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Monoclonal antibody for phosphorylated TRIO Y2681 that helps predict prognosis of post-operative colorectal cancer patients

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BACKGROUND: We previously reported that TRIO pY2681, a novel prognostic biomarker for CRC, can be detected by polyclonal antibodies (pAb). We have now developed a novel monoclonal antibody (mAb) that recognizes TRIO pY2681. This study aims to assess the utility of immunohistochemical (IHC) staining using the TRIO pY2681 mAb as a prognostic marker for CRC in clinical practice.

METHODS: IHC using TRIO pY2681 mAb was performed on surgical specimens from 357 CRC patients at Kyoto Medical Center and 320 at Kyoto University Hospital. Based on the results, we conducted a retrospective outcome analysis.

RESULTS: TRIO pY2681 mAb exhibited significantly higher titers than pAb. In both cohorts of all stages, TRIO pY2681 IHC positivity correlated with shorter disease-specific survival (DSS) (HR, 1.67; 95% CI, 1.00–2.79; $P = 0.046$, and HR, 5.84; 95% CI, 2.26–15.1; $P < 0.001$) and relapse-free survival (RFS) (HR, 1.92; 95% CI, 1.15–3.22; $P = 0.011$, and HR, 4.36; 95% CI, 2.17–8.76; $P < 0.001$). The trend persisted in stage III. Multivariate analysis confirmed TRIO pY2681 IHC positivity as an independent prognostic factor for RFS.

CONCLUSIONS: The novel TRIO pY2681 mAb identifies CRC patient subsets with poorer prognoses, enhancing prognostic precision in clinical settings.

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INTRODUCTION

Colorectal cancer (CRC) is ranked the second most lethal cancer and the third most prevalent malignant tumor worldwide [1]. Among people diagnosed with CRC, 20% has metastatic CRC, and 40% present with recurrence after previously treated localized disease. The prognosis of metastatic CRC is poor, with a 5-year survival rate of less than 20%. Despite therapeutic advances, metastatic CRC remains incurable for most patients [2]. Although survival of patients and recurrence of CRC can be comprehensively predicted by combining clinicopathological variables and genomic analysis data, these predictions remain to be improved significantly [3, 4].

A GEF protein TRIO was originally identified in 1996 as a binding partner of the transmembrane tyrosine phosphatase LAR. The TRIO family proteins are large molecules (the relative molecular mass of 350,000 Daltons), containing up to three catalytic domains, for which they are named [5]. Vertebrate TRIO family proteins contain two GEF domains (GEF1 and GEF2) and a kinase domain, each of which can activate discrete signaling output. The GEF1 domain catalyzes GTP exchange on both Rac1 and RhoG in vitro, whereas the GEF2 domain catalyzes GTP exchange on

RhoA also in vitro [6]. It is likely that TRIO plays a key role in coordinating the cell-matrix interactions and cytoskeletal rearrangements involved in cell movements [7, 8].

TRIO has been widely studied for its disease relevance in cancer [9] and neurodevelopmental disorders [10]. Higher expression of the TRIO protein is reported to be associated with a poor prognosis in individuals with glioblastoma [11], breast cancer [12], and hepatocellular carcinomas [9, 13].

We previously reported that activation of the NOTCH signaling pathway can induce phosphorylation of TRIO at its Tyr residue 2681 (TRIO pY2681), leading to increased levels of RHO-GTP and invasion of CRC cells in culture [14]. We raised polyclonal antibodies specific for TRIO pY2681 peptides, and used it to analyze pathological sections of colorectal cancer by IHC. High levels of TRIO pY2681 were found to be correlated with poor prognosis of patients with CRC after surgery [14]. To translate these findings into clinical applications, we now have developed a novel monoclonal antibody for TRIO pY2681. The aim of this study is to investigate the utility of our novel mAb as a prognostic marker for CRC.

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METHODS

Development of mAb for TRIO pY2681

In order to generate specific antibodies for the phosphotyrosine at TRIO Y2681 and its neighboring amino acid residues, we selected an undecapeptide NPNYI(pY)DVPPE (residues #2676–2686) as we used to raise polyclonal antisera [14]. The phosphopeptide was synthetically manufactured by SCRUM (Tokyo, Japan) and BEX (Tokyo, Japan), attaining a purity level exceeding 98%. Subsequently, it was conjugated to keyhole-limpet hemocyanin (KLH) with a polyethyleneglycol (PEG) spacer, also prepared by SCRUM.

After trying to raise mAb by established methods in mice several rounds in vain, we found a venture company that commercialized the mAb technology in chickens with success. In retrospect, this difficulty may be explained in part by the fact that the amino acid sequence surrounding TRIO pY2681 is identical among humans, mice, and even chickens.

The above antigenic peptide was administered to six young chickens in two rounds of immunization, and the mAb was produced at Pharma Foods Industries (PFI) (Kyoto, Japan), in close collaboration with our research team. Briefly, splenic cells were harvested from two immunized chickens, and their immunoglobulin (IgY) mRNA sequences were reverse-transcribed into a cDNA library using a phagemid vector capable of expressing the cDNA as single-chain variable fragment (scFv) proteins. The panning procedure was iteratively performed, resulting in phage clones exhibiting specific binding to the tyrosine-phosphorylated antigen peptide via enzyme-linked immunosorbent assay (ELISA), in contrast to the unphosphorylated counterpart. Through sequencing of 30 clones among them, it turned out that they consisted of essentially three independent clones, with one of them taking up 90% majority. These three clones were converted into divalent IgY by recombinant DNA technology. The mAb protein was synthesized via transient transfection in HEK293 cells at PFI. Monoclonal antibodies from these three clones were evaluated by ELISA, demonstrating high specificity for the phosphorylated antigen peptide (Supplementary Fig. S1). Subsequently, immunohistochemical staining was performed using human CRC surgical specimens to assess their diagnostic utility, and then Clone 29 was chosen, with Clone 40 as a back-up.

Immunohistochemistry (IHC)

Colorectal cancer specimens obtained through surgical procedures were systematically collected, and formalin-fixed paraffin-embedded (FFPE) blocks were prepared following standard protocols at Kyoto Medical Center (KMC) or Kyoto University Hospital (KUHP). We selected 1–3 blocks per patient that appeared most representative and indicative of actively expanding tumors. Those blocks were sectioned into 4 µm thickness. Preceding immunostaining, we executed antigen retrieval and pretreatment procedures, adhering to previously established methods [14]. In summary, deparaffinized sections were incubated with 20 µg/ml Proteinase K (Roche) at 37 °C for 30 min. Subsequently, the slides were treated with 3% H₂O₂ in methanol for 5 min, washed in PBS for 5 min, and then blocked with 10% goat serum + 3% BSA in PBS (Blocking Solution) at 37 °C for 1 h. Following this procedure, the sections were incubated with anti-TRIO pY2681 mAb at a dilution of 40–20 thousand times in the Blocking Solution at 4 °C overnight. After two washes with PBS, each lasting 5 min, the sections were incubated with biotinylated anti-chicken IgY (Vector, CA) at 1/200 dilution in 10% Blocking Solution in PBS for 30 min. Subsequent to two PBS washes, each lasting 5 min, the sections were treated with Reagents A and B, utilizing the avidin-biotin complex interaction to amplify signals of horse-radish peroxidase (Vectastain Elite® ABC Standard kit, Vector), both at 1/50 dilutions at room temperature, for 30 min. The slides were then washed in PBS twice, each time for 5 min, and colorization was achieved using the peroxidase substrate DAB (Vector DAB kit). The colorization reaction was monitored under a microscope and terminated by a swift rinse in PBS.

IHC grading

In IHC, staining patterns of tumor cells and surrounding stromal tissue were formally classified into four grades: Grade 0 to 3 (Fig. 1b, Supplementary Fig. S2). We separated them in this study because the difference showed statistical significance with respect to prognosis (Supplementary Fig. S3), although the biological significance of the distinction between Grade 2 and Grade 3 remains unclear. Specimens were considered TRIO pY2681-positive (+) when the staining intensity in malignant epithelial cells was stronger than that in the surrounding normal tissue whereas TRIO pY2681-negative (–) when the staining intensity was equal to or weaker than that of the surrounding tissue (Fig. 1b, Supplementary Fig. S2). TRIO pY2681 (+) cases exhibited

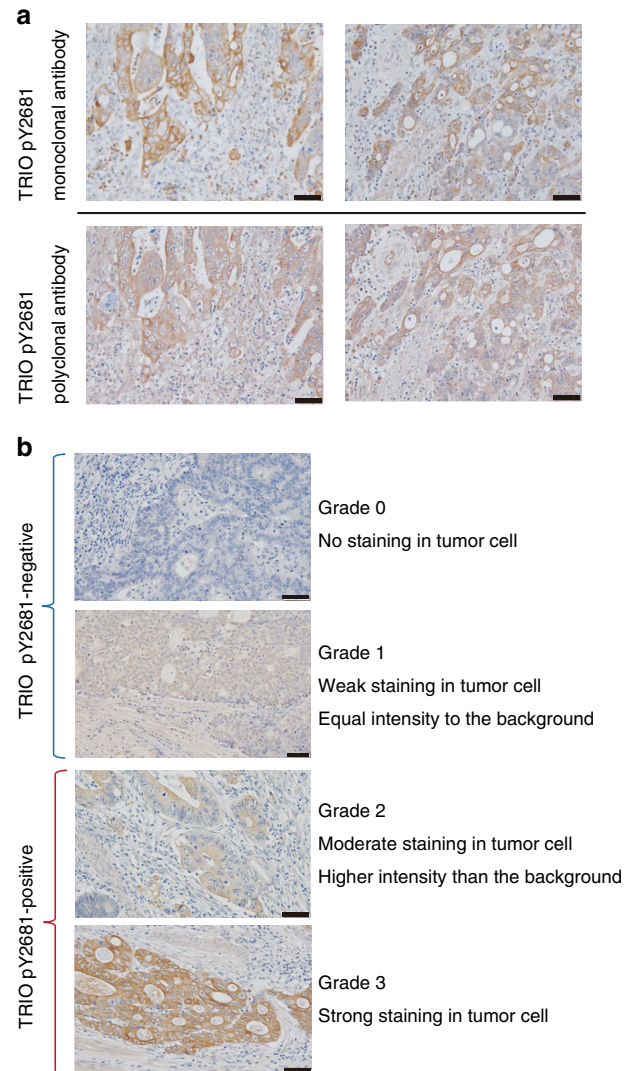


Fig. 1 Immunohistochemical assessment of colorectal cancer specimens using new monoclonal antibody for TRIO pY2681. **a** Representative examples of TRIO pY2681 immunostaining with monoclonal or polyclonal antibodies. Original magnification $\times 40$. Scale bar, 50 µm. **b** Grading criteria for TRIO pY2681 protein assessed by immunohistochemistry. The tumors were classified into four staining grades. Grade 0; No staining in tumor cells. Grade 1; Weak staining in tumor cells. Equal intensity as the background. Grade 2; Moderate staining in tumor cells. Higher intensity than the background. Grade 3; Strong staining in tumor cells. We decided to stratify the colon carcinomas staining into two classes: TRIO pY2681-negative (–) (Grade 0 and 1) or TRIO pY2681-positive (+) (Grade 2 and 3). Original magnification $\times 40$. Scale bar, 50 µm.

characteristic heterogeneous staining even within a cluster of tumor cells, which is like the staining with pAb we reported earlier that was reminiscent of some stem cell markers [14].

All specimens were scored for TRIO pY2681 expression in a blind manner. Multiple slides for a patient (i.e., samples from distinct areas of the same tumor) were scored independently and their results were combined to assess the immunopositivity of the tumor. If there were differences in scores among investigators, the grade of the tumor was determined through a consensus discussion with the final check and approval by a board-certified diagnostic pathologist (T.S.).

Patients

To explore the association between TRIO pY2681 IHC and the prognosis of CRC, we tested two separate patient cohorts retrospectively. One cohort

Table 1. Characteristics of two patients cohorts in this study

	KMC cohort				<i>P</i> value	KUHP cohort				<i>P</i> value
	TRIO pY2681 (–)		TRIO pY2681 (+)			TRIO pY2681 (–)		TRIO pY2681 (+)		
<i>N</i>	172		185			148		172		
Age (years), median (range)	71	(36–103)	68	(31–93)	0.08	68	(34-90)	69	(28-95)	0.78
Sex, <i>n</i> (%)					0.83					0.21
Male	94	(54.7)	98	(53.0)		79	(53.4)	104	(60.5)	
Female	78	(45.3)	87	(47.0)		69	(46.6)	68	(39.5)	
Location, <i>n</i> (%)					0.20					0.80
C	9	(5.2)	16	(8.7)		15	(10.1)	9	(5.2)	
A	27	(15.7)	29	(15.7)		21	(14.2)	22	(12.8)	
T	17	(9.9)	23	(12.4)		13	(8.8)	21	(12.2)	
D	8	(4.7)	12	(6.5)		8	(5.4)	10	(5.8)	
S	55	(32.0)	38	(20.5)		38	(25.7)	44	(25.6)	
R	56	(32.6)	67	(36.2)		53	(35.8)	66	(38.4)	
Pathological stage, <i>n</i> (%)					0.07					<0.01
0	18	(10.5)	6	(3.2)		5	(3.4)	0	(0)	
I	28	(16.3)	33	(17.8)		45	(30.4)	25	(14.5)	
II	53	(30.8)	55	(29.7)		50	(33.8)	50	(29.1)	
III	45	(26.2)	62	(33.5)		37	(25.0)	63	(36.6)	
IV	28	(16.3)	29	(15.7)		11	(7.4)	34	(19.8)	
Histology, <i>n</i> (%)					0.03					0.66
tub1, tub2	158	(91.9)	180	(97.3)		138	(93.9)	159	(92.4)	
por, muc	14	(8.1)	5	(2.70)		9	(6.1)	13	(7.6)	
CEA, <i>n</i> (%)										<0.01
<5						108	(73.5)	91	(52.9)	
≥5						39	(26.5)	81	(47.1)	
Preoperative therapy, <i>n</i> (%)						17	(11.5)	25	(14.7)	0.41
Postoperative therapy, <i>n</i> (%)						48	(32.4)	76	(44.2)	0.04

was the patients who underwent resection operations at Kyoto Medical Center, National Hospital Organization from 2007 to 2009 (KMC cohort), and the other was those at Kyoto University Hospital from 2015 to 2017 (KUHP cohort). The following cases were excluded from the analysis; those received treatment for other cancers or were under follow-up for recurrence at the time of surgery, as well as cases developing other types of primary cancer within the five-year postoperative observation period. Additionally, those cases were also excluded if their histological types were other than adenocarcinoma.

Statistical analysis

The primary endpoints of the statistical analyses were disease-specific survival (DSS), and the secondary endpoints were relapse-free survival (RFS). DSS was defined as the time from the first tumor surgery to death from the colorectal cancer, whereas RFS was defined as the time from the first tumor surgery to relapse or death. Both were censored at the last follow-up for patients without an event. The Kaplan–Meier method estimated DSS and RFS; comparisons were made using the log-rank test. Univariate and multivariate Cox regression analyses were performed in subgroups for DSS and RFS. Hazard ratios with 95% confidence intervals (CIs) were calculated. All *P* values are shown as the results of two-sided tests. A *P* value of ≤ 0.05 was considered statistically significant. Kaplan–Meier analysis and log-rank test were performed using GraphPad Prism version 10 (GraphPad Software, Boston, Massachusetts USA). Univariate and multivariate Cox regression analyses were performed using JMP pro version 17.0.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Immunohistochemistry evaluation

To evaluate the performance of the new TRIO pY2681 mAb, a comparison with our pAb was conducted. Using the TRIO pY2681 (+) specimens with the pAb, the cytoplasm rather than the nucleus of CRC cells was stained with the mAb in the same pattern as with the pAb. As anticipated, the mAb exhibited not only a stronger staining intensity with ~200 times titer/mg protein but also a clearer staining with lower background (Fig. 1a). Accordingly, the immunostaining results with the mAb led to a classification of two patient subgroups; TRIO pY2681 (+) and TRIO pY2681 (–) (Fig. 1b, Supplementary Fig. S2). To determine the clinical relevance of the TRIO phosphorylation in CRC, the association between positive mAb staining and patient's prognosis was assessed in two cohorts; Kyoto Medical Center and Kyoto University Hospital patients.

Kyoto Medical Center (KMC) cohort

We first performed IHC on CRC specimens excised from 357 patients who met the inclusion criteria for colorectal cancer surgery at Kyoto Medical Center from 2007 to 2009 (Supplementary Fig. S4a). Of 356 cases, 172 (48%) were classified as TRIO pY2681 (–) and 185 (52%) as TRIO pY2681 (+) (Table 1). For the cases of immunostaining positivity, a statistically significant

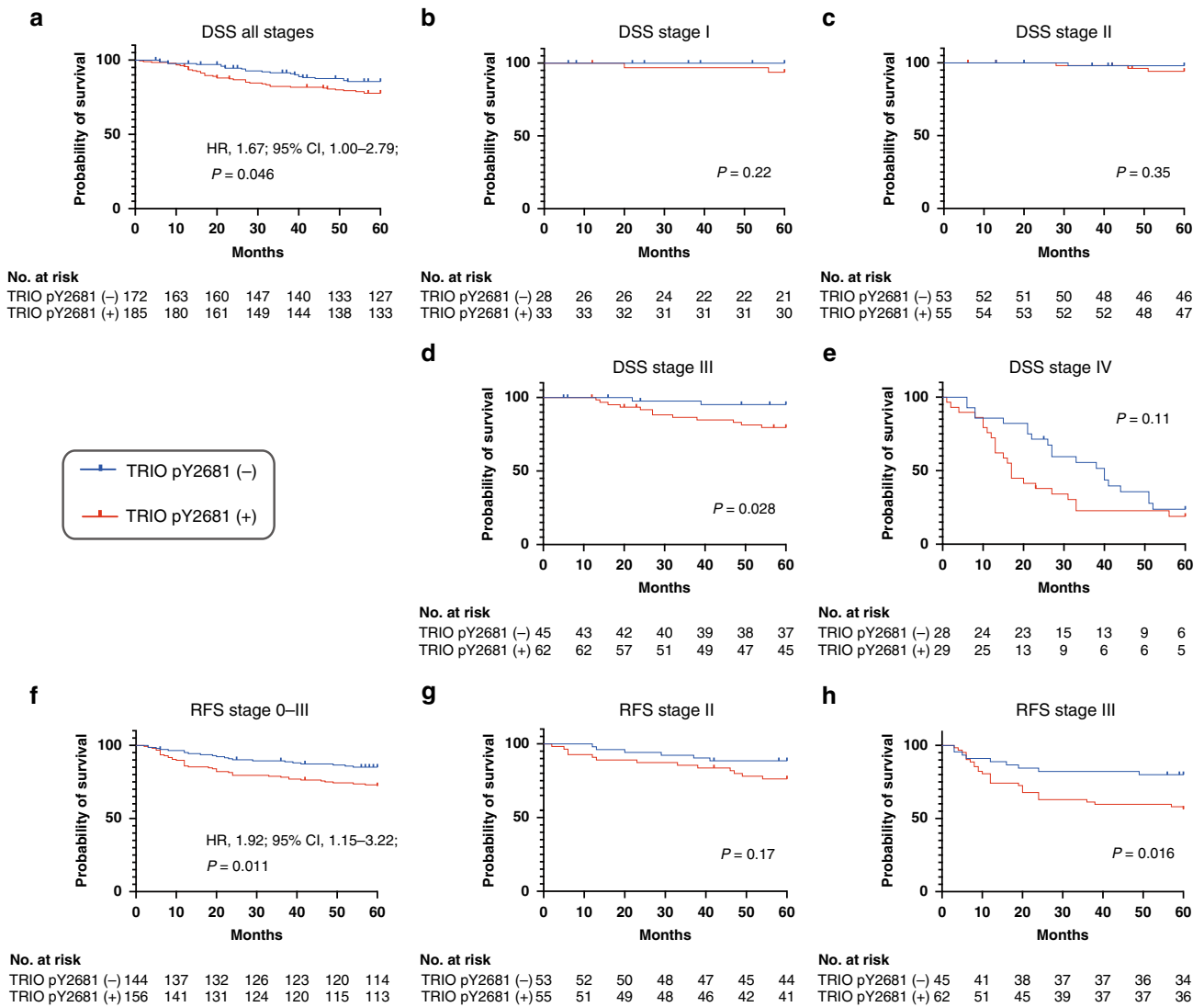


Fig. 2 Kaplan-Meier survival analysis of Kyoto Medical Center (KMC) CRC patients whose tumors had TRIO pY2681 (+) or (–). **a** Disease-specific survival (DSS) of patients. **b–e** The Analysis was stratified by stage. **f**, Relapse-free survival (RFS) of stage 0–III patients. **g, h**, The analysis was stratified by stage II (**g**) or III (**h**). HR, hazard ratio; 95% CI, 95% confidence interval

correlation was observed only with the histopathological type, whereas no associations were noted with other clinical variables (age, sex, tumor location, or pathological stage) (Table 1).

Analysis of DSS using Kaplan-Meier curves and log-rank test revealed a statistically significant shorter survival in the TRIO pY2681-positive subgroup (HR, 1.67; 95%CI, 1.00–2.79; $P = 0.046$) (Fig. 2a).

In a stratified analysis by pathological stage (Fig. 2b–e), a shorter DSS was found in stage III (HR, 4.59; 95%CI, 1.03–20.5; $P = 0.028$) (Fig. 2d). Although no significant differences were observed in other stages, there was a noticeable trend suggesting a poorer prognosis in the TRIO pY2681 (+) subgroup in the stages III and IV. To evaluate whether TRIO pY2681 positivity in IHC was also associated with recurrence, we also investigated the RFS data. In a subgroup analysis of the TRIO pY2681 (+) subgroup (excluding stage IV patients because this subgroup included those with unresected metastases), we found a significantly shorter RFS in the TRIO pY2681 (+) subgroup (HR, 1.92; 95%CI, 1.15–3.22; $P = 0.011$) (Fig. 2f). In a stratified analysis of stages II and III CRC, an association was found between TRIO pY2681 positivity and a shorter RFS in the stage III subgroup (HR, 2.44; 95%CI, 1.15–5.19; $P = 0.016$) (Fig. 2g, h).

Kyoto University Hospital (KUHP) cohort

We next examined CRC specimens excised from 320 patients at Kyoto University Hospital from 2015 to 2017 (Supplementary Fig. S4b). Of 320 cases, 148 (46%) were classified as TRIO pY2681 (–), whereas 172 (54%) as TRIO pY2681 (+). There were significantly more numbers of TRIO pY2681 (+) than TRIO pY2681 (–) tumors in patients with advanced pathological stages, high preoperative CEA levels, and high percentage of post-operative treatment (Table 1).

When data of all-stage patients were combined, the TRIO pY2681 (+) subgroup was associated with a shorter DSS than the TRIO pY2681 (–) subgroup (HR, 5.84; 95%CI, 2.26–15.1; $P < 0.001$) (Fig. 3a). Further stratified analysis by pathological stage showed that DSS tended to be shorter in the TRIO pY2681 (+) subgroup, although there was no statistically significant difference between the two groups (Fig. 3b–e). Regarding RFS (Fig. 3e–h), as in the cohort of Kyoto Medical Center (KMC), the TRIO pY2681 (+) subgroup was significantly shorter in stage III (HR, 3.59; 95%CI, 1.48–8.68; $P = 0.0024$) (Fig. 3h).

Because more detailed data were collected in the cohort of Kyoto University than that of the Kyoto Medical Center, the Cox proportional hazard model was applied to analyze subgroups

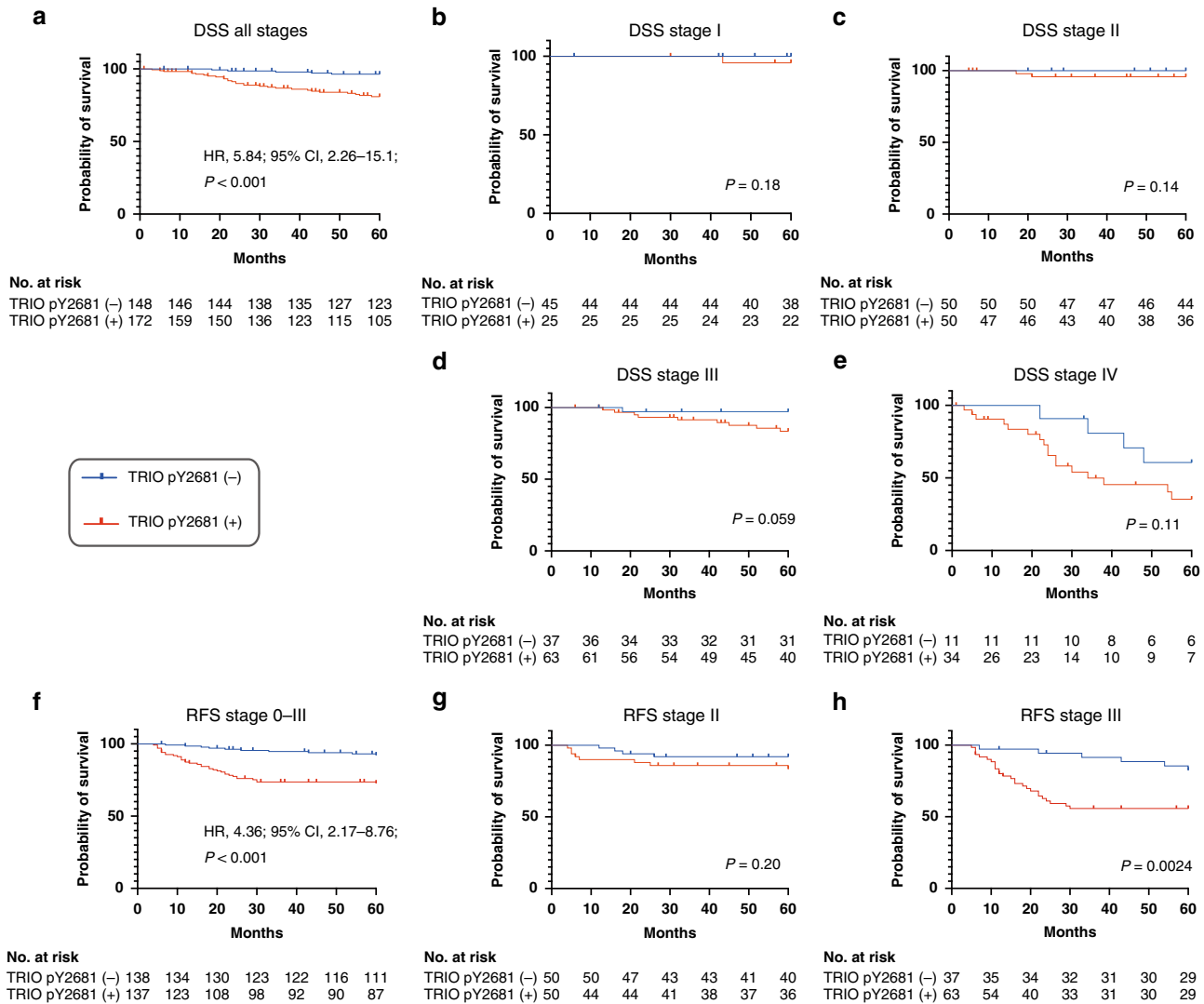


Fig. 3 Kaplan-Meier survival analysis of Kyoto University Hospital (KUHP) CRC patients whose tumors had TRIO pY2681 (+) or (–). **a** Disease-specific survival (DSS) of patients. **b–e**, The Analysis was stratified by stage. **f** Relapse-free survival (RFS) of stage 0–III patients. **g, h** The analysis was stratified by stage II (**g**) or III (**h**). HR, hazard ratio; 95% CI, 95% confidence interval

other than the stage. Although the primary endpoint was DSS, more detailed analysis was conducted using RFS, because of the small number of events. In all subgroup analyses conducted in this study, TRIO pY2681 (+) was associated with poorer prognosis in terms of RFS (Fig. 4). In a univariate analysis, TRIO pY2681 (+) IHC was an independent predictor in addition to high preoperative CEA levels, T3–4 (TNM classification), lymph node metastasis, and vascular invasion, although age or gender were not (Table 2). It was confirmed that TRIO pY2681 (+) IHC was an independent predictor of prognosis by the Cox proportional hazard analysis adjusted for CEA, T3–4, lymph node metastasis, and vascular invasion (Table 2).

DISCUSSION

We previously reported a novel biomarker TRIO pY2681 that could be detected by pAb [14]. In the present study, we have developed a novel mAb that recognizes the same phosphorylated tyrosine and neighboring residues, and characterized it in comparison with the previous pAb regarding its sensitivity, specificity, and utility in clinical diagnosis [14]. The novel mAb displayed a staining pattern like that of the pAb. Specifically, it demonstrated the capability to

detect TRIO pY2681 in CRC at dilutions ranging from 20 to 40 thousand times, in contrast to the effectiveness of pAb at ~300-fold dilutions. Due to its high potency, the anticipated cost for the testing can be reduced substantially in clinical service.

The results of TRIO pY2681 IHC were classified as positive or negative on the basis of differences in the staining intensity between tumor cells and surrounding stromal tissue, as well as the staining heterogeneity within the tumor cells. Grade 3, characterized by intense staining, showed a tendency of strong staining not only in the cytoplasm but also in the tumor cell membranes. These staining characteristics are consistent with the interpretation that TRIO is present in the membrane structures including not only plasma membrane, but also endoplasmic reticulum and Golgi vesicles of CSCs (Cancer Stem Cells) as suggested previously [14, 15]. Originally, TRIO was identified as a candidate protein binding to the cytoplasmic domain of the transmembrane protein tyrosine phosphatase LAR [5]. It has been reported to localize to the cell membrane [16–18] and cell-cell junctions [19, 20]. Our observation of uneven distribution of TRIO pY2681 in the cytoplasmic membrane is consistent with the mechanism that is activated only by the NOTCH signaling initiated at the cell surface receptor bound by its ligand JAG1/DLL4 presented by heterotypic cells such as smooth

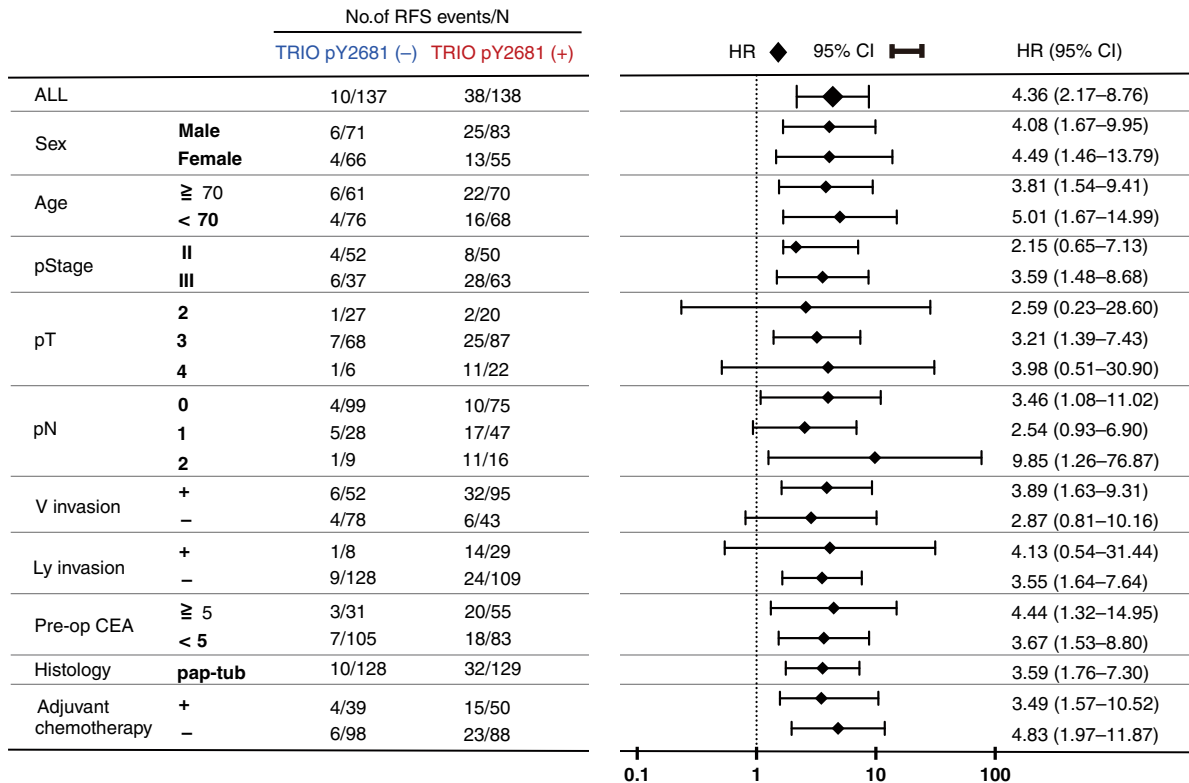


Fig. 4 Forest plot of relapse-free survival (RFS) by TRIO pY2681 of selected subgroups in Kyoto University Hospital (KUHP) cohort. RFS hazard ratio (HR) estimates are displayed based on a Cox proportional hazards model for selected subgroups and plotted on the log scale with 95% CI. pStage pathological Stage, pT pathological T, pN pathological N, V invasion venous invasion, Ly invasion lymphatic invasion

muscle, endothelial cells or macrophages [21]. Further studies are needed to clarify the significance of this localization pattern.

In the current validation study, a distinction was made between TRIO pY2681 (+) and (-) subgroups based on the IHC staining patterns. Correlations were observed between TRIO pY2681 positivity and poor prognosis in both Kyoto Medical Center (KMC) and Kyoto University Hospital (KUHP) cohorts (Figs. 2, 3). In the stratified analysis by TNM stage, TRIO pY2681 (+) subgroups exhibited a trend towards shorter DSS. Particularly in stage III, a statistically significant difference was observed between TRIO pY2681 (+) and (-) subgroups, indicating a shorter RFS in the positive subgroup. In either DSS or RFS, the lack of statistical significance may be explained by the small case numbers when broken down to stage-specific analysis and/or by scarce numbers of events in stages I/II which generally have a much better prognosis than in advanced stages. In the multivariate analysis, TRIO pY2681 (+) was identified as an independent prognostic factor for recurrence. The stage classification reflects the cancer progression at the time of surgery, but it does not indicate how the cancer has progressed or how it will progress in the future. In this study, the tendency of the TRIO pY2681 (+) subgroup within the same stage to have a poorer prognosis suggests that the phosphorylation of TRIO can serve as an indicator of cancer malignancy. This finding implies that TRIO pY2681 IHC may potentially be a valuable marker in predicting prognosis and determining postoperative treatment strategies.

We have reported previously that the phosphorylation of TRIO Y2681 does not influence the primary tumor size but is instead involved in the increased number of liver metastases by activating downstream Rho that drives directional motility associated with invasion and metastasis [14]. In the current study, there was a tendency towards a higher incidence of vascular infiltration in the TRIO pY2681 (+) subgroup (Supplementary Fig. S5). These findings are consistent with our previous results [14].

In the subgroup analysis of the stage III subgroup of KUHP cohort, adjuvant chemotherapy with agents such as fluorouracil and oxaliplatin was found to improve OS and RFS in TRIO pY2681 (+) subgroup (Supplementary Fig. S6). Therefore, more aggressive adjuvant chemotherapy can be administered for stage III TRIO pY2681 (+) cases, although standard adjuvant chemotherapy regimens have demonstrated little efficacy to overcome the TRIO pY2681 (+) status. Currently, there is no report on specific treatment established for TRIO pY2681 (+) patients. According to several reports, however, the knockdown of TRIO has suppressed cell proliferation and migration in certain cancer types [8, 13, 22]. Additionally, inhibitors targeting ROCK, a downstream effector of TRIO signaling, have been shown in preclinical studies to suppress cell motility and/or reduce metastasis in several cancer types in mice, including breast cancer, malignant melanoma, and pancreatic cancer [23–26]. Furthermore, in CRC cell lines, ROCK inhibitors have also been reported to overcome resistance to 5-fluorouracil (5-FU) and inhibit cellular invasion [27–29]. Clinically, a phase I trial of AT13148, a ROCK inhibitor, was conducted for patients with solid tumors although without favorable clinical response partly due to dose-limiting toxicities [30]. It remains to be investigated whether the toxicities were mechanism-based or compound-specific. Notably, ROCK inhibitors such as fasudil have already been approved and are safely used in other diseases such as brain vasospasms. Accordingly, their potential repurposing for cancer treatment can be a promising area of investigation [31]. These reports suggest a potential role for ROCK inhibitors and similar agents as specific therapeutic options in the future.

While acknowledging limitations in our study, it is worth noting that differences in observation periods between the two cohorts (2007–2009 and 2015–2017) may have affected the results. The rapid adoption of molecular targeted drugs for the treatment of recurrent CRC during this period in Japan may explain partly the difference in post-recurrence survival, particularly as reflected in

Table 2. Univariate and multivariate analyses for RFS

Variables		Univariate			Multivariate		
		HR	(95%CI)	P	HR	(95%CI)	P
Age	≥70: <70	1.71	(0.97–3.04)	0.07			
Sex	Male: Female	1.47	(0.81–2.65)	0.20			
CEA	≥5: <5	2.21	(1.25–3.89)	0.006	1.31	(0.73–2.34)	0.36
Histological type	por, muc; tub	2.58	(1.10–6.08)	0.03			
Vascular invasion	(+): (–)	3.4	(1.65–7.03)	<0.001	1.86	(0.89–3.91)	0.10
pT	3–4: 0–2	6.34	(2.28–17.7)	<0.001	2.96	(1.03–8.53)	0.05
pN	(+): (–)	5.03	(2.70–9.38)	<0.001	3.32	(1.75–6.29)	<0.001
Adj chemotherapy	(+): (–)	1.37	(0.77–2.45)	0.28			
TRIO pY2681	(+): (–)	4.36	(2.17–8.76)	<0.001	2.55	(1.25–5.24)	0.011

DSS. It is also possible that the relatively small sample size and unknown potential selection biases and/or variations in adjuvant chemotherapy protocols across the cohorts influenced our analysis results.

Further work should aim to validate the prognostic role of TRIO pY2681 IHC in prospective clinical trials. Using the evaluation criteria for IHC established in this study, the proportion of TRIO-negative and TRIO-positive cases is expected to be roughly equal, or with a slight predominance of TRIO-positive cases (i.e., 1 to ~1.3). Thus, the required sample size was estimated to be ~200 subjects including the leeway for potential dropouts using Schoenfeld's formula assuming a HR of 2 in comparing 5-year DSS. Through prospective studies, further characterization of TRIO-positive patients, such as their relationship with existing therapeutic agents, can lead to improvements in current treatment strategies. Additionally, the development of novel therapies targeting the TRIO signaling pathway may further enable personalized treatment regimens.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

TA, MS, KK, YS, MMT conceptualized the study. TA, FK, HM, YI developed the methodology. TA, HM, YI, KK, RM, IL, KM, KO, YS acquired patient samples and clinical data. TA, HM, ST, MMT evaluated the IHC. TA, TS, MMT analyzed the data. TA, MMT discussed and interpreted the data, and wrote and edited the manuscript. All authors were involved in writing the paper and had final approval of the submitted and published version.

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COMPETING INTERESTS

MMT has ownership interest in and consultant/advisory board relationship with KyoDiagnostics KK (Kyoto, Japan) that executes patent rights owned by Kyoto

University for diagnostic use of TRIO pY2681 antibodies. Other authors have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was performed in accordance with the Declaration of Helsinki. The study protocol involving human samples was approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee as well as the Ethics Committee of Kyoto Medical Center. All participants provided their written informed consent for study participation, including the use of their tissue samples and data analysis (Approval No. G0550, FY 2013-2024).

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