Clinical and biological significance of TROY (TNFRSF19) splice variants in colorectal cancer

MITSUAKI NISHIOKA[1], YUKI KUNIMUNE[1], YUTAKA SUEHIRO[1,2], MASAKI KODAMA[1], AKIYO ISHIGURO[2], TATSUYA TAKAGI[3], JUN NISHIKAWA[3], HIDENORI TAKAHASHI[4], HIROTO MATSUI[4], SHINOBU TOMOCHIKA[4], KOICHI HAMABE[5], SHINICHI HASHIMOTO[5], HIROAKI NAGANO[4], TARO TAKAMI[5] and TAKAHIRO YAMASAKI[1,2]

[1]Division of Laboratory, Yamaguchi University Hospital, Ube, Yamaguchi 755-8505, Japan; [2]Department of Oncology and Laboratory Medicine, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan; [3]Faculty of Laboratory Science, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan; [4]Department of Gastroenterological, Breast, and Endocrine Surgery, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan; [5]Department of Gastroenterology and Hepatology, Yamaguchi University Graduate School of Medicine, Ube, Yamaguchi 755-8505, Japan

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Abstract. Colorectal cancer (CRC) remains a major cause of cancer-related mortality worldwide, and the development of reliable biomarkers for postoperative recurrence risk stratification is urgently required. TROY (also known as tumor necrosis factor receptor superfamily member 19) is a Wnt/β-catenin-regulated receptor implicated in tumor progression; however, to the best of our knowledge, its isoform-specific biological functions and clinical importance have not been defined. The present study examined the roles of the two major splice variants, TROY variant 1 (TROYv1) and TROY variant 2 (TROYv2), in CRC through integrated functional assays and clinical analyses. *In vitro*, HCT116 cells overexpressing TROYv1 or TROYv2 exhibited significantly greater proliferation than control cells by day 3 (P<0.0001 vs. mock for TROYv1; P=0.001 vs. mock for TROYv2), with TROYv1 promoting a stronger proliferative effect than TROYv2 (P=0.018), whereas no differences in migration or invasion were observed. Clinically, droplet digital PCR enabled quantitative assessment of TROY isoforms in 108 surgically resected tumor specimens obtained from patients with Stage I-III CRC, among whom 20 experienced recurrence and 27 died. High TROYv1 expression was strongly associated with recurrence (P=0.01) and was significantly associated with shorter recurrence-free survival time (P=0.014). By contrast, TROYv2 exhibited no significant prognostic association. Notably, Cox proportional hazards analyses identified TROYv1 as an independent molecular predictor of recurrence (hazard ratio=1.360; P=0.047). These findings suggested that isoform-specific expression of TROY may serve as a prognostic biomarker in CRC, and that TROYv1 expression is associated with tumor progression. Isoform-specific assessment of TROY, particularly TROYv1, may contribute to refining postoperative risk stratification and could represent a potential therapeutic target in CRC.

Correspondence to: Dr Mitsuaki Nishioka, Division of Laboratory, Yamaguchi University Hospital, 1-1-1 Minami-Kogushi, Ube, Yamaguchi 755-8505, Japan
E-mail: mnishi@yamaguchi-u.ac.jp

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Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. According to the latest estimates from the International Agency for Research on Cancer (IARC) on global regional cancer statistics for 2022, CRC ranks third in morbidity and second in mortality among all cancers (1). Its development is strongly linked to dysregulation of the Wnt signaling pathway, which promotes tumorigenesis and maintenance of cancer stem cell properties (2). The Wnt signaling pathway is primarily divided into two branches: the canonical pathway and the non-canonical pathway. Both play important roles in cell fate, proliferation, and tumor formation, and are involved in the development of various cancers (3,4). In the canonical pathway, β-catenin is stabilized through the activation of Frizzled and LRP5/6 receptors, thereby promoting the transcription of TCF/LEF target genes associated with proliferation and invasion (5). In contrast, non-canonical pathways do not depend on β-catenin and instead regulate calcium signaling, cytoskeletal organization, and cell migration (6,7). Aberrant canonical and non-canonical Wnt signaling is observed in CRCs (8,9).

Tumor necrosis factor (TNF) receptor superfamily member 19 [TROY, also called TNF receptor superfamily member 19 (TNFRSF19)] is a type I cell surface receptor

protein containing the highly conserved TNF receptor cysteine-rich motifs in the extracellular domain and a TNF receptor-associated factor (TRAF)-binding sequence in the large cytoplasmic domain required for signaling (10). TROY is a β-catenin target gene (11). Aberrant expression of TROY has been reported in several malignancies, including CRC (12), glioma (13), and melanoma (14), where it is thought to contribute to tumor cell proliferation, invasion, and resistance to therapy. Its expression pattern and regulation by Wnt signaling suggest that TROY may serve as a key mediator linking extracellular cues to intracellular pathways that drive malignant progression.

TROY exists in two main splice variants, TROY variant 1 (TROYv1) and TROY variant 2 (TROYv2), which are implicated in CRC through the canonical Wnt signaling pathway (12). In addition, TROYv1 is negatively regulated by adipogenic transcription factor CCAAT/enhancer-binding proteins (C/EBP) (15). TROYv2 contains a major TRAF2-binding consensus sequence, (SLQE at amino acid 413-416), which is absent in TROYv1 (15). TROYv2, induced by canonical Wnt signaling, differentiates human multipotent mesenchymal stem cells into osteoblasts (15). While our previous studies have implicated TROY in CRC prognosis (16), the specific roles of each isoform remain to be clarified.

The objective of this study was to examine the contributions of TROYv1 and TROYv2 to CRC tumor progression in patients with Stage I-III CRC.

Materials and methods

Cell culture. Human colon cancer cell line HCT-116 was purchased from the American Type Culture Collection. HCT-116 cells were cultured in McCoy's 5A medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific), 100 IU/ml penicillin, and 100 mg/ml streptomycin.

Establishment of gene-overexpressing cells via plasmid transfection. To generate TROYv1- and TROYv2-overexpressing cells, two types of plasmids containing TROYv1 or TROYv2 inserts were used: TROY (TNFRSF19) (NM_018647) Human Tagged open reading frame (ORF) Clone (RC214944), which corresponds to TROYv1, and TROY (TNFRSF19) (NM_148957) Human Tagged ORF Clone (RC208389), which corresponds to TROYv2, both purchased from OriGene Technologies, Inc. The ORF and tag regions of the plasmids (TROYv1 or TROYv2 + DDK + Myc tag) were subcloned into the pcDNA3.1[+] vector (Promega Corporation). The fragments were excised using *Kpn*I and *Sma*I (New England BioLabs, Inc.) restriction enzymes, purified with a QIAquick Gel Extraction Kit (Qiagen GmbH), and ligated into similarly digested pcDNA3.1[+] using T4 DNA ligase (Takara Bio Inc.). The ligation products were transformed into *Escherichia coli* DH5α (Takara Bio Inc.), amplified, and then plasmid DNA was purified using a QIAamp® Spin Miniprep Kit (Qiagen GmbH). The resulting plasmids [pcDNA3.1(+)-TROYv1 and pcDNA3.1(+)-TROYv2] were transfected into HCT116 cells using Effectene® Transfection Reagent (Qiagen GmbH) to generate the TROY-overexpressing cell lines HCT116_TROYv1 and HCT116_TROYv2. Stable cell lines were established by selection with G418 (FUJIFILM Wako Pure Chemical Corporation). Mock cells were generated by transfecting HCT116 cells with the empty pcDNA3.1[+] vector (Promega Corporation) using the same transfection procedure, and stable cell lines were similarly established by selection with G418.

Proliferation, migration, and invasiveness assays. We used the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation) according to the manufacturer's instructions. Briefly, cells ($5x10^3$) were seeded into 96-well plates, and cell proliferation was measured daily for 3 days using CellTiter 96® AQueous One Solution. Absorbance was measured at 492 nm using a Infinit® 200PRO (Tecan Group, Ltd.). Results are presented as the average absorbance of three wells per experiment. Cell migration and invasion abilities were assessed using a CytoSelect™ 96-Well Cell Migration and Invasion Assay Kit (8 μm pore size, fluorometric format; Cell Biolabs, Inc.) following the manufacturer's instructions. For the migration assay, cells were suspended in serum-free medium at a concentration of $1.0x10^6$ cells/ml. Then, 100 μl of the cell suspension was added to the upper chambers of the assay plate, while the lower chambers were filled with 150 μl of medium containing 10% FBS as a chemoattractant. After incubation for 24 h at 37˚C in a 5% CO_2 incubator, migrated cells on the bottom surface of the membrane were detached using Cell Detachment Solution and stained with CyQuant® GR Dye. Fluorescence intensity was measured at 480 nm excitation and 520 nm emission using a fluorescence microplate reader. For the invasion assay, the basement membrane-coated inserts were first rehydrated with serum-free medium for 1 h at room temperature. Cells were then seeded into the upper chambers in serum-free medium, and the lower chambers were filled with chemoattractant-containing medium. After incubation for 24 h at 37˚C in a 5% CO_2 incubator, invasive cells were dissociated, stained, and quantified in the same manner as described for the migration assay. All experiments were conducted in quadruplicate, and relative fluorescence units were used to evaluate the migratory and invasive capacities of the cells.

Study participants. In total, 133 CRC tissue samples were collected from patients who underwent surgical treatment at the Department of Gastroenterological, Breast and Endocrine Surgery, Yamaguchi University Graduate School of Medicine (Ube, Japan) between February 2007 and October 2014. Of these patients, 25 with Stage IV disease were excluded from the analyses, because the distinction between true recurrence and persistence or progression of residual disease is inherently difficult in this setting, even after apparently curative resection of metastatic lesions. The final study cohort thus comprised 108 patients with Stage I-III CRC, all of whom underwent curative resection and completed follow-up (100% follow-up completeness). The cohort included 55 male and 53 female patients, with an overall median age of 71 years (range: 30-92). The median age was 71 years (range: 37-87) for male patients and 69 years (range: 30-92) for female patients. During the follow-up period, 20 recurrence events and 27 deaths were recorded. No patients were lost to follow-up; patients who had

Table I. Clinicopathologic characteristics of the participants.

		Recurrence status			Survival status		
Factor	n	Recurrence-free	Recurrence	P-value	Survival	Non-survival	P-value
Sex, n							
Male	55	42	13	0.16[a]	36	19	0.02[a]
Female	53	46	7		45	8	
Median age, years (range)	71.0 (30.0-92)	72.5 (30-92)	69.5 (54-86)	0.35[b]	67.0 (30-92)	76.0 (54-86)	0.0072[b]
Histopathological stage, n							
Stage I	25	22	3	0.64[c]	21	4	0.35[a]
Stage II	27	22	5		18	9	
Stage III	56	44	12		42	14	
Adjuvant chemotherapy							
Received	67	53	14	0.42[c,d]	54	13	0.086[c]
Stage I	8	7	1		6	2	
Stage II	14	11	3		11	3	
Stage III	45	35	10		37	8	
Not received	41	35	6		27	14	
Stage I	17	15	2		15	2	
Stage II	13	11	2		7	6	
Stage III	11	9	2		5	6	

[a]χ^2 test; [b]Mann-Whitney U test; [c]Fisher's exact test; [d]χ^2 test was performed between received and not received groups in adjuvant chemotherapy.

not experienced the event of interest by the end of the observation period (last hospital visit or January 2024, whichever came first) were censored at that time point. All samples were immediately frozen in liquid nitrogen after sample collection from surgically resected materials and then stored at -80˚C until use. The clinicopathologic characteristics of the CRC patients are summarized in Table I. The histopathological stage of each patient was determined according to the International Union Against Cancer staging system. The administration status of postoperative adjuvant chemotherapy was evaluated. Of the 108 patients, 67 received postoperative adjuvant chemotherapy, and 41 did not (Table I). Recurrence-free survival (RFS) was defined as the time from the date of surgery to the date of radiologically confirmed recurrence, as determined by imaging modalities including computed tomography and/or magnetic resonance imaging. Patients who died without prior confirmed recurrence were censored as recurrence free at the time of death. This study was conducted in compliance with the ethical principles of The Declaration of Helsinki. The study protocol was approved by the Institutional Review Boards of Yamaguchi University Hospital (approval no. 2024-165).

Isolation of nucleic acids from cell lines and tissues. Genomic DNA and total RNA from cell lines and tissues were isolated using the DNeasy Blood and Tissue Kits for DNA Isolation Kit (Qiagen GmbH) and a QIAmp RNA Blood Mini Kit (Qiagen GmbH), respectively. The extracted total RNA for 2 μg was reverse transcribed into single-stranded cDNA using a High-Capacity cDNA Archive Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.).

Gene expression analysis. Measurement of the copy numbers of the target cDNA for droplet digital PCR (ddPCR) was quantified using a QX200™ Droplet Digital™ PCR System (Bio-Rad Laboratories, Inc.). The TaqMan® Gene Expression Assays used in this study were as follows: Hs99999903_m1 (cat. no. 4326315E) for β-actin, Hs00973825_m1 (cat. no. 4351372) for TROYv1, and Hs00969483_m1 (cat. no. 4351372) for TROYv2 (all from Thermo Fisher Scientific, Inc.). As the primer and probe sequences are proprietary to the manufacturer, they cannot be disclosed. However, Hs00973825_m1 and Hs00969483_m1 were validated for isoform-specific detection of TROY, and Hs99999903_m1 was validated for specific detection of β-actin. The PCR reaction solution consisted of 2 μl of cDNA, 10 μl of 2X ddPCR Supermix for Probes (Bio-Rad Laboratories, Inc.), and 1 μl of each probe in a total volume of 20 μl. After droplets were generated by an automated droplet generator (Bio-Rad), PCR was performed. PCR cycling conditions included preheating at 95˚C for 10 min followed by 40 cycles of denaturation at 94˚C for 30 sec and annealing at 56˚C for 60 sec. Final heating was performed at 98˚C for 10 min. Following amplification, the PCR plate was transferred to a QX200 droplet reader, and fluorescence amplitude data were obtained using QuantaSoft software (both Bio-Rad Laboratories, Inc.). Each sample was analyzed in duplicate. Although ddPCR provides absolute quantification without the need for a standard curve, normalization to β-actin was performed to correct for inter-sample variability in RNA input amount, RNA integrity, and cDNA synthesis efficiency, as recommended

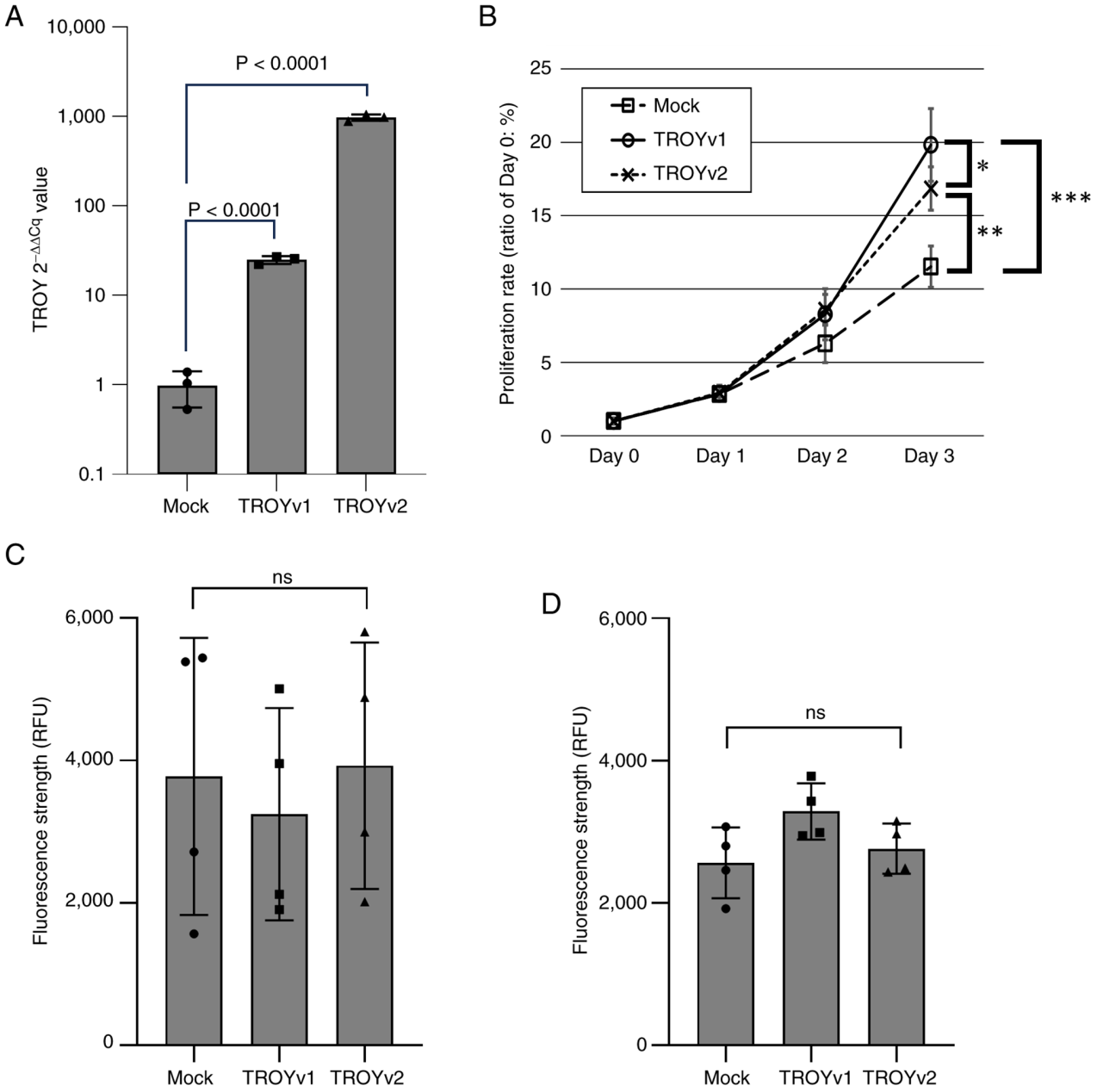

Figure 1. Effects on cell proliferation, migration, and invasion in cells overexpressing TROYv1 and TROYv2 (*in vitro*). (A) Total TROY mRNA expression levels in HCT116 cells overexpressing TROYv1 or TROYv2, and Mock cells. mRNA expression was measured using the TaqMan® assay Hs00218634_m1, which targets a region common to all TROY isoforms. As the isoform-specific assays target regions including the 5'non-coding region outside the open reading frame, which are not present in the overexpression vectors, total TROY expression was assessed to confirm successful overexpression. TROYv1 and TROYv2 expression levels were significantly increased in the respective overexpressing cell lines compared with Mock cells (both P<0.0001). (B) Cells overexpressing TROYv1 and TROYv2 exhibited significantly higher proliferation capacity than mock cells by day 3. Additionally, TROYv1 exhibited a significantly higher proliferation capacity than TROYv2 by Tukey's multiple comparison test. *P=0.018, **P=0.001, ***P<0.0001. (C) No significant differences in cell migration ability were observed among the three cell types. (D) Likewise, no significant differences in cell invasion ability were observed among the three cell types. Statistical significance determined by one-way analysis of variance followed by Tukey's multiple comparison test. ns, not significant; RFU, relative fluorescence units; TROYv, TROY variant.

for clinical tissue samples (17,18). The copy numbers of TROYv1 and TROYv2 were normalized to that of β-actin by dividing each target gene copy number by the corresponding β-actin copy number.

Quantitative PCR was performed using TaqMan Gene Expression Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The TaqMan® Gene Expression Assays used in this study were Hs99999903_m1 (cat. no. 4326315E) for β-actin and Hs00218634_m1 (cat. no. 4331182) for TROY (both Thermo Fisher Scientific, Inc.). Hs00218634_m1 targets a region common to all TROY isoforms and therefore detects total TROY expression. Quantitative PCR was performed on a QuantStudio® 5 Real-Time PCR System (Thermo Fisher Scientific, Inc.). PCR conditions were as follows: preheating at 50˚C for 2 min and 95˚C for 10 min, 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 $2^{-\Delta\Delta Cq}$ method, in which

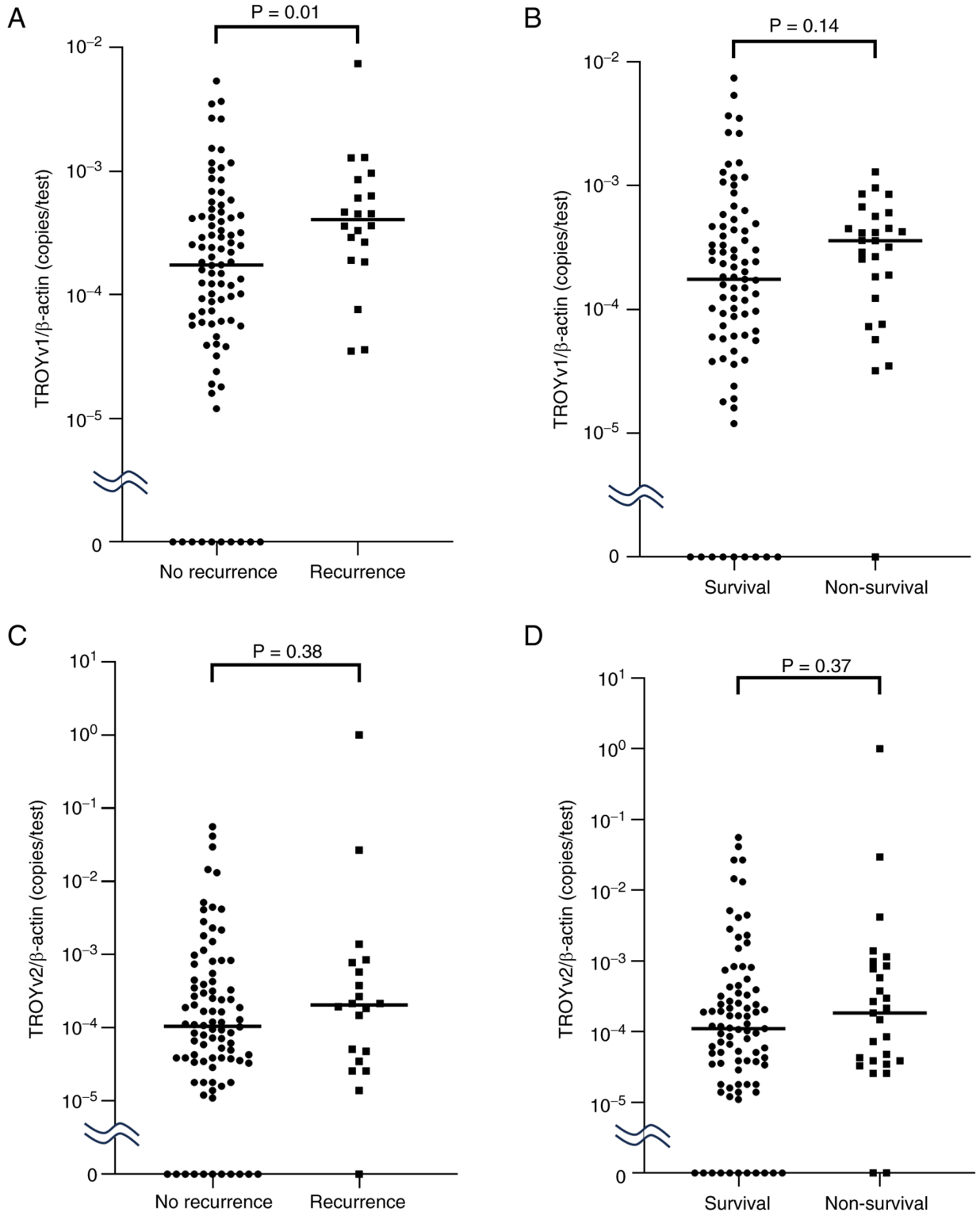

Figure 2. Relationship between the mRNA expression levels of TROYv1 and TROYv2, as measured by droplet digital PCR, and prognosis in patients with Stage I-III CRC. (A) When patients with and without recurrence were compared, TROYv1 expression levels were significantly higher in the recurrence group. (B) In contrast, no significant difference in TROYv1 expression levels was observed between the survival and non-survival groups. Regarding TROYv2 expression levels, no significant differences were observed (C) between patients with and without recurrence and (D) between the survival and non-survival groups. Statistical significance determined by Mann-Whitney U test. TROYv, TROY variant.

relative quantification of mRNA expression level was calculated using β-actin as the internal reference (19).

Statistical analysis. The Mann-Whitney U test, χ^2 test, Fisher's exact test, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, Kaplan-Meier survival analysis with the log-rank test and Cox proportional hazards analysis were performed. $P<0.05$ was considered to indicate a statistically significant difference. For Kaplan-Meier survival analysis, patients were divided into high- and low-expression

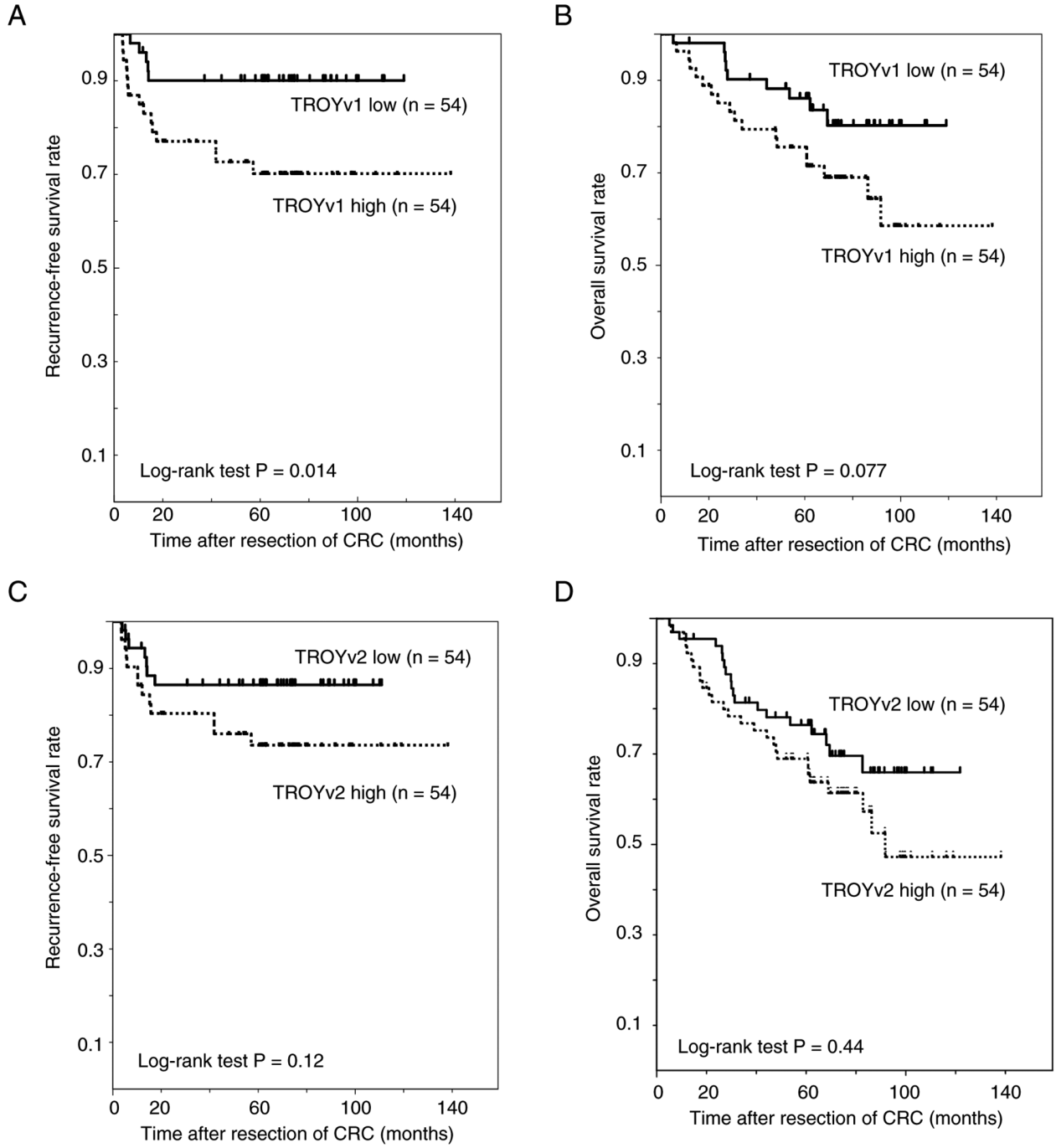

Figure 3. Comparison of recurrence-free survival and overall survival based on mRNA expression levels of TROYv1 and TROYv2 in patients with Stage I-III CRC. The mRNA expression levels of TROYv1 and TROYv2 were divided into a high expression group and low expression group based on the median value, and Kaplan-Meier analysis was performed. (A) For TROYv1, the high group had a significantly shorter recurrence time compared to the low group, (C) whereas no significant difference was observed for TROYv2. There were no significant differences in overall survival time between the high and low groups for either (B) TROYv1 or (D) TROYv2. TROYv, TROY variant.

groups based on the median expression values of TROYv1 and TROYv2 for illustrative purposes only. The primary analysis was conducted using TROYv1 and TROYv2 expression as continuous variables in the Cox proportional hazards model, which preserves statistical power and avoids the limitations associated with arbitrary dichotomization. The above statistical analyses were performed with StatFlex Ver. 7 (Artech Co., Ltd.), GraphPad Prism (ver. 9; Dotmatics) and JMP® Student Edition 18.2.1 (SAS Institute Inc.). To validate the prognostic value of TROYv1 and assess model robustness, bootstrap optimism correction (300 resamples), sensitivity analyses, proportional hazards assessment (Grambsch-Therneau test), linearity evaluation (Martingale residual plots), calibration, likelihood ratio tests and decision curve analysis were performed using R (version 4.5.1; R Foundation for Statistical Computing). To evaluate the influence of individual observations on the estimated regression coefficients in the Cox proportional hazards model, difference in β (DFβ)-based influence diagnostics were performed using a leave-one-out approach. Specifically, for each patient, the Cox proportional hazards model was refitted

Table II. Results of Cox proportional hazards regression analysis of risk factors for recurrence in patients with stage I-III of colorectal cancer (n=108).

Variable	β	SE(β)	z	P-value	HR (95% CI)
Histopathological stage	0.185	0.288	0.642	0.521	1.203 (0.684-2.116)
TROYv1 mRNA level	0.307	0.155	1.982	0.047	1.360 (1.003-1.843)
TROYv2 mRNA level	0.033	0.093	0.350	0.726	1.033 (0.861-1.240)

CI, confidence interval; HR, hazard ratio; SE, standard error; TROYv, TROY variant.

Table III. Results of Cox proportional hazards regression analysis of risk factors for overall survival in patients with stage I-III of colorectal cancer (n=108).

Variable	β	SE(β)	z	P-value	HR (95% CI)
Age	0.074	0.022	3.342	0.001	1.077 (1.031-1.125)
Sex[a]	-1.071	0.434	2.468	0.014	0.343 (0.146-0.802)
Histopathological stage	0.020	0.275	0.074	0.941	1.021 (0.596-1.748)
TROYv1 mRNA level	0.109	0.120	0.909	0.364	1.115 (0.882-1.409)
TROYv2 mRNA level	0.046	0.072	0.649	0.516	1.048 (0.911-1.205)

[a]Female is the reference category. CI, confidence interval; HR, hazard ratio; SE, standard error; TROYv, TROY variant.

after excluding that individual observation, and the difference in the estimated regression coefficient (β) for each covariate between the full model and the leave-one-out model was calculated. This difference was then standardized by dividing by the standard error of the coefficient from the full model to compute DFβ residuals. A threshold of $\pm 2/\sqrt{n}$ was applied to identify highly influential observations. This analysis was performed using the survival package (https://cran.r-project.org/package=survival) in R (version 4.5.1).

Results

TROYv1 and TROYv2 overexpression enhances cell proliferation but not migration or invasion. The results of measuring the expression levels of TROY in three cell lines, TROYv1-overexpressing cells (TROYv1 cells), TROYv2-overexpressing cells (TROYv2 cells), and Mock, are shown in Fig. 1A. One-way ANOVA followed by Tukey's multiple comparison test confirmed that TROYv1 and TROYv2 expression levels were significantly increased in the respective overexpressing cell lines compared with Mock cells (both P<0.0001; Fig. 1A). When cell proliferative capacity was assessed using these cells, TROYv1 cells and TROYv2 cells had significantly higher proliferative capacity compared to Mock on day 3 of culture. Furthermore, TROYv1 cells showed significantly higher proliferative capacity compared to TROYv2 cells (Fig. 1B). In contrast, there were no significant differences in cell migration and invasive capacity between the three groups (Fig. 1C and D).

TROYv1 overexpression predicts recurrence in CRC. To assess the clinical significance of TROYv1 and TROYv2, 108 surgical specimens of CRC (Table I) were analyzed for their association with recurrence and survival. The follow-up period ranged from 0.5 to 138.2 months (median, 68.0 months; mean, 65.5 months). During this period, 27 patients (19 men and 8 women) died, and 20 patients (13 men and 7 women) experienced recurrence (Table I). TROYv1 expression was significantly higher in the recurrence group compared with the no recurrence group (Fig. 2A), whereas no significant difference was observed between the survival group and non-survival group (Fig. 2B). For TROYv2, there were no significant differences in expression levels both recurrence and survival between the two groups (Fig. 2C and D). Survival curve analysis showed RFS to be significantly shorter in the TROYv1 high-expressing group (Fig. 3A), whereas no significant difference in overall survival (OS) was observed between the two groups (Fig. 3B). No significant differences were observed for TROYv2 in either comparison (Fig. 3C and D). Furthermore, Cox proportional hazards analysis showed the expression level of TROYv1 to be an independent predictor of recurrence (Table II). TROYv1 mRNA expression level was identified as a significant predictor of RFS [β=0.307; hazard ratio (HR)=1.360; 95% confidence interval: 1.003-1.843; P=0.047). In contrast, age and sex were the independent predictors for OS, whereas, TROYv1 mRNA level was not significantly associated with the risk of death (Table III).

To correct for overestimation of model performance (optimism) and estimate more realistic predictive performance, bootstrap optimism correction (300 resamples) showed that addition of TROYv1 improved the optimism-corrected C-index for RFS from 0.614 to 0.670 (ΔC=+0.056), whereas no improvement was observed for OS from 0.718 to 0.715 (ΔC=-0.003). The Grambsch-Therneau test confirmed that the proportional hazards assumption was satisfied for all variables except histopathological stage in the RFS model (P=0.006).

Martingale residual plots confirmed adequate linearity for all continuous predictors. DFβ-based influence diagnostics identified 5 potentially influential observations exceeding the size-adjusted threshold of $\pm 2/\sqrt{n} = \pm 0.192$ (where n=108) for TROYv1. Sensitivity analyses confirmed the robustness of the TROYv1 association with RFS across the following conditions: Exclusion of the 5 influential observations (HR=1.447; P=0.021), a reduced model including TROYv1 and histopathological stage only (HR=1.401; P=0.023), and substitution of TROYv1 with TROYv2, which abolished the prognostic effect (HR=1.103; P=0.278). For OS, exclusion of influential observations also yielded consistent results (TROYv1 mRNA level HR=1.142; P=0.262), confirming that the reported HRs and P-values were not materially affected by influential observations. Calibration at 60 months was good for OS (slope ~≈1.0), whereas a slight tendency toward overprediction was noted for RFS at 60-120 months. A likelihood ratio test confirmed a significant improvement in model fit upon TROYv1 addition for RFS (P=0.011; ΔAIC=-4.552), but not for OS (P=0.223; ΔAIC=+0.514), where AIC denotes Akaike's Information Criterion. Decision curve analysis revealed that the full model (base model + TROYv1 mRNA level) provided a net benefit comparable to the base model (stage + age + sex) for RFS at 24 months, but it showed greater net benefit across a range of risk thresholds at 60 and 120 months. For OS, no meaningful difference in net benefit was observed between the full and base models at any time point.

Discussion

This study shows that the two main splice variants of TROY, TROYv1 and TROYv2, have distinct roles in CRC. Analysis of 108 clinical CRC specimens revealed that high TROYv1 expression was significantly associated with recurrence, shorter RFS, and was an independent predictor of recurrence. In contrast, TROYv2 expression showed no significant relationship with recurrence or prognosis. These findings suggest that evaluating TROY expression at the isoform level can provide additional prognostic information beyond that obtained from total TROY expression. TROYv1 was identified as an independent predictor of recurrence in CRC.

Previous studies have shown that TROY is a β-catenin target gene and can activate NF-κB signaling in CRC cells in a β-catenin-dependent manner (12). TROYv1 and TROYv2 exhibit a remarkably high amino acid sequence identity (98%), differing only in the presence or absence of a TRAF2-binding motif at the C-terminus (15). This structural difference has functional implications: TROYv2 has been reported to regulate osteogenic differentiation of human mesenchymal stem cells via canonical Wnt signaling (15), whereas TROYv1 lacks this TRAF2-binding sequence and may engage alternative downstream pathways. Clinically, we previously identified total TROY expression as a prognostic biomarker in CRC (16) but had not performed isoform-specific analysis. In other malignancies, such as glioblastoma, TROYv2 is markedly upregulated and promotes cell invasion via Pyk2-Rac1 signaling (13), whereas TROYv1 expression is less prominent, suggesting that isoform function may be tissue dependent.

We conducted functional assays to investigate their underlying biological functions. Both isoforms enhanced proliferation of CRC cells *in vitro*, but TROYv1 exhibited a stronger proliferative effect than TROYv2, consistent with its stronger prognostic association. Neither isoform significantly affected migration or invasion in our experimental setting, suggesting that their primary contribution may be to tumor growth rather than motility under the conditions tested. Given the differences in downstream signaling motifs, it is plausible that TROYv1 and TROYv2 engage distinct molecular pathways in CRC, leading to their divergent clinical associations.

In the Cox proportional hazards analysis of recurrence, histopathological stage was not significant predictive factor. In contrast, TROYv1 expression remained significant, indicating that higher TROYv1 expression was associated with an increased risk of recurrence. This suggests that TROYv1 expression is associated with an increased risk of recurrence in addition to conventional clinicopathological factors, although the underlying mechanisms remain to be elucidated. For OS, however, age and sex were identified as independent prognostic factors (Table III), whereas TROYv1 expression showed no significant independent predictive value for OS in the present cohort. Recent studies have shown that circulating tumor DNA (ctDNA) is a robust prognostic marker for postoperative recurrence (20,21). Integrating TROYv1 expression with ctDNA status may provide more accurate risk assessment in these patients. Such a combined strategy could refine prediction of recurrence and inform individualized follow-up after surgery, potentially leading to more personalized postoperative surveillance and selection of adjuvant therapy.

This study has several limitations. First, the functional analyses were limited to overexpression experiments in a single CRC cell line (HCT116), and no loss-of-function experiments (e.g., knockdown or CRISPR-based approaches), validation in additional CRC cell lines with diverse molecular backgrounds, or *in vivo* studies were performed. These limitations preclude causal conclusions regarding the role of TROYv1 in tumor progression. Second, the downstream signaling pathways modulated by TROYv1 and TROYv2 were not investigated in detail, leaving the mechanistic basis for the observed phenotypic and clinical differences unresolved. Future studies incorporating mechanistic readouts of NF-κB and Wnt downstream signaling consistent with the isoform differences will be essential. Third, this was a retrospective single-center analysis and did not include an independent validation cohort. Thus, external validation will be essential to establish the clinical utility of TROYv1. In addition, although publicly available datasets such as The Cancer Genome Atlas provide gene-level expression data, they do not allow reliable isoform-level quantification required to distinguish between TROYv1 and TROYv2. Therefore, external validation using these resources was not considered appropriate in the present study. We are currently preparing a prospective, multi-institutional study and evaluating whether integrating TROYv1 with postoperative ctDNA surveillance could further improve recurrence prediction. Additionally, variability in follow-up duration is an inherent limitation of this retrospective study and may have introduced some degree of heterogeneity in the survival analyses. Fourth, immunohistochemical validation of isoform-specific TROY expression cannot be performed in this study. Because TROYv1 and TROYv2 share 98% amino acid sequence identity, antibodies raised

against one isoform are highly likely to cross-react with the other, making reliable immunohistochemical discrimination between the two isoforms technically challenging (22,23). Instead, isoform-specific TaqMan assays with ddPCR were used for accurate transcript-level quantification. Development of isoform-specific antibodies remains an important goal for future studies. Fifth, the Cox proportional hazards models were subject to a limited events-per-variable ratio (EPV), with EPV=7.3 for Table II and EPV=5.4 for Table III. Although simulation studies have shown that acceptable levels of bias and confidence interval coverage can be achieved with EPV values in the range of 5-9 under certain conditions (24), we acknowledge that the stability of our regression estimates may be limited, and external validation in a larger cohort remains essential. Several clinicopathological variables were incomplete, limiting their inclusion in multivariable analyses. MSI/MMR status was available for only 42/108 cases (MSI-H: n=9, MSS: n=33) and was excluded because of multicollinearity. Data on perineural invasion (2/108), radial margin status (76/108, all negative), and histological grade (92/108) were also limited. Proximal and distal margins were negative in nearly all evaluable cases (107/108, R0). Although adjustment for adjuvant chemotherapy (67/108) and sensitivity analyses incorporating lymphatic/venous invasion data (92/108) did not alter the significance of TROYv1 as an independent predictor of RFS, residual confounding due to missing data remains a limitation. Despite these limitations, our data provide important insights into isoform-specific biology, suggesting that TROYv1 is a potential prognostic biomarker warranting further investigation.

In conclusion, TROYv1 was identified as a clinically relevant biomarker for predicting postoperative outcomes in CRC. Future studies should investigate isoform-specific signaling pathways, explore their interaction with the tumor microenvironment, and validate their clinical utility in larger, prospective cohorts.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MN conceived and designed the study, performed data curation (management, annotation and maintenance of research data for initial use and re-use) and formal analysis, acquired funding, developed the methodology, administered the project and was the major contributor in writing the original draft. YK, MK, TTakagi, HT, KH and SH conducted the investigation. AI contributed to data curation, and preparation, creation and presentation of published figures. JN and HN provided resources and contributed to acquisition of data. YS, TTakami and TY contributed to conception and design of the study, supervised the study, and contributed to writing, review and editing of the manuscript. MN and YS contributed to analysis and interpretation of data, and review and editing of the revised manuscript. HM and ST contributed to analysis and interpretation of data, and review and editing of the manuscript. MN and YK confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted according to the principles of The Declaration of Helsinki and was approved by the Ethics Committee of Yamaguchi University (approval number: 2024-165). The clinical samples were originally collected between 2007 and 2014 as part of a prospective clinical study, during which written informed consent was obtained from each participant at the time of enrollment. The present study is a retrospective secondary analysis of those archived samples. In accordance with the institutional guidelines for retrospective research, broad consent for this secondary analysis was obtained through an opt-out procedure published on the hospital website.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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