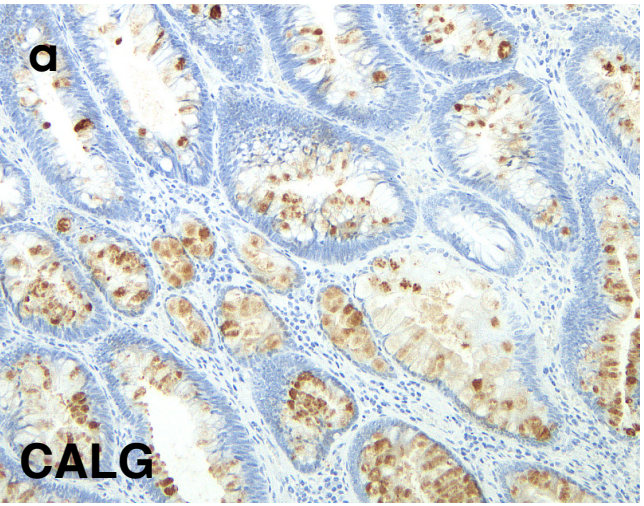
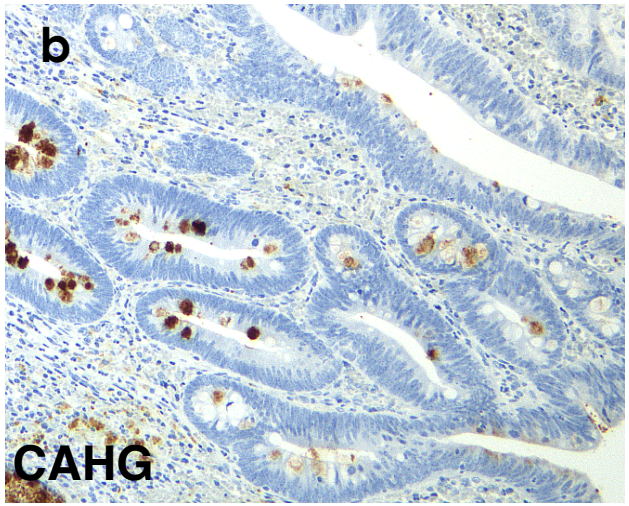
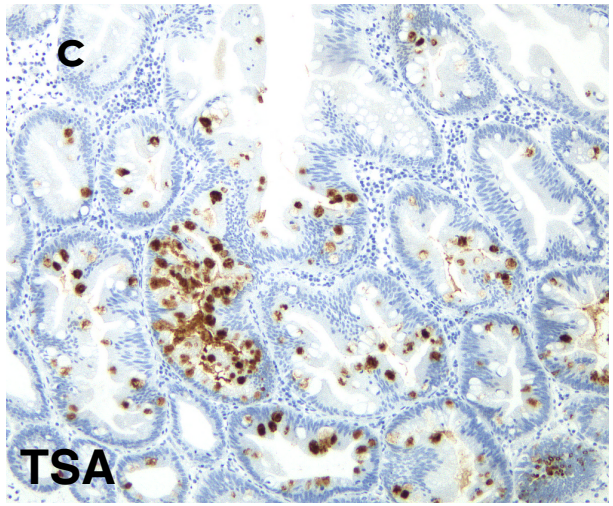
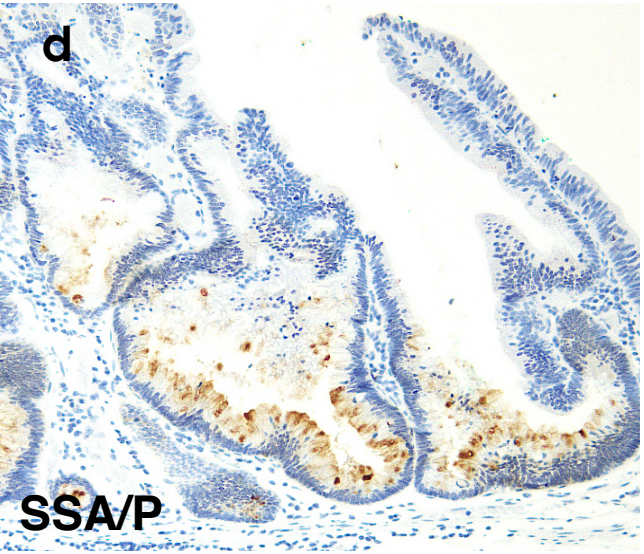


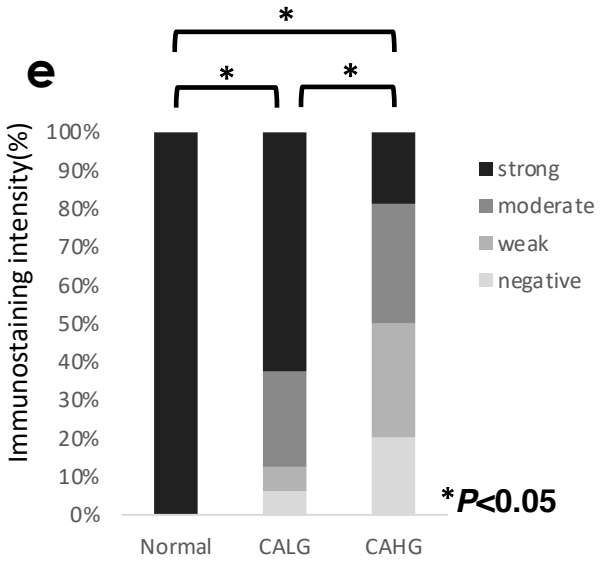
Figure 5

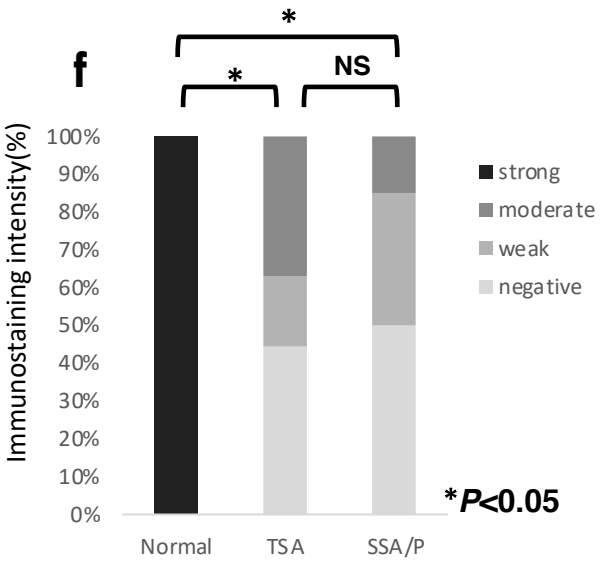

a
CALG


b
CAHG


c
TSA


d
SSA/P


e
*
*
*
Immunostaining intensity(%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Normal
CALG
CAHG
strong
moderate
weak
negative
*P<0.05


f
*
*
NS
Immunostaining intensity(%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Normal
TSA
SSA/P
strong
moderate
weak
negative
*P<0.05

Supporting Figure 1