Differential effects of KRAS mutational status on long-term survival according to the timing of colorectal liver metastases

Nozomu Sakai, Katsunori Furukawa, Tsukasa Takeyashiki, Satoshi Kuboki, Shigetsugu Takano and Masayuki Ohtsuka*

Abstract

Background: The relationship between KRAS mutational status and timing of colorectal liver metastasis (CRLM) remains unclear. This study evaluated the relationship between KRAS mutational status and long-term survival in patients with synchronous CRLM.

Methods: Of the 255 patients who underwent initial hepatic resection for CRLM between January 2001 and December 2018, the KRAS mutational status was examined in 101 patients. Medical records of these patients were reviewed to evaluate recurrence and survival outcomes.

Results: KRAS mutant status was identified in 38 patients (37.6%). The overall survival (OS) was significantly better in patients with wild-type KRAS than in those with mutant KRAS status. In patients with synchronous metastases, the OS of patients with wild-type KRAS was significantly better than those with mutant KRAS. Multivariate analyses indicated shorter OS to be independently associated with positive primary lymph node, and large tumor size and R1 resection in patients with metachronous metastasis, whereas to be independently associated with mutant KRAS status in patients with synchronous metastasis. Furthermore, in the subgroup of patients with synchronous metastases, the repeat resection rate for hepatic recurrence was significantly high in those with wild type KRAS than in those with mutant KRAS.

Conclusion: KRAS mutation is an independent prognostic factor in patients with synchronous CRLM, but not in patients with metachronous CRLM.

Keywords: Colorectal cancer, Liver metastases, KRAS, Synchronous metastasis, Hepatectomy

Introduction

Colorectal cancer (CRC) is one of the common causes of cancer-related mortality worldwide. Distant metastasis is strongly associated with poor prognosis in patients with CRC. During the course of CRC, colorectal liver metastases (CRLM) occur in approximately half of the patients [1]. Surgical resection is the primary treatment modality for CRLM, which ensures complete restoration or long-term survival in the patients. The 5-year OS rate in patients with CRLM after surgical resection is currently 33–51% [1–3]. Historically, hepatic resection for CRLM was indicated for tumors isolated only in the liver with less aggressive features (i.e., low number, small size) [4]. However, the indication for hepatic resection for CRLM has been extended over the past decades to include more patients with aggressive disease features. Recent studies demonstrated that the presence of extrhepatic metastases is no longer a contraindication for surgical resection in patients with colorectal metastases [5–7].
the past, one centimeter was considered an adequate surgical margin [8, 9]. However, recent data have shown that a 1 cm margin is not a requisite for curative resection, and that margin width does not affect long-term survival [10, 11]. One reason for these changes might be attributable to the use of perioperative chemotherapy with molecular targeted agents [12]. In fact, systemic chemotherapy has been evolving since the late 2000s, especially after the EPOC study [13]. Moreover, several randomized control trials demonstrated the clinical implication of KRAS mutational status and RAS mutational status is now commonly used as a determinant of anti-EGFR antibody administration in modern chemotherapy regimens [14, 15].

Synchronous metastasis is associated with shorter disease-free survival duration and may correlate with the more disseminated disease than that with metachronous metastasis [16]. A recent nationwide survey in Japan demonstrated that adjuvant chemotherapy is associated with a favorable prognosis in patients with synchronous CRLM, but not in those with metachronous CRLM [17]. Another study suggested that adjuvant chemotherapy is more effective in cases with synchronous metastases than in those with metachronous metastases [18]. These data highlight the differences in tumor biology between synchronous and metachronous metastases. Recent studies have revealed that somatic mutations in genes such as KRAS and BRAF are associated with poor clinical outcomes in patients with CRLM [19–24]. Mutations in KRAS are found in about 30% of the patients with CRLM [20]. However, fewer studies have studied the association between the mutational status of KRAS and the timing of CRLM.

The aim of the present study was to evaluate the relationship between the mutational status of KRAS and long-term survival in patients with synchronous CRLM.

**Patients and methods**

**Patients**

A total of 255 patients underwent initial hepatic resection for CRLM at the Department of General Surgery, Chiba University, between January 2001 and December 2018. Of these, the mutation status of KRAS was examined in 101 patients. The medical records of these consecutive patients were reviewed retrospectively. This study was approved by the Institutional Ethics Committee of the College of Medicine, Chiba University, Japan. Informed consent was obtained from all the patients after explaining the extent of the disease, and the benefits and risks associated with the treatments.

**Surgical procedure**

Pringle’s maneuver was used, whenever possible, to decrease intraoperative bleeding from the cut surface of the liver. Transection of the liver parenchyma was performed in all patients using a cavitron ultrasonic surgical aspirator.

**KRAS mutation profiling**

DNA was extracted from the paraffin blocks of primary CRC or CRLM. Polymerase chain reaction (PCR)-based primer extension assay was performed to screen for genomic mutations encoding residues 12 and 13 of the KRAS protein.

**Definition of synchronous metastases**

Synchronous metastases was defined as the metastases that are clinically and/or radiologically detected when the primary cancer is diagnosed.

**Definition of surgical margin**

All the resected specimens were subjected to a routine pathological examination. The cases with R1 resection were identified based on microscopically incomplete resection with the presence of tumor invasion on the cut surface (i.e., a tumor-free margin of 0 mm).

**Statistical analysis**

All data were retrospectively collected and differences were considered statistically significant at 0.05. Relationships between categorical variables were assessed using either the chi-square test or Fisher’s exact test, as appropriate. Survival outcomes after the initial hepatectomy for CRLM were evaluated using the Kaplan-Meier method and log-rank test. Survival data were evaluated using univariate and multivariate Cox proportional regression analyses. All the statistical analyses were performed using the JMP Pro software (version 13; SAS Institute Japan, Tokyo, Japan).

**Results**

**Patients’ characteristics and perioperative data**

The demographic and clinicopathological characteristics of patients are summarized in Table 1. Of the 101 patients, 63 (62.4%) had wild-type KRAS status (KRAS-wt), whereas 38 (37.6%) had mutant KRAS status (KRAS-mut). As indicated in Table 1, there were no significant differences in patient characteristics based on the mutational status of KRAS.

**Oncological outcomes**

The 3-year recurrence-free survival (RFS) and 5-year OS rates were 14.9 and 41.2%, respectively, for all the patients. In KRAS-wt patients, the median RFS was 11 months and the 3-year RFS rate was 24.2%. In KRAS-mut patients, the median RFS was 8 months and the 3-year RFS rate was 8.2% (Fig. 1a). Further, in KRAS-wt patients, the median OS was 71 months and the 5-year
OS rate was 50.1%. In KRAS-mut patients, the median OS was 36 months and the 5-year OS rate was 26.8% (Fig. 2a). There was no significant difference between RFS for KRAS-wt and KRAS-mut patients (P = 0.139). Whereas the OS rate was found to be significantly better in patients with KRAS-wt than in those with KRAS-mut status (P = 0.021). Next, in the subgroup analysis, RFS was not significantly different based on the KRAS mutational status in patients with metachronous metastases (3-year RFS rates and median RFS: 21.5% and 11 months in KRAS-wt, while 18.8% and 10 months in KRAS-mut patients, respectively; P = 0.567) (Fig. 1b). In patients with synchronous metastases, the RFS of KRAS-wt patients was relatively better, but not significant, than those with KRAS-mut status (3-year RFS rate and median RFS: 27.4% and 11 months in patients with KRAS-wt, and 0.0% and 10 months in those with KRAS-mut status, respectively; P = 0.076) (Fig. 1c). The OS was not significantly different based on the KRAS mutational status in patients with metachronous metastases (5-year OS rate and median OS: 36.8% and 50 months in KRAS-wt versus 45.3% and 58 months in KRAS-mut; P = 0.294)

Table 1 Patient characteristics

| Characteristics                              | KRAS-wt (n = 63) | KRAS-mut (n = 38) | P-value |
|----------------------------------------------|------------------|-------------------|---------|
| Sex, male/female                             | 36/27 (57.1/42.9) | 22/16 (57.9/42.1) | 1.000   |
| Median age (range) (years)                   | 66 (33–83)       | 69 (35–82)        | 0.821   |
| Primary tumor                                |                  |                   |         |
| Right-sided/Left-sided/rectum                | 11/31/21 (17.5/49.2/33.3) | 7/18/13 (18.4/47.4/34.2) | 0.983   |
| pT1–3 / T4                                   | 36/27 (57.1/42.9) | 23/15 (60.5/39.5) | 0.738   |
| Node positive / node negative                | 48/15 (76.2/23.8) | 30/8 (78.9/21.1)  | 0.811   |
| Initial liver metastases                     |                  |                   |         |
| Synchronous / metachronous diagnosis         | 34/29 (54.0/46.0) | 22/16 (57.9/42.1) | 0.837   |
| Unilobar / bilobar                           | 36/27 (57.1/42.9) | 18/20 (47.4/52.6) | 0.412   |
| Mean number of tumors, (range)               | 3.7 (1–26)       | 3.6 (1–19)        | 0.910   |
| Solitary / multiple metastases               | 23/40 (36.5/63.5) | 12/26 (31.6/68.4) | 0.670   |
| Largest tumor’s mean size, cm                | 3.8 (0.5–13)     | 3.5 (0.8–10)      | 0.531   |
| Serum carcinoembryonic antigen (ng/mL), < 5 / ≥ 5 | 17/46 (27.0/73.0) | 13/25 (34.2/65.8) | 0.503   |
| Hepatectomy, minor-major                     | 43/20 (68.3/31.8) | 27/11 (71.1/28.9) | 0.827   |
| Surgical margin, R0/R1                       | 44/19 (69.8/30.2) | 26/12 (68.4/31.6) | 1.000   |
| Preoperative chemotherapy, yes/no            | 37/26 (58.7/41.3) | 23/15 (60.5/39.5) | 1.000   |
| Adjuvant chemotherapy, yes/no                | 41/22 (65.1/34.9) | 25/13 (65.8/34.2) | 1.000   |

Values are number of patients or mean/median values, as indicated

Fig. 1 Kaplan-Meier survival curves for recurrence-free survival. a RFS according to the KRAS mutational status in patients with colorectal liver metastases (CLRM). Subgroup analyses for RFS in patients with (b) metachronous and (c) synchronous metastases. RFS, recurrence-free survival.
Whereas, the OS of patients with synchronous metastases harboring \textit{KRAS}-wt was significantly better than those harboring \textit{KRAS}-mut status (5-year OS rate and median OS: 60.4\% and 81 months in \textit{KRAS}-wt versus 14.9\% and 31 months in \textit{KRAS}-mut; \(P < 0.001\)) (Fig. 2c). Furthermore, the univariate analyses revealed that shorter OS duration in patients was associated with synchronous metastasis (\(P = 0.050\)), bilobar tumor distribution (\(P = 0.005\)), increased number of tumors (\(P = 0.001\)), large tumor size (\(\geq 5\text{ cm}, P < 0.001\)), high serum carcinoembryonic antigen levels (\(\geq 5\text{ ng/mL}, P = 0.029\)), major hepatectomy (\(P = 0.002\)), R1 resection (\(P = 0.002\)), and mutant \textit{KRAS} (\(P = 0.026\)) (Table 2). In multivariate analyses, shorter OS duration in patients was found to independently associate with R1 resection (hazard ratio [HR]: 2.554, \(P = 0.003\)), and mutant \textit{KRAS} status (HR: 2.409, \(P = 0.003\)) (Table 2). In patients with metachronous metastasis, shorter OS duration was found to be independently associated with positive primary lymph node (HR: 1.779, \(P = 0.039\)), large tumor size (\(\geq 5\text{ cm}, \ldots\))

![Fig. 2 Kaplan-Meier survival curves for overall survival.](Sakai et al. BMC Cancer (2021) 21:412)

**Table 2** Predictive factors of shorter OS (\(n = 101\))

| Variables                                      | Univariate analysis | Multivariate analysis |
|------------------------------------------------|---------------------|-----------------------|
| Sex, male                                      | 1.209               | 1.186                 |
| Age                                            | 1.007               | 0.907                 |
| Primary tumor                                  | 0.888               | 0.812                 |
| Location, colon/rectum                         | 0.818               | 1.274                 |
| Location, right-sided/left-sided               | 1.426               | 0.062                 |
| pT stage                                       | 1.405               | 0.050                 |
| Positive lymph node                            | 1.642               | 1.106                 |
| No. of tumors                                  | 1.072               | 1.074                 |
| No. of tumors (solitary/multiple): multiple    | 2.050               | 2.122                 |
| Tumor size \(\geq 5\text{ cm}\)               | 2.122               | 0.001                 |
| Serum carcinoembryonic antigen \(\geq 5\text{ ng/mL}\) | 2.122               | 0.001                 |
| Type of hepatectomy: major                     | 1.812               | 1.812                 |
| Surgical margin: R1                            | 1.805               | 1.805                 |
| \textit{KRAS} mutational status: \textit{KRAS}-mut | 1.860               | 1.860                 |

**HR** Hazard ratio; **CI** Confidence interval
### Table 3 Predictive factors of shorter OS (metachronous) \((n = 45)\)

| Variables                              | Univariate analysis | Multivariate analysis |
|----------------------------------------|---------------------|-----------------------|
|                                        | HR                  | P-value               | HR                  | 95% CI               | P-value |
| Sex, male                              | 1.060               | 0.824                 |                      |                      |         |
| Age                                    | 1.119               | 0.854                 |                      |                      |         |
| Primary tumor                          |                     |                       |                      |                      |         |
| Location, colon/rectum                 | 1.035               | 0.899                 |                      |                      |         |
| Location, right-sided/left-sided       | 0.784               | 0.473                 |                      |                      |         |
| pT stage                               | 1.204               | 0.350                 |                      |                      |         |
| Positive lymph node                    | 1.756               | 0.042                 | 1.779               | 1.028–3.223          | 0.039   |
| Initial liver metastases               |                     |                       |                      |                      |         |
| Tumor distribution: bilobar            | 1.256               | 0.420                 |                      |                      |         |
| No. of tumors                          | 1.053               | 0.127                 |                      |                      |         |
| No. of tumors (solitary/multiple): multiple | 1.489               | 0.123                 |                      |                      |         |
| Tumor size \(\geq 5\) cm              | 3.023               | < 0.001               | 3.010               | 1.734–5.131         | < 0.001 |
| Serum carcinoembryonic antigen \(\geq 5\) ng/mL | 2.075               | 0.023                 | 1.701               | 0.882–3.612         | 0.117   |
| Type of hepatectomy: major             | 1.377               | 0.328                 |                      |                      |         |
| Surgical margin: R1                    | 1.898               | 0.034                 | 2.060               | 1.122–3.632         | 0.021   |
| KRAS mutational status: KRAS-mut       | 0.608               | 0.289                 |                      |                      |         |

HR: Hazard ratio; CI: Confidence interval

### Table 4 Predictive factors of shorter OS (synchronous) \((n = 56)\)

| Variable                              | Univariate analysis | Multivariate analysis |
|----------------------------------------|---------------------|-----------------------|
|                                        | HR                  | P-value               | HR                  | 95% CI               | P-value |
| Sex, male                              | 1.421               | 0.139                 |                      |                      |         |
| Age                                    | 1.016               | 0.133                 |                      |                      |         |
| Primary tumor                          |                     |                       |                      |                      |         |
| Location, colon/rectum                 | 0.783               | 0.303                 |                      |                      |         |
| Location, right-sided/left-sided       | 0.818               | 0.529                 |                      |                      |         |
| pT stage                               | 1.232               | 0.344                 |                      |                      |         |
| Positive lymph node                    | 1.083               | 0.765                 |                      |                      |         |
| Initial liver metastases               |                     |                       |                      |                      |         |
| Tumor distribution: bilobar            | 1.857               | 0.009                 | 1.312               | 0.562–3.138         | 0.530   |
| No. of tumors                          | 1.081               | 0.006                 | 1.063               | 0.961–1.164         | 0.223   |
| No. of tumors (solitary/multiple): multiple | 2.930               | < 0.001               | 0.920               | 0.298–3.230         | 0.890   |
| Tumor size \(\geq 5\) cm              | 1.511               | 0.117                 |                      |                      |         |
| Serum carcinoembryonic antigen \(\geq 5\) ng/mL | 1.205               | 0.514                 |                      |                      |         |
| Type of hepatectomy: major             | 1.976               | 0.005                 | 1.221               | 0.535–2.765         | 0.632   |
| Surgical margin: R1                    | 1.641               | 0.047                 | 1.718               | 0.740–3.887         | 0.202   |
| KRAS mutational status: KRAS-mut       | 4.517               | < 0.001               | 4.316               | 1.973–9.845         | < 0.001 |

HR: Hazard ratio; CI: Confidence interval
Table 5 Recurrence after initial hepatectomy

|                      | KRAS-wt n = 63 | KRAS-mut n = 38 | P-value |
|----------------------|----------------|-----------------|---------|
| Total cohort         | 49/63 (77.8%)  | 33/38 (86.8%)   | 0.304   |
| Synchronous metastases | 26/34 (76.5%)  | 20/34 (60.6%)   | 0.285   |
| Metachronous metastases | 23/39 (69.2%)  | 13/34 (38.2%)   | 1.000   |

HR: 3.010, \( P < 0.001 \), and R1 resection (HR: 2.060, \( P = 0.021 \)) (Table 3). In patients with synchronous metastasis, shorter OS duration was found to independently associate with mutant \( KRAS \) status (HR: 4.316, \( P < 0.001 \)) (Table 4).

**Recurrence after initial hepatectomy**

Disease recurrence after the initial hepatectomy was observed in 82 patients (81.2%). There was an insignificant difference in recurrence rates between patients with \( KRAS \)-wt and \( KRAS \)-mut status (77.8% vs. 86.8%) (Table 5). Repeat resection was performed in 25 (51.0%) and 15 (45.5%) patients with \( KRAS \)-wt and \( KRAS \)-mut status, respectively (\( P = 0.658 \)) (Table 6). In the subgroup of synchronous metastases, the repeat resection rate for all recurrence was 57.7% in patients with \( KRAS \)-wt and 30.0% in those with \( KRAS \)-mut status (\( P = 0.079 \)) (Table 6). Hepatic recurrence was observed in 26 (41.3%) and 23 (60.5%) patients with \( KRAS \)-wt and \( KRAS \)-mut status, respectively (\( P = 0.068 \)) (Table 7). Repeat hepatectomy was performed in 15 (57.7%) and 8 (34.8%) patients with \( KRAS \)-wt and \( KRAS \)-mut status, respectively (\( P = 0.154 \)) (Table 8). Whereas, in the subgroup of synchronous metastases, the repeat resection rate for hepatic recurrence was 66.7% in patients with \( KRAS \)-wt and 14.3% in those with \( KRAS \)-mut status (\( P = 0.008 \)) (Table 8). Repeat hepatectomy was not recommended in patients with synchronous metastases harboring \( KRAS \)-mut status due to multiple hepatic recurrence in 3 patients, and both hepatic and extrahepatic recurrence to the lung, peritoneal dissemination and bone in 9 patients. Of these, 9 patients developed recurrence within 12 months after initial hepatectomy. Moreover, extrahepatic recurrence (including both extrahepatic and hepatic recurrences) was observed in 18 (68.2%) and 15 patients (52.9%) with synchronous metastases harboring \( KRAS \)-wt and \( KRAS \)-mut status, respectively (\( P = 0.282 \)).

**Discussion**

We aimed to delineate the relationship between \( KRAS \) mutational status and long-term survival in patients with CRLM, and assess whether there were any differences in the effect of \( KRAS \) mutational status on the disease outcome based on the timing of metastasis. The present study clearly demonstrated that the effect of the mutation status of \( KRAS \) varied according to the timing of liver metastasis. The \( KRAS \)-mut status was significantly associated with poor prognosis in patients with synchronous metastasis, but not in those with metachronous metastasis.

The reason for poor prognosis in patients with synchronous metastasis and \( KRAS \)-mut tumors may be partially explained by the recurrence patterns after initial hepatectomy. As reported previously, repeat hepatectomy for hepatic recurrence can yield survival benefit similar to that after initial hepatectomy [25]. Therefore, tumor recurrence itself is not always associated with poor prognosis, although unresectable recurrence is considered a distinct poor prognostic factor [26–28]. We found that the rate of re-resection for hepatic recurrence was significantly low in patients with synchronous metastases and \( KRAS \)-mut tumors, which might lead to poor prognosis in these patients than in those with synchronous metastases and \( KRAS \)-wt tumors.

Moreover, the rate of extrahepatic recurrence was relatively high in patients with synchronous metastases and \( KRAS \)-mut tumors than in those with synchronous metastases and \( KRAS \)-wt tumors. Since extrahepatic metastasis is also known to be associated with poor prognosis [6], this trend may affect the poor prognosis in patients.
Another reason could be the difference in response to systemic chemotherapy according to the \textit{KRAS} mutation status. In the context of systemic chemotherapy, Mise et al. investigated the association between \textit{RAS} mutational status and response to preoperative chemotherapy in patients with CRLM. They revealed that \textit{RAS} mutations were significantly associated with minor pathological and suboptimal morphological responses, which were assessed using computed tomographic scans [29]. Other studies also demonstrated that \textit{KRAS} mutations were significantly associated with minor response to chemotherapy in patients with CRLM, and that \textit{RAS} mutation status may serve as a biomarker for response to chemotherapy [30–32]. In the present study, the effect of preoperative chemotherapy was not assessed since various treatment regimens were used during the extended study period. However, preoperative and adjuvant chemotherapy were administered to almost 60% of the patients. Therefore, the variable responses to chemotherapy may lead to prognostic differences observed in the present study. Moreover, about 70% of the patients with synchronous metastases and \textit{KRAS}-mut tumors received perioperative chemotherapy. Despite the high rate of administering chemotherapy, a poor prognosis was observed in this subgroup, suggesting that unfavorable response to chemotherapy may have resulted in disseminated and/or unresectable recurrences.

Other than the \textit{KRAS} mutation status, the univariate and multivariate analyses revealed several differences between synchronous and metachronous metastases in terms of clinicopathological factors associated with patients’ prognoses. In the context of the timing of metastasis, Tsai et al. demonstrated that synchronous metastasis was significantly associated with low disease-free survival, and proposed that it may represent a highly disseminated disease than that with metachronous metastasis [16]. Additionally, other studies have demonstrated that response to chemotherapy may vary with the timing of metastasis [18, 32]. Together, these results support the hypothesis that tumor biology may be influenced by the timing of metastasis. However, further studies would be required to ascertain the role of mutational statuses influencing the biological differences according to the timing of metastases.

Here, surgical margin status was not found to be a prognostic factor of synchronous metastasis. The clinical significance of surgical margin has been debated in past decades. Moreover, it has been reported that the extent of optimal tumor-free margin may vary according to the \textit{KRAS} mutation status [33], with 1–4 mm margins being considered sufficient in patients with wild-type \textit{KRAS}, whereas 1-cm margins being considered insufficient for those with mutant \textit{KRAS} status. Furthermore, other studies reported that the \textit{KRAS} mutation status was associated with a narrow width of surgical margin [34, 35]. We defined R1 resection as microscopically incomplete resection with the presence of tumor invasion at the cut surface without considering margin width. Additionally, in all cases, parenchymal transection was performed using the cavitron ultrasonic surgical aspirator, which is known to ablate or aspirate parenchyma along the transection plane [36, 37]. Therefore, there may be a certain number of patients who were inappropriately categorized on the basis of surgical margin status and our definition may have failed to stratify R0 and R1 resection, especially in patients with synchronous metastasis.

The present study has several limitations. First, the data were retrospectively collected from the database at a single center and comprised a small sample size. Second, the background data of the patients were not synchronized, indicating that selection bias may exist and affect the RFS and OS. Third, while the mutation status of \textit{KRAS} was assessed, the differential effect of codons 12 and 13 was not considered. Moreover, somatic mutations in other genes such as \textit{NRAS} and \textit{BRAF} were examined only in a few patients and were not analyzed in the present study. Therefore, further studies should be conducted to assess the relationship of somatic mutations in multiple genes, such as \textit{KRAS}, \textit{NRAS}, and \textit{BRAF}, with the clinical outcome of patients with CRLM.

**Conclusion**

In conclusion, our study found \textit{KRAS} mutational status as an independent prognostic factor in patients with synchronous CRLM, but not in those with metachronous CRLM. We propose that the treatment strategy should be planned on the basis of the timing of metastasis, considering its influence on the biological behavior of CRLM.

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**Authors’ contributions**

Study design: Sakai N., Ohtsuka M. Data acquisition: Sakai N. Analysis and interpretation: Sakai N., Furukawa K., Takayashiki T., Kuboki S., Takano S. Drafting of the manuscript: Sakai N. Critical revision of the manuscript: Furukawa K., Otsuka M. Final approval for the publication: all authors.

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**Availability of data and materials**

The datasets used during this study are available from the corresponding author on reasonable request.

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Institutional Review Board of Chiba University (approval number 3300) and was performed according to the ethical principles of the Declaration of Helsinki. Informed consent was obtained from all the patients after explaining the extent of the disease, and the benefits and risks associated with the treatments.
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