NOTCH gene alterations in metastatic colorectal cancer in the Nationwide Cancer Genome Screening Project in Japan (SCRUM-Japan GI-SCREEN)

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Received: 27 March 2022 / Accepted: 9 May 2022 / Published online: 27 May 2022 © The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

Abstract

Purpose Activated Notch receptor signaling has been implicated in tumor growth and progression in colorectal cancer (CRC). However, the pathogenic relevance of NOTCH gene alterations remains unclear. The aim of this study was to clarify mutational landscapes and assess their clinical significance in patients with metastatic CRC.

Methods Pre-chemotherapy tumor tissues obtained from 1154 metastatic CRC patients in the Nationwide Cancer Genome Screening Project in Japan between April 2017 and March 2019 were studied using the Oncomine Comprehensive Assay.

Results The frequencies of NOTCH1, NOTCH2, and NOTCH3 nonsynonymous sequence variants were 11.5%, 4.4%, and 10.4%, respectively. The majority of variants were missense of unknown significance that were distributed across all domains of all three NOTCH genes. The gain-of-function mutations in NOTCH reported in multiple malignancies were not identified. The NOTCH amplification rate was less than 1%. No NOTCH fusions were detected. In patients who were registered before, or within 1 year of, first-line chemotherapy, overall survival for 51 patients with only NOTCH3 variants was significantly longer than for 540 patients with no NOTCH variants (median, 40.2 months vs 27.7 months; P = 0.04). Multivariate analysis revealed that variant NOTCH3 was an independent prognostic factor for increased survival (hazard ratio 0.61, 95% confidence interval, 0.39–0.94; P = 0.03) besides poor prognostic factors associated with mutant TP53, KRAS, and BRAF, as well as amplified MYC.

Conclusion NOTCH genes are unlikely to harbor driver mutations and amplifications in patients with metastatic CRC. NOTCH3 variant should be further investigated as a favorable prognostic marker.

Keywords NOTCH gene · Colorectal cancer · Next-generation sequencing · Prognosis

Abbreviations

ADAM A zinc-dependent disintegrin and metalloprotease
AKT AKT serine/threonine kinase, protein kinase B
BRAF B-rapidly accelerated fibrosarcoma
CI Confidence interval
CRC Colorectal cancer
DLL Delta-like ligand
EGF Epidermal growth factor
FBXW7 F-box and WD repeat domain containing 7
FISH Fluorescence in situ hybridization
FLT3 Fms-related receptor tyrosine kinase 3
HES1 Hairy and enhancer of split 1
HR Hazard ratio
JAG Jagged ligand
KRAS Kirsten rat sarcoma viral oncogene homolog
MYC Myelocytomatosis proto-oncogene
mCRC Metastatic colorectal cancer
NGS Next-generation sequencing
NICD Notch intracellular domain
The Notch receptor signaling pathway is complex; it can inhibit head and neck cancer, esophageal cancer, and skin squamous-cell carcinoma (Lobry et al. 2011; Zhang et al. 2016). In colorectal cancer (CRC), Notch activation has been reported to play a major role in the epithelial-to-mesenchymal transition. It also is involved in tumor growth through activation of the PI3K-AKT signaling pathway, inactivation of the TP53 signaling pathway, activation of the TGF-β signaling pathway, and upregulation of MYC (Tyagi et al. 2020; Jackstadt et al. 2019; Varga et al. 2020; Koch and Radtke 2020). JAG1 is upregulated by β-catenin in the WNT signaling pathway, and DLL4 is upregulated by vascular endothelial growth factor activating Notch (Reedijk et al. 2008; Estrach et al. 2006; Katoh and Katoh 2006).

It has been reported that Notch1 and Notch3 promote tumor growth, invasion, metastasis, and angiogenesis; its overexpression is a poor prognostic factor for patients with mCRC (Varga et al. 2020; Zhang et al. 2010; Chu et al. 2011; Piecuch et al. 2020; Ozawa et al. 2014). These results were predominantly derived from basic research or immunohistochemical analyses of protein expression, and it has been unclear how NOTCH variants are involved in the prognosis of mCRC patients. In this study, we identified NOTCH gene alterations in mCRC and assessed their clinical relevance.

### Methods

#### Study design and patients

This was an observational, retrospective, multicenter study of patients with mCRC. A total of 1777 patients with mCRC were enrolled in the SCRUM-Japan GI-SCREEN. Tumor tissue samples were sequenced using next-generation sequencing (NGS). The included patients had (1) a pathologically confirmed colorectal adenocarcinoma, (2) received systemic chemotherapy for metastatic disease, (3) RAS mutational status identified by PCR, (4) an Eastern Cooperative Oncology Group performance status of 0–1 and adequate bone marrow, renal, and hepatic function at the initiation of chemotherapy; (5) no other severe medical conditions; and (6) provided written informed consent. The ethical, medical, and scientific aspects of the study were reviewed and approved by the institutional review board of each institution. This trial was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN000016343). The study was conducted in accordance with the Declaration of Helsinki, as revised in 2000.

#### Targeted sequencing

Formalin-fixed and paraffin-embedded biopsy or surgically resected samples were sent to the clinical laboratory improvement amendments-certified Life Technologies Clinical Services Laboratory (910 Riverside Parkway, Helsinki, as revised in 2000.)
West Sacramento, CA 95605, USA). Tumor DNA and RNA were extracted and used for multiplex PCR-based amplicon sequencing using the Ion Torrent™ Oncomine™ Comprehensive Assay v3 (Thermo Fisher Scientific, Waltham, MA, USA). This assay covers 161 of the most relevant cancer-related genes and detects relevant single nucleotide variants, copy-number variations, gene fusions, and indels in 1 streamlined workflow (Supplementary Table 1). The annotated genome variant call format files and the binary version of the sequence alignment files were stored at the SCRUM-Japan Data Center.

NGS

A variant was called if its allele frequency was more than 5% and its depth of coverage was more than 200 reads, excluding synonymous variants. Amplification was defined as a copy-number of ≥ 4.0. Driver mutation classifications, such as gain-of-function or loss-of-function, were determined using the Oncomine Knowledgebase and were annotated using Ion Reporter™ software.

Sequence data were mapped onto NOTCH1, NOTCH2, and NOTCH3 using the eBioPortal tool MutationMapper. We evaluated the frequency of co-alteration status of NOTCH1, NOTCH2, and NOTCH3 genes, including VUS and potential driver genes, in receptor tyrosine kinase, RAS-MAPK, PI3K-AKT, TP53, WNT, TGF-β, and DNA damage repair signaling pathways. Variant allele frequency (VAF) status was divided into two categories, high or low, using the median for each NOTCH gene.

Statistical analyses

For mutual exclusivity, P values were derived from the one-sided Fisher’s exact test, and Q values were derived from the Benjamini–Hochberg false-discovery rate correction procedure. The significance of differences in age was estimated using the Mann–Whitney U test. Differences in proportions were evaluated using a two-sided Fisher’s exact test. Overall survival (OS) was defined as the time from the initiation of first-line chemotherapy to death by any cause. Survivors were censored at the last contact. OS was calculated using the Kaplan–Meier method and compared using the log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using the Cox proportional hazards model. In multivariate analyses, the Cox proportional hazards model was used to evaluate the prognostic significance of survival. All statistical analyses were conducted using JMP software (version 16.0; SAS Institute, Cary, NC, USA). Statistical significance was set at P < 0.05.

Results

Gene alterations and mutual relationships

Between April 2017 and March 2019, 1777 patients were enrolled, with sequencing for 1613 completed (Fig. 1). Of these, 1154 patients who met the study inclusion criteria and whose tumor samples were obtained before chemotherapy initiation were defined as the gene analysis population. The frequencies of NOTCH1, NOTCH2, and NOTCH3 sequence variants were 11.5%, 4.4%, and 10.4%, respectively (Fig. 2). The frequencies of NOTCH1, NOTCH2, and NOTCH3 amplifications were 0.8%, 0.9%, and 0.4%, respectively. No NOTCH fusions were detected. For the relationship between NOTCH and other major gene alterations (> 5%), there were no mutually exclusive gene alterations in NOTCH and other genes (Fig. 3). NOTCH alterations overlapped in 14.1% of cases (Fig. 4). NOTCH1 and NOTCH3 alterations overlapped with each other in 21.8% (31/142) and 24.8% (31/125), respectively.

Variant loci were mapped onto Notch domain organization (Fig. 5). No oncogenic mutations previously reported in the literature were detected. Missense variants were diffusely distributed across all domains of NOTCH1, NOTCH2, and NOTCH3. In NOTCH1, two missense variants of VUS (p.Gln1134Arg and p.Arg2263Gln) were found in more than 10 patients. Truncating variants (nonsense or frameshift) were mostly observed in the NOTCH3 extracellular domain (n = 22, 1.9%). In particular, p.Asp847Ter/fs was found in 14 patients (1.2%). These are all considered loss-of-function variants.

Clinicopathological relationships with NOTCH alterations

The relationships between clinicopathological features and sequence variants of NOTCH1, NOTCH2, and NOTCH3 are shown in Table 1. The proportions of right-colon and non-tubular (poorly differentiated, mucinous, and signet ring cell) adenocarcinoma were significantly higher in patients with NOTCH3 variants than in those without (P < 0.01, and P = 0.02, respectively). These differences were not observed in patients with the NOTCH3 truncating variant (Supplementary Table 2). NOTCH1, NOTCH2, and NOTCH3 amplifications were not associated with any clinicopathological features (Supplementary Table 3).

Prognostic significance of NOTCH alterations

To avoid survivor bias, we excluded patients registered for more than one year after the initiation of first-line
chemotherapy. The remaining 713 patients were analyzed for survival as a prognostic population (Fig. 1). The median follow-up time was 23.6 months (range 0.7–54.6 months); 400 patients (56.1%) died. There were no differences in OS between the \( \text{NOTCH1} \)-variant and -wildtype patients (median OS 25.2 months vs. 28.2 months; HR 1.07, \( P = 0.63 \)), the \( \text{NOTCH2} \)-variant and -wildtype patients (median OS 26.0 months vs 28.1 months; HR 1.29, \( P = 0.23 \)), and the \( \text{NOTCH3} \)-variant and -wildtype patients (median OS 31.3 months vs 27.6 months; HR 0.77, \( P = 0.13 \)) (Fig. 6). No difference in OS was observed between patients with the \( \text{NOTCH3} \) truncating variant and VUS (\( P = 0.98 \)) (Supplementary Fig. 1). In the comparison between patients with high and
low VAF, OS was not significantly different among the three NOTCH variants (Supplementary Fig. 2). However, the patients having high VAF of NOTCH3 had longer, but not statistically significant OS than those having low VAF (median OS 40.2 months vs 31.2 months; HR 0.70, \(P = 0.30\)).

Since the prognostic trend of variants and their allele frequencies is consistent for NOTCH3, but not for NOTCH1 or NOTCH2, we separated the patients with NOTCH covariants to exclude their combined influence on OS, and found a significant difference between the NOTCH3-variant patients without other NOTCH covariants and all NOTCH-wildtype patients (median OS 40.2 months vs 27.7 months; HR 0.64, \(P = 0.04\)) (Fig. 7).

Multivariate analysis showed that male sex, non-tubular adenocarcinoma, mutant TP53, mutant KRAS, mutant BRAF, and amplified MYC were independent prognostic factors for poorer prognosis, while variant NOTCH3 without covariants in NOTCH1 and NOTCH2 was an independent prognostic factor for better prognosis (HR 0.61, 95% CI 0.39–0.94, \(P = 0.03\)) (Table 2). In the subgroup OS analyses for each NOTCH variant without the NOTCH covariants (Supplementary Fig. 3), significant interactions were observed for BRAF and MYC status with NOTCH1 variants (\(P < 0.01\), and \(P = 0.02\), respectively), and for FLT3 status with NOTCH3 variants (\(P = 0.04\)).
Discussion

In this study, we observed 5–10% NOTCH variants and less than 1% NOTCH amplifications in patients with mCRC. We identified no pathogenic mutational hotspots reported in other cancers, such as T cell acute lymphoblastic leukemia, adenoid cystic carcinoma, and triple-negative breast cancer (Aster et al. 2017; Ferrarotto et al. 2017; Stoeck et al. 2014; Weng et al. 2004). Our most interesting finding was that variant NOTCH3 without covariants in NOTCH1 and NOTCH2 was an independent favorable prognostic factor. To the best of our knowledge, this is the first report to demonstrate the genetic status and clinical relevance of NOTCH gene alterations in a large-scale mCRC study. The frequencies and patterns of gene alterations were similar to those of the Cancer Genome Atlas project of human CRC and its Japanese version (Cancer Genome Atlas Network 2012; Nagashima et al. 2020), suggesting that our cohort is representative of mCRC patients. The occurrence of NOTCH1 and NOTCH3 alterations was frequently observed in our mCRC patients (Fig. 4); this appeared to affect patient prognosis, as there was a significant difference in OS between the sole NOTCH3 variant and all NOTCH wildtype (Fig. 7). This co-occurrence may be biologically relevant. As the reason for no significant difference between NOTCH3 variants including covariants and NOTCH3 wildtype, we considered that the NOTCH covariants having a worse trend of survival than all NOTCH wildtype (HR 1.20 in Fig. 7) interfered with the favorable significance of NOTCH3 variants.

Driver NOTCH mutations predominantly occur in the NRR and PEST domains (Pagliaro et al. 2020; Aster et al. 2017; Ferrarotto et al. 2017; Stoeck et al. 2014; Weng et al. 2004). Missense and in-frame insertion/deletion mutations in NRR disrupt its structure, resulting in ligand-independent proteolysis by metalloproteases, NICD release, and activation of Notch-targeted genes. Nonsense and frameshift mutations in the PEST domain, which binds the ubiquitin ligase FBXW7, lead to the inhibition of NICD degradation, NICD accumulation, and activation of Notch-targeted genes. We did not find any such mutations in the NRR and PEST domains in any NOTCH gene in our patients. This suggests that NOTCH gene alterations are unlikely to be associated with the progression of mCRC.

We showed that the proportions of right-sided primary tumors and non-tubular adenocarcinomas were higher in patients with NOTCH3 variants than in those without. It is well known that right-sided tumors are undifferentiated more frequently than left-sided tumors and exhibit more aggressive progression. The Notch receptor signaling pathway in the colon and rectum is necessary to maintain intestinal homeostasis and inhibit the differentiation of colonic epithelial cells (Katoh and Katoh 2007; Miyamoto and Rosenberg 2011). Thus, increased expression of NICD induces this differentiation. Unfortunately, we did not examine the expression of NICD in the same samples, so we could not explain how these might be related to each other in this study. NOTCH3 sequence variations rather repressed Notch3 activity and were favorable events for the prognosis of patients with mCRC.

In this study, non-tubular adenocarcinoma, mutant TP53, mutant KRAS, mutant BRAF, and amplified MYC were independent poor prognostic factors. These results were consistent with those of other prognostic studies (Yaeger et al. 2018). In addition, variant NOTCH3 was significantly associated with a better prognosis in the absence of other NOTCH covariants. A plausible explanation for this might be truncating and missense variations in NECD; truncating variations lead to a loss of NICD, and missense variations in the EGF-like domain could modify fringe regions to bind ligands. These may cause the loss-of-function of Notch3 and subsequent decrease of its downstream signaling, and result in a favorable prognosis of variant NOTCH3.

Biochemical and genetic studies have indicated that the glycosylation of NECD plays a critical role in Notch signaling (Luca et al. 2017; Yamamoto et al. 2012; Urata et al. 2020). A previous study showed that fringe modifications

Fig. 4 Venn diagram illustrating overlaps between NOTCH1, NOTCH2, and NOTCH3 gene alterations
Fig. 5 Variant loci across NOTCH genes mapped onto a NOTCH1, b NOTCH2, and c NOTCH3 with the cBioPortal tool MutationMapper. Domains of NOTCH genes are shown beneath. Green circles, missense variants; black circles, truncations (nonsense point variants, frameshift deletions and frameshift insertions); orange circles, in-frame variants. NECD Notch extracellular domain, NICD Notch intracellular domain, TM transmembrane, EGF epidermal growth factor, NRR negative regulatory region, ANK ankyrin repeat, PEST peptide sequence rich in proline, glutamine, serine, and threonine
at EGF8 and EGF12 in EGF-like repeats enhanced Notch1 binding to, and activation by, DLL1, while modifications at EGF6 and EGF36 inhibited Notch1 activation by JAG1 (Kakuda and Haltiwanger 2017). In our study, there were no patients with NOTCH1 variants in EGF8 and EGF12, and only four patients with variants in EGF6 and EGF36 (Fig. 5). The ligand-binding regulation by the glycosylation of NECD may not be affected by NOTCH1 variations in mCRC. However, these phenomena for NOTCH3 are not well understood; therefore, further investigation of NOTCH3 variations and NECD glycosylation is needed.

As for our other analyses, NOTCH1, NOTCH2, and NOTCH3 amplifications were less than 1%, and the number of patients was too small to analyze their effects on survival. A previous study found that NOTCH1 copy-number gain was present in approximately 20% of patients with metastatic CRC and was associated with lower relapse-free survival after liver resection, but not OS (Arcaroli et al. 2016). This discrepancy is difficult to interpret; it may be due to the copy-number cutoff values (4 vs. 3), detection methods (PCR-based amplicon sequencing vs. FISH tissue microarray), and differences in study subject selection (metastatic patients vs. liver-resected patients). NOTCH amplifications in the publicly available cBioPortal database are also rare (data not shown); therefore, a comparison with the FISH method should be investigated further. In the subgroup analyses for OS, interactions were observed with BRAF and MYC for NOTCH1 and with FLT3 for NOTCH3. These results suggest the presence of synergistic or antagonistic actions between NOTCH gene alterations and those in other genes. Since the number of subgroup patients was small, these interactions should be further validated and studied to identify their mechanisms. The reason for the different prevalence of NOTCH3 variant in sidedness was unclear. However, in the subgroup analyses, the NOTCH3 variant showed a favorable trend in both right- and left-sided CRC (interaction test, $P = 0.39$), regardless of the difference.

### Table 1 Patient characteristics as a function of NOTCH gene variation

|                  | NOTCH1 | P value | NOTCH2 | P value | NOTCH3 | P value |
|------------------|--------|---------|--------|---------|--------|---------|
|                  | Wildtype | Variant n = 133 | Wildtype | Variant n = 51 | Wildtype | Variant n = 120 |
|                  | n = 1021 | N (%) | n = 1103 | N (%) | n = 1034 | N (%) |
| Age              | 62 (23–88) 64 (28–82) | 0.65 | 63 (23–88) 65 (28–80) | 0.29 | 63 (23–88) 61 (28–82) | 0.34 |
|                  | < 65 years | 565 (55.3) 68 (51.1) | 0.40 | 608 (55.1) 25 (49.0) | 0.39 | 559 (54.1) 74 (61.7) | 0.12 |
|                  | ≥ 65 years | 456 (44.7) 65 (48.9) | 0.84 | 495 (44.9) 26 (51.0) | 0.22 | 475 (45.9) 46 (38.3) | <0.01 |
| Gender           | Male | 561 (54.9) 67 (50.4) | 0.35 | 601 (54.5) 27 (52.9) | 0.89 | 569 (55.0) 59 (49.2) | 0.25 |
|                  | Female | 460 (45.1) 66 (49.6) | 0.50 | 502 (45.5) 24 (47.1) | 0.18 | 465 (45.0) 61 (50.8) | 0.75 |
| Primary site     | Right colon | 320 (31.3) 43 (32.3) | 0.84 | 343 (31.1) 20 (39.2) | 0.22 | 308 (29.8) 55 (45.8) | <0.01 |
|                  | Left colon, rectum | 690 (67.6) 89 (66.9) | 0.35 | 749 (67.9) 30 (58.8) | 0.22 | 714 (69.1) 65 (54.2) | 0.02 |
|                  | Unknown | 11 (1.1) 1 (0.8) | 0.05 | 11 (1.0) 1 (2.0) | 0.05 | 12 (1.2) 0 (0.0) | 0.00 |
| Histology        | Tubular adenocarcinoma | 908 (88.9) 115 (86.5) | 0.31 | 977 (88.6) 46 (90.2) | 1.00 | 924 (89.4) 99 (82.5) | 0.02 |
|                  | Non-tubular adenocarcinomaa | 109 (10.7) 18 (13.5) | 0.02 | 122 (11.1) 5 (9.8) | 0.22 | 106 (10.3) 21 (17.5) | 0.02 |
|                  | Unknown | 4 (0.4) 0 (0.0) | 0.05 | 4 (0.4) 0 (0.0) | 0.05 | 4 (0.4) 0 (0.0) | 0.00 |

*Poorly differentiated, mucinous, and signet ring cell adenocarcinomas*

$P$ values were calculated using a two-sided Fisher’s exact test, with the exception of those for age, which were calculated using the Mann–Whitney U test.
In Notch-targeted drug development, multiple drugs and biologics have been investigated not only for hematological tumors, but also for solid tumors (Katoh and Katoh 2020; Strosberg et al. 2012; Ferrarotto et al. 2018; Wang et al. 2015; Massard et al. 2018; Kieran et al. 2021). However, no Notch-targeted drugs have been approved for any malignancy. A study of γ-secretase inhibitor monotherapy in patients with mCRC showed no objective response and short progression-free survival, indicating a lack of clinical activity (Strosberg et al. 2012). Since NOTCH amplifications were rare and there were no driver-mutation hotspots in 1154 patients with mCRC, NOTCH gene alterations are unlikely to be therapeutic targets for mCRC therapeutics. However, if Notch is overexpressed, it may be a therapeutic target, and the sole NOTCH3 variant can be considered as one of the stratification factors for Notch-targeted clinical trials in patients with mCRC.

Our study has several limitations. We did not use a validation cohort because of the exploratory nature of our study. The information about metastatic organs was not collected in this study. Immunohistochemical investigation of Notch expression was necessary, as mentioned before, because wildtype NOTCHs may be upregulated like EGFR in mCRC. Although there have been several reports on the protein expression levels of Notch1, Notch2, and Notch3 in CRC, the relationship between these expression levels and genetic alterations remains unclear (Varga et al. 2020; Zhang et al. 2010; Chu et al. 2011; Piecuch et al. 2020; Ozawa et al. 2014). The patients within 1 year from the initiation of first-line chemotherapy were included, and this was still a survival bias. However, most patients meeting the eligibility criteria would survive for more than 1 year, and the bias seems to be small.

In summary, this study provides insights into NOTCH gene alterations in mCRC. Although some patients with mCRC harbored NOTCH1, NOTCH2, and NOTCH3 variants, no driver mutations were identified, and NOTCH gene amplifications were also rare. These findings suggest that NOTCH gene alterations are not involved in the progression of mCRC. In contrast, the NOTCH3 variant is considered to be a favorable prognostic factor, and further study is needed.

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**Fig. 6** Kaplan–Meier plots of overall survival in patients with a NOTCH1 variant versus wildtype, b NOTCH2 variant versus wildtype, and c NOTCH3 variant versus wildtype. OS overall survival, HR hazard ratio, CI confidence interval. P values were calculated using the log-rank test.
Fig. 7 Kaplan–Meier plots of overall survival as a function of NOTCH variant status. Patients were divided into 5 groups: NOTCH1 variant without other NOTCH covariants, NOTCH2 variant without other NOTCH covariants, NOTCH3 variant without other NOTCH covariants, NOTCH covariants, and all NOTCH wildtype. OS overall survival, HR hazard ratio, CI confidence interval. P values were calculated using the Wald test.
Table 2  Overall survival analyses

| Category                        | Univariate                     | Multivariate                  |
|---------------------------------|---------------------------------|-------------------------------|
|                                 | \( N \)  | Median OS (months) | Hazard ratio | 95% CI | \( P \) value | Hazard ratio | 95% CI | \( P \) value |
| Age                             |                                  |                               |
| < 65 years                      | 389 | 26.2 | 1.00 |       |       | 1.00 |       |       |
| \( \geq 65 \) years             | 324 | 30.7 | 0.80 | 0.66–0.98 | 0.03 | 0.84 | 0.68–1.03 | 0.09 |
| Gender                          |                                  |                               |
| Male                            | 402 | 27.1 | 1.00 |       |       | 1.00 |       |       |
| Female                          | 311 | 29.7 | 0.88 | 0.72–1.07 | 0.19 | 0.76 | 0.62–0.94 | 0.01 |
| Primary site                    |                                  |                               |
| Right colon                     | 243 | 25.3 | 1.00 |       |       | 1.00 |       |       |
| Left colon, rectum              | 464 | 29.2 | 0.80 | 0.65–0.98 | 0.03 | 0.87 | 0.70–1.09 | 0.22 |
| Histology                       |                                  |                               |
| Tubular adenocarcinoma          | 614 | 29.4 | 1.00 |       |       | 1.00 |       |       |
| Non-tubular adenocarcinoma\(^a\) | 97  | 16.5 | 2.15 | 1.66–2.79 | <0.01 | 2.19 | 1.66–2.88 | <0.01 |
| **NOTCH1**                      |                                  |                               |
| Wildtype or NOTCH2/NOTCH3 covariant | 640 | 28.2 | 1.00 |       |       | 1.00 |       |       |
| Variant                         | 73  | 27.2 | 0.99 | 0.71–1.38 | 0.97 | 0.97 | 0.69–1.36 | 0.84 |
| **NOTCH2**                      |                                  |                               |
| Wildtype or NOTCH1/NOTCH3 covariant | 687 | 28.1 | 1.00 |       |       | 1.00 |       |       |
| Variant                         | 26  | 26.0 | 1.28 | 0.79–2.09 | 0.31 | 1.26 | 0.77–2.08 | 0.36 |
| **NOTCH3**                      |                                  |                               |
| Wildtype or NOTCH1/NOTCH2 covariant | 662 | 27.6 | 1.00 |       |       | 1.00 |       |       |
| Variant                         | 51  | 40.2 | 0.63 | 0.41–0.97 | 0.04 | 0.61 | 0.39–0.94 | 0.03 |
| **TP53**                        |                                  |                               |
| Wildtype                        | 266 | 30.8 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 447 | 27.0 | 1.21 | 0.99–1.49 | 0.07 | 1.39 | 1.12–1.73 | <0.01 |
| **KRAS**                        |                                  |                               |
| Wildtype                        | 387 | 30.7 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 326 | 26.1 | 1.23 | 1.01–1.50 | 0.04 | 1.44 | 1.15–1.80 | <0.01 |
| **PIK3CA**                      |                                  |                               |
| Wildtype                        | 601 | 28.2 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 112 | 27.0 | 1.10 | 0.85–1.43 | 0.45 | 1.07 | 0.81–1.42 | 0.64 |
| **FLT3**                        |                                  |                               |
| Non-amplification               | 619 | 27.6 | 1.00 |       |       | 1.00 |       |       |
| Amplification                   | 94  | 30.0 | 0.81 | 0.60–1.09 | 0.16 | 0.80 | 0.59–1.09 | 0.16 |
| **BRAF**                        |                                  |                               |
| Wildtype                        | 630 | 28.5 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 83  | 21.8 | 1.55 | 1.16–2.07 | <0.01 | 1.55 | 1.11–2.17 | 0.01 |
| **MYC**                         |                                  |                               |
| Non-amplification               | 646 | 28.3 | 1.00 |       |       | 1.00 |       |       |
| Amplification                   | 67  | 22.4 | 1.38 | 1.01–1.89 | 0.04 | 1.39 | 1.00–1.92 | 0.05 |
| **FBXW7**                       |                                  |                               |
| Wildtype                        | 666 | 27.9 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 47  | 28.5 | 0.94 | 0.62–1.43 | 0.78 | 0.80 | 0.52–1.23 | 0.32 |
| **SMAD4**                       |                                  |                               |
| Wildtype                        | 668 | 28.2 | 1.00 |       |       | 1.00 |       |       |
| Mutant                          | 45  | 26.7 | 1.29 | 0.87–1.89 | 0.20 | 1.16 | 0.78–1.72 | 0.46 |

Kaplan–Meier estimates of median overall survival

\( P \) values were calculated using the Wald test

\( OS \) overall survival, \( CI \) confidence interval

\(^a\)Poorly differentiated, mucinous, and signet ring cell adenocarcinomas
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00432-022-04064-4.

Acknowledgements This work was supported by SCRUM-Japan funds (http://www.scrum-japan.ncc.go.jp/index.html). We thank the patients and members who participated in this study. We owe special thanks to Yasunori Asano for helping with the data analysis. We would like to thank Editage for English language review.

Author contributions TK and IH designed the study; AN, YN, MS, SY, HT, HH, TO, TE, ES, AT, TMo, TD, KO, YS, YH, HK, TKat, TS, KA, TMI, HY, MG, and HQ performed the clinical research; TK, NY, and RY analyzed the data; and TN, KY, TY, and IH supervised the study. All the authors approved the manuscript.

Funding This work was supported by SCRUM-Japan funds (http://www.scrum-japan.ncc.go.jp/index.html).

Data availability The authors declare that all variant data used in the conduct of the analyses are available within the article and its supplementary information. To protect the privacy and confidentiality of patients in this study, clinical data are not made publicly available in a repository or the supplementary material of the article, but will be made available following reasonable request to the corresponding author.

Declarations

Conflict of interest Takeshi Kajiwara received honoraria for lectures from Taiho Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., and Eli Lilly Japan K.K. Tomohiro Nishina received honoraria for lectures from Taiho Pharmaceutical Co., Ltd., and Ono Pharmaceutical Co., Ltd. Yoshiaki Nakamura received honoraria for lectures from Chugai Pharmaceutical Co., Ltd., and research grants from Chugai Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd. Satoshi Yuki received honoraria for lectures from Eli Lilly Japan K.K., Chugai Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Bristol-Myers Squibb K.K. Hiroya Taniguchi received honoraria for lectures from Chugai Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Taiho Pharmaceutical Co., Ltd., and research grants from Takeda Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd. Satoshi Yuki received honoraria for lectures from Eli Lilly Japan K.K., Chugai Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., and Bristol-Myers Squibb K.K. Hiroki Hara received research grants from AsaZeneca K.K., Bayer Yakuhin, Ltd., BeiGene, Ltd., Chugai Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., MSD K.K., and Ono Pharmaceutical Co., Ltd. Takeshi Ohta received research grants from Takeda Pharmaceutical Co., Ltd., and research grants from Ono Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., MSD K.K., Eisai Co., Ltd., Bayer Yakuhin, Ltd., Bristol-Myers Squibb K.K., and BeiGene, Ltd. Tadamichi Denda received research grants from Symsex Corporation and Ono Pharmaceutical Co., Ltd., fees from Sawai Pharmaceutical Co., Ltd., and research grants from MSD K.K., and Ono Pharmaceutical Co., Ltd. Hisato Kawakami received honoraria for lectures from Bristol-Myers Squibb K.K., Ono Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd., and research grants from Bristol-Myers Squibb K.K., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Kobayashi Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd. Takeshi Kato received honoraria for lectures from Chugai Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Yakult Honsha Co., Ltd., and Taiho Pharmaceutical Co., Ltd., and research grants from Chugai Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd. Taroh Satoh received honoraria for lectures from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Bristol-Myers Squibb K.K., Taiho Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd., research grants from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Bristol-Myers Squibb K.K., Taiho Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd., MSD K.K., Gilead Sciences, Inc., and Parexel International Corporation, scholarship grants from Taiho Pharmaceutical Co., Ltd., and Daiichi Sankyo Co., Ltd., and endowed chair from Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., and Yakult Honsha Co., Ltd. Kentaro Yamazaki received honoraria for lectures from Chugai Pharmaceutical Co., Ltd., and Takeda Pharmaceutical Co., Ltd. Takayuki Yoshino received honoraria for lectures from Taiho Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Eli Lilly Japan K.K., Takeda Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., Bayer Yakuhin, Ltd., and Ono Pharmaceutical Co., Ltd., and research grants from MSD K.K., Ono Pharmaceutical Co., Ltd., Sanofi K.K., Daiichi Sankyo Co., Ltd., Chugai Pharmaceutical Co., Ltd., Parexel International Corporation, Taiho Pharmaceutical Co., Ltd., Amgen K.K., and Sumitomo Dainippon Pharma Co., Ltd. Ichinosuke Hyodo received honoraria for lectures from Yakult Honsha Co., Ltd., and Taiho Pharmaceutical Co., Ltd., and participated in Data Safety Monitoring Boards or Advisory Boards of Ono Pharmaceutical Co., Ltd., Chugai Pharmaceutical Co., Ltd., Daiichi Sankyo Co., Ltd., Taiho Pharmaceutical Co., Ltd., Merck Biopharma Co., Ltd., and Asahi Kasei Pharma Corporation. The other authors declare no conflicts of interest.

Ethics approval The ethical, medical, and scientific aspects of the study were reviewed and approved by the institutional review board of each institution. The study was conducted in accordance with the Declaration of Helsinki, as revised in 2000.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent to publish This manuscript contains no individual data.

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