TROY is a promising prognostic biomarker in patients with colorectal cancer

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Abstract. Tumor necrosis factor receptor superfamily member 19 (*TROY*) is involved in the Wnt/β-catenin signaling pathway and interacts with leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5), which is a well-known biomarker of cancer stem cells and a prognostic marker of colorectal cancer (CRC). Because there have been no studies to evaluate the prognostic significance of *TROY*, we performed the present study to determine whether *TROY* can be a prognostic biomarker in CRC patients. We evaluated *TROY* expression levels in 100 CRC tissues by quantitative real-time PCR and investigated the association of *TROY* expression levels with clinicopathologic features. Cancer stage and *TROY* expression level were found to be independent prognostic factors of disease-free survival. Moreover, *TROY* overexpression was the sole independent prognostic factor of disease-free survival in patients with stage II and III CRC. These results suggest that analysis of *TROY* might help predict clinical outcome in patients with CRC. To support our findings, confirmatory studies using independent data sets are needed.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related deaths worldwide, accounting for roughly 1.36 million new people and 690,000 deaths per year in 2012 (1). The outcome of patients with CRC is related to the stage at diagnosis: The 5-year relative survival is 95.0% for CRC patients with stage I, 83.3% for those with stage II, 77.4% for those with stage III, and 16.9% for those with stage IV (2). Although adjuvant chemotherapy is recommended for CRC with high risk of relapse, its administration to patients with stage II and III CRC, in which the 5-year relapse rates are approximately 20 and 35-50%, respectively, remains controversial (3,4). Because patients with CRC that is identical in grade and stage often have significantly different clinical outcomes or responses to therapy and there are no established biomarkers to predict relapse, improved tumor classification is needed for CRC, especially for stage II and III CRC.

The Wnt/β-catenin signaling pathway plays an essential role in the development of various tumors (5,6). It is well known that the Wnt/β-catenin signaling pathway is activated in most sporadic CRC (up to 80%) (7). Recently, LGR5 (leucine-rich repeat containing G-protein-coupled receptor 5) was identified as the target gene of the Wnt/β-catenin signaling pathway (8,9). LGR5 is reported to be a biomarker of cancer stem cells of CRC and stem cells in adult intestinal crypts (10,11). TROY (tumor necrosis factor receptor superfamily member 19, TNFRSF19, TAJ) is a type I cell surface receptor protein containing the highly conserved TNFR cysteine-rich motifs in the extracellular domain and a TNF receptor-associated factor (TRAF)-binding sequence in the large cytoplasmic domain required for signaling (12). Recently, *TROY* was reported to be a β-catenin target gene and to form a complex with LGR5 in cellular membranes (13). Although overexpression of TROY is observed in CRC cell lines (14-17), its clinical significance for CRC is poorly understood. Because TROY might be a possible prognostic biomarker of CRC, we performed this study to

investigate the clinical significance of TROY in patients with CRC and compared its clinical usefulness to that of LGR5.

Materials and methods

Materials. We collected 100 CRC tissues from 100 patients who underwent surgical treatment at the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine between March 2000 and May 2008. From them, 36 matched normal-appearing mucosa tissues were also collected from a site distal to the resected materials. All samples were immediately frozen in liquid nitrogen after sample collection from surgically resected materials and then stored at -80˚C. Total RNA was isolated using the AllPrep DNA/RNA Mini Kit (QIAGEN). The extracted total RNA was reverse transcribed into single-stranded cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The clinicopathologic characteristics of the CRC patients are shown in Table I. The patient population consisted of 51 men and 49 women with a mean age of 66.9 years (range, 38-92 years). According to the staging system of the International Union Against Cancer (UICC) (18), 14 patients were stage I, 36 patients were stage II, 30 patients were stage III, and 20 patients were stage IV. After surgical treatments, 66 patients were treated with adjuvant chemotherapies using tegafur/uracil, cisplatin, or irinotecan, and 34 patients did not receive any adjuvant chemotherapy. This study was approved by the Institutional Review Board of Yamaguchi University Hospital (approval number: H28-073), and written informed consent was obtained from each patient before inclusion in the study.

Quantitative real-time PCR. Quantitative real-time PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The Applied Biosystems catalog numbers of the primer and probe set for the endogenous control (β-actin) and target genes were 4326315E for β-actin, Hs00969422_m1 for *LGR5*, and Hs00218634_m1 for *TROY*. Quantitative real-time PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR cycling conditions included preheating at 50˚C for 2 min for optimal UDG (uracil-DNA glycosylase) enzyme activity and at 95˚C for 10 min for AmpliTaq Gold DNA polymerase and UP enzyme activation followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 60˚C for 1 min. All reactions were carried out in a 20-μl reaction volume in triplicate. The mRNA expression level was determined using the $2^{-\Delta\Delta CT}$ method, in which relative quantification of mRNA expression level was calculated using β-actin as the internal reference (19).

Statistical analysis. Data analyses were performed using StatFlex v6 (Artech Co., Ltd., Osaka, Japan). Student's t-test, Fisher's exact test, one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test, two-tailed Spearman's test, Kaplan-Meier analysis, stepwise multiple regression analysis, or Cox proportional hazard regressions analysis was used. Disease-free survival (DFS) time was defined as the length of time after the primary surgical treatment for CRC during which
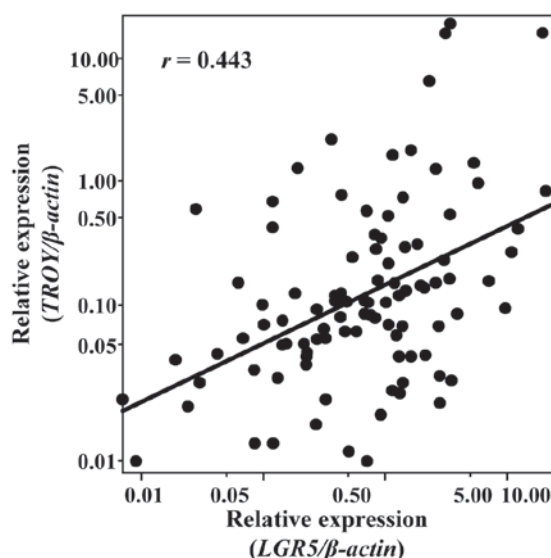

Figure 1. Correlation between TROY and LGR5 expression levels. Each sample is indicated by a black circle. TROY, tumor necrosis factor receptor superfamily member 19; LGR5, leucine-rich repeat containing G-protein-coupled receptor 5.

no recurrence was detected. The follow-up period ended on June 30, 2016. Before the analysis, a logarithmic transformation was performed on *LGR5* and *TROY* mRNA expression values to obtain normally distributed data sets. P<0.05 was considered to indicate a statistically significant difference.

Results

LGR5 and TROY mRNA expression levels in the CRC and non-tumor specimens. There was a positive correlation of expression level between *TROY* and *LGR5* (r=0.443, P<0.001 by two-tailed Spearman's test) (Fig. 1). *LGR5* mRNA expression levels were significantly higher in the CRC tissues of each stage except for stage I than those in the normal-appearing mucosa tissues (P<0.001 by one-way ANOVA; both P<0.01 by Tukey multiple comparison test) (Fig. 2A). *TROY* mRNA expression levels were significantly higher in the CRC tissues of each stage than those in the normal-appearing mucosa tissues (P<0.001 by one-way ANOVA; both P<0.01 by Tukey multiple comparison test) (Fig. 3A).

Prognostic significance of LGR5. *LGR5* expression levels were slightly higher in the relapse group than in the disease-free group (P=0.058 by Student's t-test) (Fig. 2B). Receiver operator characteristic (ROC) curves and sensitivity and specificity curves were plotted using LGR5 mRNA expression values for differentiating between the disease-free condition and relapse. As the crossover of the sensitivity and specificity curves was 0.739, we divided the CRC patients into 2 groups on this basis: the *LGR5*-high group (≥0.739) and the *LGR5*-low (<0.739) group. Univariate analysis showed CRC patients in the *LGR5*-low group tended to have improved DFS as compared with those in the *LGR5*-high group (P=0.055 by log-rank test) (Fig. 2C). In contrast, there was no association between the *LGR5* expression level and overall survival status (P=0.37 by Student's t-test) (data not shown).

Table I. Relation between clinicopathologic factors and patient outcomes.

Factor	n	Relapse status			Survival status		
		Disease-free (n)	Relapse (n)	P-value	Survival (n)	Non-survival (n)	P-value
Sex							
Male	51	36	15	0.9000	38	13	0.5500
Female	49	34	15		39	10	
Age in years (mean ± SD/range)	66.9±11.4/ 38-92	67.3±11.6/ 38-92	66.1±11.2/ 48-89	0.4900	67.3±11.4/ 38-92	65.5±11.7/ 49-89	0.5100
pStage							
I	14	14	0	NA	14	0	NA
II	36	27	9	0.0390	30	6	0.1700
III	30	21	9	0.0220	24	6	0.0720
IV	20	8	12	0.0003	9	11	0.0007
pT							
1	8	8	0	NA	8	0	NA
2	9	8	1	0.3300	8	1	0.3300
3	70	48	22	0.0610	54	16	0.1300
4	13	6	7	0.0110	7	6	0.0230
pN							
0	55	43	12	NA	47	8	NA
1	23	17	6	0.6800	17	6	0.2300
2	22	10	12	0.0051	13	9	0.0120
pM							
0	80	62	18	NA	68	12	NA
1	20	8	12	0.0011	9	11	0.0001
Lymphatic vessel invasion							
Negative	12	12	0	NA	12	0	NA
Positive	88	58	30	0.0160	65	23	0.0440
Vascular invasion							
Negative	31	26	5	NA	27	4	NA
Positive	69	44	25	0.0430	50	19	0.1100
Liver metastasis							
Negative	86	63	23	NA	70	16	NA
Positive	14	7	7	0.0780	7	7	0.0096
Peritoneal metastasis							
Negative	91	66	25	NA	74	17	NA
Positive	9	4	5	0.0800	3	6	0.0011
Histopathological type							
Well differentiated adenocarcinoma	29	25	4	NA	25	4	NA
Others	71	44	26	0.0210	51	19	0.1500
Postoperative adjuvant chemotherapy							
Not performed	34	31	3	NA	30	4	NA
Performed	66	39	27	0.0009	47	19	0.0550

pStage, pathological stage; pT, pathological tumor classification; pN, lymph node metastasis; pM, distant metastasis; NA, not applicable; SD, standard deviation.
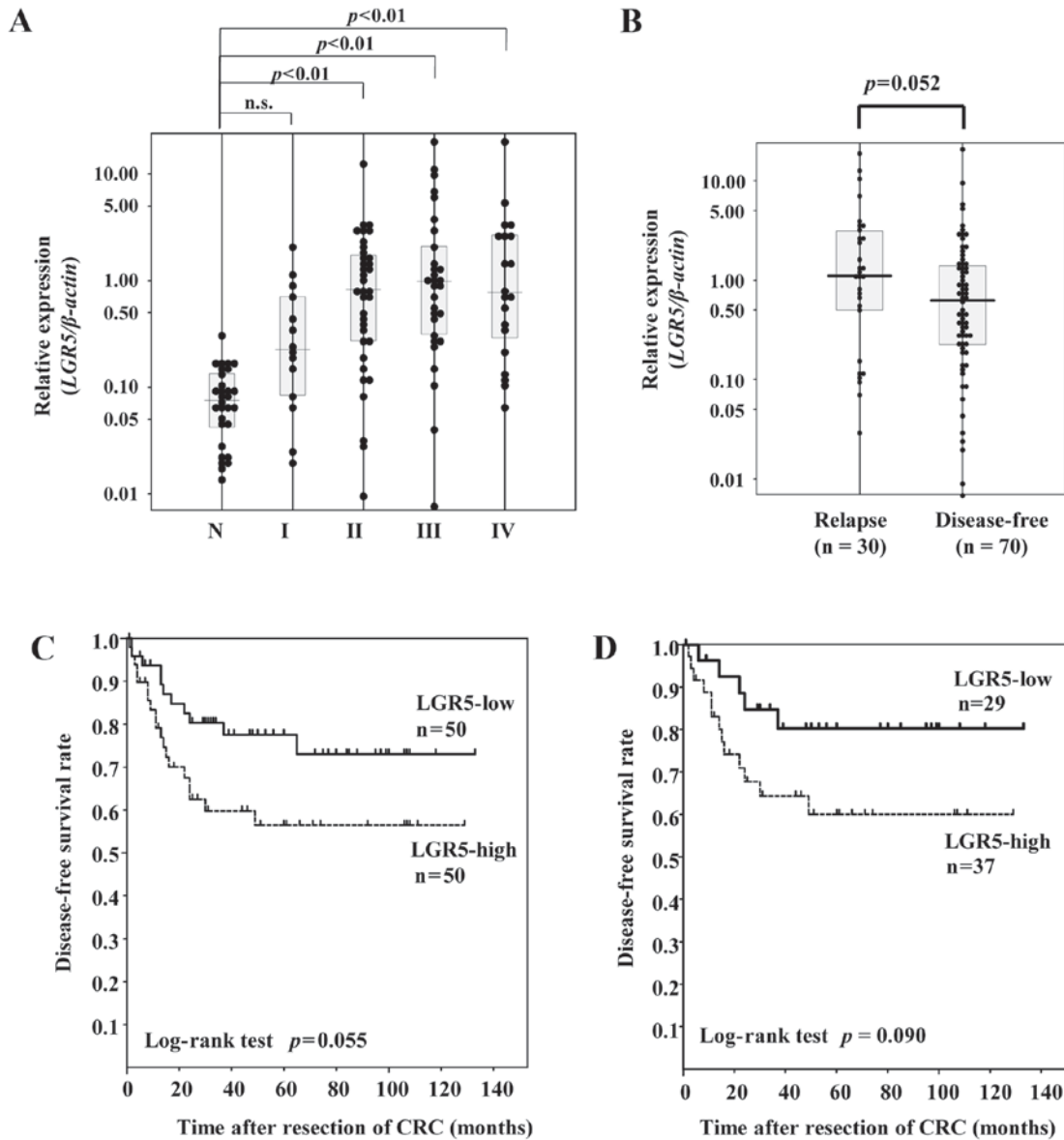
Figure 2. (A) Distribution of LGR5 mRNA expression levels in normal-appearing mucosa specimens and CRC specimens of each stage. (B) LGR5 levels in the relapse group and the disease-free group. (C) Disease-free survival in all CRC patients according to LGR5 expression status. (D) The horizontal lines represent the median level in each group. N, non-tumor specimens; n.s., not significant; I, II, III, and IV, pathological stages of CRC. CRC, colorectal cancer; LGR5, leucine-rich repeat containing G-protein-coupled receptor 5.

TROY as a prognostic biomarker of CRC. There was a significant difference in the expression level of *TROY* between the disease-free and recurrence groups (P=0.0004 by Student's t-test) (Fig. 3B). ROC analysis revealed the area under the ROC curve to be 0.694, and an optimal cut-off point to discriminate between the disease-free and recurrence groups was 0.15 according to the crossover of the sensitivity and specificity curves, resulting in a sensitivity of 60.0% and a specificity of 77.1%. Univariate survival analysis showed patients with high *TROY* expression (n=36) had a significantly shorter duration of DFS than those with low *TROY* expression (n=64) (P=0.0003 by log-rank test) (Fig. 3C), and this association was also observed when patients were limited to stage II and III CRC (P=0.0026 by log-rank test) (Fig. 3D). Multivariate analysis identified stage and *TROY* mRNA level as the independent predictive factors of DFS (Table II). In contrast, there was no association between *TROY* mRNA

Table II. Results of Cox proportional hazards regression analysis of risk factors for recurrence in patients with all stages colorectal cancer (n=100).

Variable	P-value	HR	95% CI
Sex[a]	0.6378	0.837	0.399-1.757
Age	0.5711	1.010	0.976-1.046
pStage	0.0004	2.288	1.444-3.624
LGR5 mRNA level	0.6209	0.856	0.463-1.583
TROY mRNA level	0.0039	2.364	1.317-4.241

CI, confidence interval; HR, hazard ratio. [a]Female is the reference category. LGR5, leucine-rich repeat containing G-protein-coupled receptor 5; TROY, tumor necrosis factor receptor superfamily member 19.
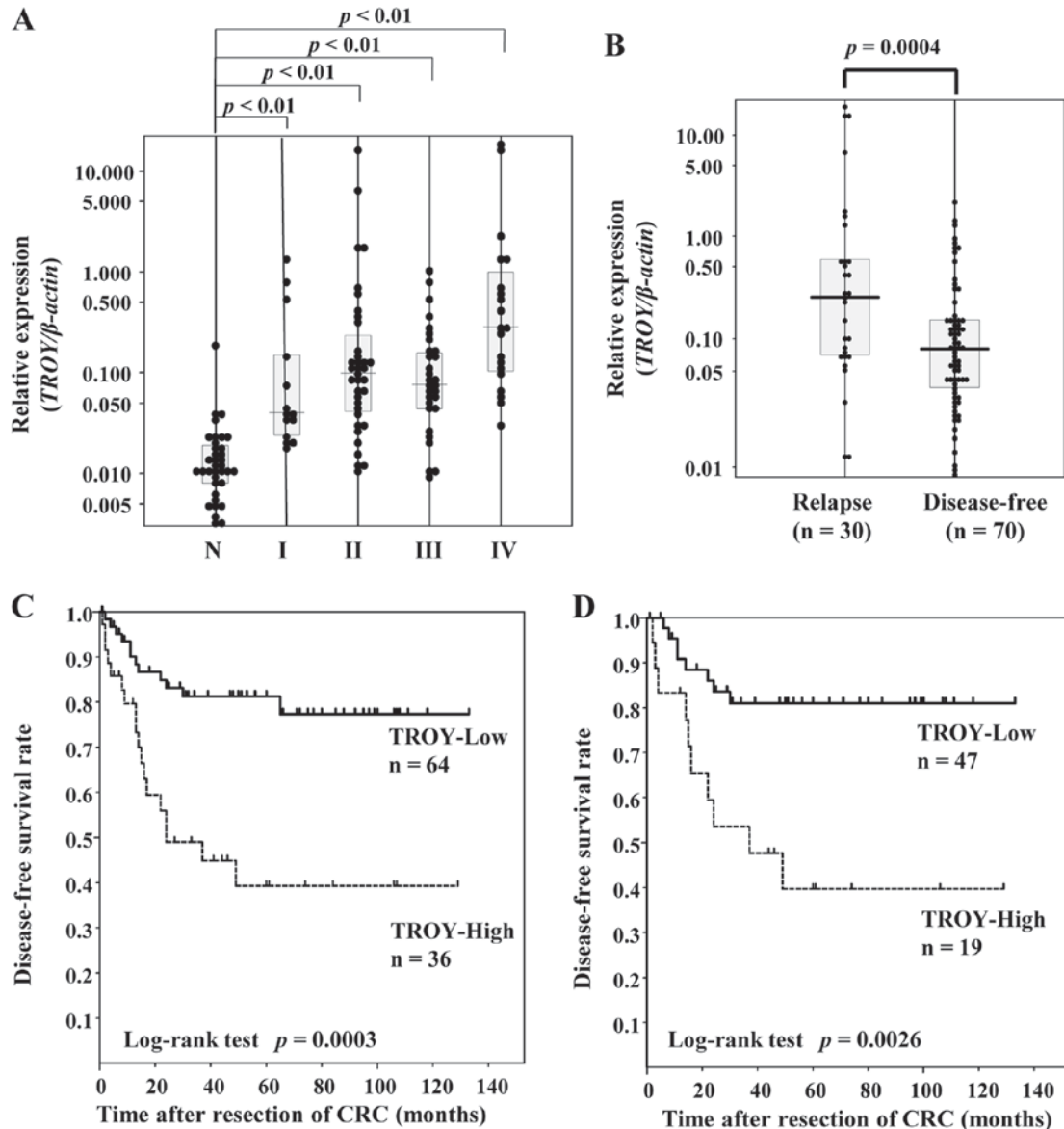
Figure 3. (A) Distribution of TROY mRNA expression levels in normal-appearing mucosa specimens and CRC specimens of each stage. (B) TROY levels in the relapse group and the disease-free group. (C) Kaplan-Meier plots of disease-free survival in patients with all stages of CRC (D) and in patients with stage II and III CRC according to TROY expression levels. TROY, tumor necrosis factor receptor superfamily member 19; CRC, colorectal cancer.

level and overall survival status (P=0.14 by Student's t-test) (data not shown).

Discussion

In the present study, *LGR5* mRNA expression levels were significantly higher in the CRC tissues of stages II-IV than those in the normal-appearing mucosa tissues, and CRC patients with higher *LGR5* expression tended to have unfavorable outcomes compared with those with lower *LGR5* expression. *LGR5* overexpression has been reported in several cancers including hepatocellular carcinoma, ovarian cancer, cervical cancer, and basal cell carcinoma (20-23). Regarding CRC, many study groups reported that LGR5 is overexpressed in CRC tissues and is associated with unfavorable outcome in CRC patients (24-27). In the present study, although univariate analysis showed overexpression of *LGR5* to be a possible

biomarker of unfavorable outcome of CRC, its clinical usefulness in predicting relapse of CRC was not as expected. Thus, we continued searching for other biomarkers including *TROY*.

In the present study, the overexpression of *TROY* was found to be an independent prognostic marker of DFS in all stages of CRC and even when limited only to stage II and III CRC. To date, this is, to our knowledge, the first report in the world of *TROY* as an independent prognostic marker of DFS of CRC. *TROY* has unique aspects in the Wnt signaling pathway. *TROY* has the potential of being a negative feedback mechanism in the canonical Wnt pathway. Because *TROY* is one of the β-catenin/TCF4 target genes, *TROY* expression is increased in a Wnt-signaling-dependent manner; however, *TROY* also reduces Wnt signaling by destabilizing LRP6 (28). Thus, we speculate that the overexpression of *TROY* observed in the present study may reflect a failure of the negative feedback of *TROY* in Wnt signaling or a protective effect of TROY to suppress Wnt signaling. The

function of *TROY* in CRC is poorly understood. One research group reported that TROY is significantly up-regulated in neoplastic tissues from mice during intestinal tumorigenesis and is produced specifically by fast-cycling intestinal stem cells (13). Another research group suggested that TROY can contribute to the initiation or progression of colorectal tumors with deregulated β-catenin activity (17). Regarding the prognostic significance of TROY, one research group reported no increase in *TROY* mRNA in CRC specimens in a small sample of patients (n=20) (13). In contrast, another group reported an increase in *TROY* mRNA expression in 4 of 8 CRC tissues but did not investigate its prognostic significance (17). Because the number of samples analyzed in both reports was small, they were insufficient to evaluate the prognostic significance of the expression level of TROY. Further studies are required to confirm the association of the higher expression of TROY with outcome in patients with CRC. In cancers other than CRC, higher expression of TROY correlates with increasing glial tumor grade (15), glioblastoma cell invasion, and unfavorable outcome of glioblastoma (16,29). These reports may support our finding of TROY as a possible prognostic marker in CRC. However, we realize that limitations exist in our study: We did not measure protein levels of TROY and did not investigate its localization because of an insufficient amount of tissue samples. Thus, further studies incorporating investigation of these issues are required to prove TROY as a true prognostic factor in CRC.

In conclusion, *TROY* may be a promising and better biomarker of predictive relapse of CRC than *LGR5*. To support our findings, confirmatory studies using independent data sets are needed. In addition, to improve the outcome of patients with CRC, further studies are required to investigate whether the evaluation of *TROY* expression level can be useful in determining whether to introduce adjuvant chemotherapy especially in patients with stage II and III CRC.

Acknowledgements

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