chemotherapy after curative operation of stage II CRC. Several prognostic markers or predictors of chemosensitivity for stage II patients have been reported such as allelic imbalance,[7] gene expression profiling by cDNA microarray,[8] or microsatellite instability,[9] respectively. However, such molecular markers have been unsuitable for routine application at present because they have not been finally validated yet and are still costly and time-consuming.

Anastomotic leakage (Lk) is thought to occur at a rate ranged from 3% to 18% and has been reported to be a risk factor for local recurrences in curatively operated CRC patients.[10–12] In this meaning, at least patients with Lk may be potential candidate for adjuvant chemotherapy. However, these results were based upon curatively operated patients with CRC of several stages, and the impact of Lk on long-term survival remains controversial,[10–14] especially in stage II CRC. Accordingly, clinicopathological factors including Lk were prognostically analyzed within stage II patients to evaluate whether Lk could be a clinically available parameter for predicting long-term prognosis.

Patients and Methods

Characteristics of Patients with Stage II CRC

A total of 1,101 patients having electively undergone surgical resection of primary CRC at the Kitasato University Hospital from January 1, 1990 to March 31, 2000, were reviewed. Patients with colorectal multiple cancer, malignant disease of other organ, familial adenomatous polyposis, or inflammatory bowel diseases, patients who underwent resections without anastomosis, and patients undergone emergency resection for perforation or one-stage resection for obstruction were excluded. Among the remaining 946 patients of sporadic CRC, 207 patients were diagnosed (21.9%) as stage II CRC disease and were operated on with curative intent. Preoperative chemotherapy or radiation therapy had not been performed in this cohort. Patients without obstruction received mechanical bowel preparation with polyethylene glycol electrolyte solution the day before surgery, and patients with obstruction and patients with rectal cancer received bedside orthograde colorectal lavage with lukewarm water. Prophylactic intravenous antibiotics were administered at the induction of anesthesia and 3 h after the beginning of operation. Patients were followed up until the recurrence of cancer or end point (April 30 2007). All patients were followed up at least every 3 months for the first year and every 6 months thereafter. Follow-up assessment involved a medical history-taking, physical examination, biologic tests, measurement of the serum CEA and CA19-9 levels, colonoscopy, chest radiography, abdominal ultrasonography (US), and chest/abdominal computed tomography (CT). Serum CEA and CA19-9 were usually evaluated every visit, and abdominal US and CT were performed every 6 months. Chest CT and colonoscopy were examined every year. Recurrence was diagnosed on the basis of imaging and, if necessary, either cytologic analysis or biopsy was performed. Patient demographics, tumor characteristics, and postoperative course were recorded and analyzed. Perioperative transfusion was defined as allogeneic blood transfusion during surgery or in the first two postoperative days, as in previous press,[15] and was performed at the discretion of the treating surgeons and anesthesiologists. The number of total dissected lymph nodes was also classified according to previous press.[16] Pathological TNM classification was made according to the UICC (*Unio Internationalis Contra Cancrum*) staging system.

Patients who received adjuvant chemotherapy for more than 3 months were defined as adjuvant chemotherapy "Yes" group. Adjuvant chemotherapy was consisted of oral administration of 5-fluorouracil (5FU)-based regimens: 5FU, Tegafur/uracil (UFT), or Furtulon (5′-deoxy-5-fluorouridine) alone, or one of them plus PSK (protein-bound polysaccharide K). Although curative operation alone is a standard therapy in stage II CRC at present, oral adjuvant chemotherapy had been recommended to patients with stage II CRC during the term of this patient cohort if they fulfilled the following eligibility criteria: age of 20 to 75 years; the absence of prior chemo-immunotherapy or radiotherapy, and the absence of severe liver dysfunction, heart failure, renal dysfunction, or other severe systemic complications. Therefore, patients who received oral adjuvant chemotherapy reached 180 cases, and the remaining 27 patients declined or did not fulfill the above criteria.

Lk was defined as any clinical or radiological evidence of dehiscence of the anastomosis: the presence of peritonitis caused by anastomosis dehiscence, the presence of feculent discharge from the drainage tube, or the presence of abscess with demonstration of Lk. These were also confirmed by radiography from drainage tube, hydrosoluble enema, or CT-guided abscess drainage except the cases with obvious feculent discharge from the drainage tube (Supplemental Table 1). Anastomotic dehiscence, which was basically diagnosed by, later, routine imagings prior to closure of diverting ileostomy, was not included. We performed routine imagings only for patients with diverting ileostomy prior to ileostomy closure more than 3 months after primary operation. Four patients underwent diverting ileostomy, but no anastomotic dehiscence was detected in such routine diagnosis.

Statistical Analysis

The relationship between Lk and clinicopathological parameters were assessed by Pearson's chi-square test or

Fisher's exact test, as appropriate, and multivariate logistic regression analysis were performed to obtain an adjusted effect of each factor. The time of follow-up was calculated from the operation date for the primary lesion to the date of recurrence. Cumulative disease-free survival (DFS) of patients was estimated using the Kaplan–Meier method, and statistical significance of the difference of the survival rate between groups was tested using the log-rank test. For the Kaplan–Meier estimate of the survival curves, we truncated the data at a follow-up period of 5 years to avoid the number at risk to be too small. Those with a survival time of more than 5 years were reported to be 5 years, and events occurring after the end of the 5-year follow-up period were computed as censored data. Five-year cumulative DFS probability was estimated using the life table method with the interval length set at 1 month. Multivariable analysis was performed by employing the Cox proportional hazards model to examine the interaction between Lk and other clinicopathological variables and estimate the independent prognostic effect of Lk on survival by adjusting for confounding factors. For ordinal variable, when zero event was detected in the lowest exposure group, analyses was designed to be performed by grouping categories together, treating it as ordinal data to get an average effect, or by confounding sensitivity analyses excluding it from analysis. Within the present study population, there were 27 recurrences of stage II CRC which allows up to three variables to be included in a multivariable regression model. To avoid over-fitting, all potential confounding factors of Lk were reduced to one single composite characteristic by applying a propensity score.[17] The conventional P value of 0.05 or less was used to determine the level of statistical significance. All reported P values are two-sided. Analyses were performed independently at our clinical research center using SPSS version 17.0 software (SPSS Inc., Chicago, IL).

Results

Patients' Characteristics and Their Association with Lk

The clinicopathological characteristics were shown in Table 1. One hundred twenty-seven males and 80 females were analyzed with age being 61.0±11.1 years. Lk occurred in 12 (5.8%) cases, and, among them, only one patient had a particularly preoperative complication (diabetes mellitus). The diabetes of this patient was well-controlled by insulin from preoperation through postoperation. And, there was no patient with other factors for poor nourishment such as medication of steroids. Lk occurred in 22.2% of patients with perioperative blood transfusion and in 1.2% of those without perioperative blood transfusion. Lk was significantly related to perioperative blood transfusion ($P<0.001$, Fisher's exact test), followed by T4 factor (direct invasion into other organ; $P=0.071$), the elevation of preoperative CEA ($P=0.110$), and tumor position ($P=0.129$). Preoperative obstruction was observed in only one patient with Lk (Table 1). There was also no significance in relationship between Lk and obstruction in the present study population. Lk occurred in five cases (3.8%) in colon cancer and seven in rectal cancer (9.2%). Among them, two patients required ileostomy (reoperation) for Lk in colon cancer and five in rectal cancer, and one patient (colon cancer) underwent ileostomy before curative resection (two-stage operation) for obstruction, one patient (rectal cancer) underwent diverting ileostomy, and the remaining three patients were conservatively observed with percutaneous drainage and finally cured. The multivariable logistic regression analysis of these factors indicated that Lk was independently associated with perioperative blood transfusion ($P<0.001$).

Kaplan–Meier Estimate of 5-Year DFS

All the patients were included in the survival analysis. The overall follow-up period ranged from 2 to 207 months (median, 116 months), and the mean DFS was 55.4 months corresponding to a 5-year follow-up. Because a cumulative DFS probability of 50% was not yet reached by the end of 5-year follow-up, the overall median DFS time was not determined. The overall DFS rate was 87.0% (27 cases with recurrence and 180 cases without recurrence). Five-year cumulative DFS of patients with Lk was remarkably worse (58.3%), which corresponded to stage III CRC (63.2%), compared with those without Lk (88.7%; $P<0.001$, Fig. 1a). Lymphatic involvement (ly; $P=0.119$) and vascular involvement (v; $P=0.086$) tended to indicate poor prognosis (Supplemental Fig. 1a, b), and patients with both ly and v involvement ($n=28$) showed significantly poor prognosis (DFS, 84.9%) compared with the counterpart ($n=179$; 100.0%; $P=0.033$; Supplemental Fig. 1c).

When separately analyzed on tumor position, Lk still significantly affected adversely on long-term prognosis in both colon and rectum (Fig. 1b, c), and there was no significant difference between DFS of patients with Lk in colon cancer (60.0%) and that in rectal cancer (57.1%). In addition, Lk was the only significant prognostic factor, and there was no factor which had prognostic potential ($P<0.1$) both in colon and rectum when separately analyzed (data not shown).

Contribution of Lk to the Risk of Recurrence with Multivariable Analysis

Cox proportional hazards model was applied to estimate the effect of Lk on DFS. Lk was the only significant prognostic

Table 1 Characteristics and those in correlation with anastomotic leakage (Lk)

Variables	No. of patients	Percentage	Lk			
			Present	Absent	Present rate (%)	P^a values
Gender						
Male	127	61	10	117	7.9	0.13
Female	80	39	2	78	2.5	
Age (years)						
<60	94	45	4	90	4.3	0.55
>60	113	55	8	105	7.1	
Tumor position						
Ccolon	131	63	5	126	3.8	0.13
Rectum	76	37	7	69	9.2	
Differentiation						
Non-poor	194	94	12	182	6.2	0.36
Poor[b]	13	6	0	13	0.0	
T factor						
T3	199	96	10	189	5.0	0.07
T4	8	4	2	6	25.0	
Lymphatic involvement (ly)						
Negative	16	8	0	16	0.0	0.61
Positive	191	92	12	179	6.3	
Vascular involvement (v)						
Negative	19	9	1	18	5.3	0.92
Positive	188	91	11	177	5.9	
Preoperative CEA						
Normal (<2.5 ng/ml)	138	67	5	133	3.6	0.110
Elevated (>2.5 ng/ml)	69	33	7	62	10.1	
Preoperative CA19-9						
Normal (<37 ng/ml)	183	88	10	173	5.5	0.64
Elevated (>37 ng/ml)	24	12	2	22	8.3	
Obstruction						
Yes	16	8	1	15	6.3	0.94
No	191	92	11	180	5.8	
Lk						
Yes	12	6	n/a	n/a	n/a	n/a
No	195	94	n/a	n/a	n/a	
Number of total dissected lymph node						
<6	5	2	0	5	0.0	0.78
6–10	27	13	1	26	3.7	
11–15	34	17	3	31	8.8	
>15	141	68	8	133	5.7	
Laparoscopy-assisted operation						
Yes	8	4	0	8	0.0	0.47
No	199	96	12	187	6.0	
Adjuvant chemotherapy						
Yes	180	87	9	171	5.0	0.2
No	27	13	3	24	11.1	
Perioperative transfusion						
Yes	45	22	10	35	22.2	<0.001
No	162	78	2	160	1.2	

OR odds ratio, *LNDE* lymph node dissection extent, *n/a* not applicable

[a] Compared by Fisher's exact test or chi-square test

[b] Poor consists of poorly differentiated, mucinous, and undifferentiated types

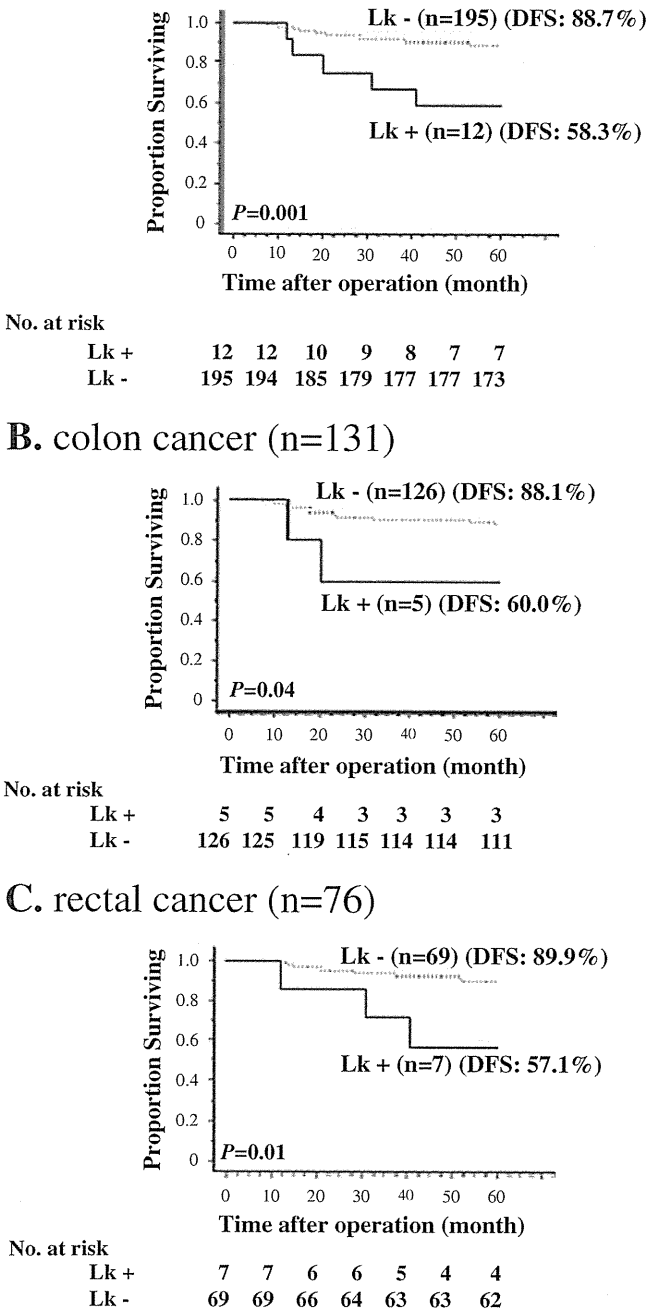

Fig. 1 Kaplan–Meier curve of 5-year DFS according to anastomotic leakage (Lk): **a** Total stage II CRC (n=207). **b** Colon cancer (n=131). **c** Rectal cancer (n=76)

factor, and there was no other factor which had prognostic potential (P<0.1). The crude hazard ratio (HR) of Lk-positive compared to Lk-negative was 4.38 (95% confidence interval (CI), 1.66–11.58; P=0.003), which indicated Lk increased the risk of recurrence of CRC and cancer-related death by more than four times that of without Lk. The effect of Lk on recurrence in colon and rectal cancer group gave similar results: crude HR (95%CI) was 4.1 (0.9–17.9) for the colon group and 4.9 (1.3–19.0) for the rectal group.

Before multivariable analyses were adopted to estimate adjusted effect of Lk on DFS, we further confirmed that there was no interaction effect between cancer position (colon or rectum) and Lk (P=0.874); taking into account that evaluation in each group would result in a small sample size and thus decrease the power of the study, we finally combined them together. Potential confounders of variables were included in the multivariable analysis (Table 2). The adjusted HR of Lk became 5.27 (95%CI, 1.54–18.10; P=0.008) in comparison to Lk-negative. We also performed an analysis by using propensity score to adjust the effect of Lk by transforming all other confounding variables into a single estimator and revealed that, after the adjustment, the HR of Lk became 4.21 (95%CI, 1.24–14.33; P=0.021). These findings suggested that Lk seems to be an independent and significant risk factor of poorer DFS (Table 2).

Lk was Associated with Hematogenic Recurrence Rather than Local or Peritoneal Recurrence in Stage II CRC

Next, first recurrence site in patients with stage II CRC was analyzed according to Lk. Interestingly, Lk was correlated with hematogenic recurrence (P=0.003 by Fisher's exact test) rather than local recurrence or peritoneal dissemination (P=0.605; Table 3). Therefore, Lk may cause systemic micrometastasis, leading to systemic recurrence.

Effect of Lk on DFS When Taking Systemic Inflammatory Response into Account

Recently, a systemic inflammatory response, as evidenced by raised circulating levels of C-reactive protein (CRP), has been reported to be associated with poor survival in patients who underwent potentially curative resection for CRC.[18] These reports may explain the above implication of Lk in systemic recurrences, hence circulating level of CRP was analyzed, which was measured as a part of routine blood examination either before or after potentially curative resection for stage II CRC. CRP level was classified as raised (≥1.0 mg/dl) or normal (<1.0 mg/dl) from a clinical practice view. Lk was significantly correlated with CRP level at 1 or 2 weeks after curative operation (P=0.018, 0.003, respectively, by Fisher's exact test; Supplemental Table 2). Moreover, the sustained elevation of CRP level at 2 weeks after operation predicted significantly worse prognosis (DFS, 75.0%) than its counterpart (89.3%; P=0.022, compared by log-rank test, Supplemental Fig. 2), while preoperative CRP and CRP at 1 week after operation did not show prognostic significance (data not shown). The multivariable prognostic analysis including CRP at 2 weeks

Table 2 Prognostic analysis of stage II patients according to 5-year DFS (n=207)

Variables	Univariable analysis		Multivariable analysis			
			Model 1		Model 2	
	HR (95%CI)	P^b values	HR (95%CI)	P^b values	HR (95%CI)	P^b values
Lk	4.38 (1.66–11.58)	0.003	5.27 (1.54–18.10)	0.008	4.21 (1.24–14.33)	0.021
Gender (male)	1.87 (0.79–4.43)	0.154	1.76 (0.71–4.34)	0.221	n/d	n/d
Age >60	1.26 (0.58–2.71)	0.559	1.24 (0.56–2.73)	0.603	n/d	n/d
Tumor position (colon)	0.99 (0.46–2.17)	0.988	1.12 (0.47–2.69)	0.797	n/d	n/d
Poor differentiation[c]	0.56 (0.08–4.14)	0.572	0.59 (0.07–5.29)	0.637	n/d	n/d
T factor (T4)	1.02 (0.14–7.51)	0.985	0.65 (0.07–5.66)	0.693	n/d	n/d
Lymphatic involvement (ly)	22.90 (0.05–9651.67)	0.310	n/d	n/d	n/d	n/d
Vascular involvement (v)	23.51 (0.09–6204.78)	0.267	n/d	n/d	n/d	n/d
Preoperative CEA elevation	1.21 (0.55–2.64)	0.636	1.13 (0.48–2.68)	0.783	n/d	n/d
Preoperative CA19-9 elevation	0.59 (0.14–2.48)	0.470	0.57 (0.13–2.55)	0.458	n/d	n/d
Obstruction	1.54 (0.46–5.11)	0.482	1.89 (0.47–7.56)	0.368	n/d	n/d
Number of total dissected lymph node					n/d	n/d
<6	reference		reference		n/d	n/d
6–10	1.60 (0.21–12.01)	0.649	0.50 (0.05–5.53)	0.570	n/d	n/d
11–15	1.26 (0.43–3.75)	0.674	0.48 (0.05–5.05)	0.542	n/d	n/d
>15	1.29 (0.48–3.50)	0.615	0.40 (0.04–3.68)	0.416	n/d	n/d
Laparoscopy-assisted operation	0.96 (0.13–7.05)	0.956	1.15 (0.15–8.79)	0.895	n/d	n/d
Adjuvant chemotherapy	0.90 (0.31–2.59)	0.838	0.95 (0.29–3.08)	0.928	n/d	n/d
Perioperative transfusion	1.28 (0.54–3.03)	0.575	0.70 (0.22–2.24)	0.547	n/d	n/d
Propensity score	n/d	n/d	n/d	n/d	1.16 (0.07–18.50)	0.918

DFS disease-free survival, *HR* hazard ratio, *CI* confidence interval, *n/d* not determined

[a] End-point: date of death or April 30, 2007, no patient was lost to follow-up

[b] Significance based on Cox's proportional hazard model

[c] Poor consists of poorly differentiated, mucinous, and undifferentiated types

There was no event in ly or v negative cases, so that these variables were excluded from multivariable analysis

Multivariable model 2 indicates the adjusted effect of Lk by applying propensity score which is a conditional probability of presenting Lk given by other clinicopathological factors including gender, age, tumor position, differentiation, vascular involvement, preoperative CEA elevation, and perioperative transfusion

after operation (n=175) showed that Lk still indicated prognostic potential (HR, 3.70, P=0.075; Table 4). This result suggests that Lk is more strongly associated with recurrence independent of sustained systemic inflammation.

Discussion

The present study showed that an anastomotic leakage (Lk) was closely associated with an adverse impact on long-term DFS (5-year DFS, 58.3%) in patients who underwent potentially curative resection for stage II CRC, and it was the most robust independent prognostic factor. This DFS was comparable to that of patients with stage III CRC. Although intramural vessel involvement may be available for the selection of low-risk patients (DFS, 100.0%), it was insufficient for the patient selection who have high risk of recurrence and would be rather low-risk selection (Supplemental Fig. 1). Therefore, with regard to patient selection, Lk alone may be potential classifier of stage II CRC. Lk has

Table 3 Association of Lk with first recurrence site in stage II patients

Lk	Local or peritoneal recurrence		P^a values	Hematogenic recurrence		P^a values
	Present	Absent		Present	Absent	
Yes	1	11	0.605	4	8	0.003
No	14	181		8	187	

[a] Significance based on Fisher's exact test

Table 4 Multivariate analysis of Lk effect on 5-year DFS in stage II CRC patients taken CRP into account (n=175)

Variables	Model 1		Model 2	
	HR (95%CI)	P^b values	HR (95%CI)	P^b values
Lk	3.05 (0.79–11.83)	0.106	3.70 (0.88–15.62)	0.075
Post-CRP (2w)	0.53 (0.21–1.35)	0.182	n/d	n/d
Gender (male)	1.97 (0.73–5.30)	0.178	n/d	n/d
Age>60	1.34 (0.59–3.14)	0.464	n/d	n/d
Tumor position (colon)	1.12 (0.43–2.91)	0.823	n/d	n/d
Poor differentiation[c]	1.02 (0.12–8.45)	0.986	n/d	n/d
T factor (T4)	0.53 (0.05–5.14)	0.583	n/d	n/d
Preoperative CEA elevation	1.30 (0.52–3.22)	0.572	n/d	n/d
Preoperative CA19-9 elevation	0.21 (0.03–1.66)	0.139	n/d	n/d
Obstruction	1.50 (0.33–6.90)	0.602	n/d	n/d
Number of total dissected lymph node			n/d	n/d
<6	Reference		n/d	n/d
6–10	6863.02	0.938	n/d	n/d
11–15	10138.02	0.935	n/d	n/d
>15	7343.4	0.937	n/d	n/d
Laparoscopy-assisted operation	1.17 (0.15–9.12)	0.884	n/d	n/d
Adjuvant chemotherapy	0.79 (0.23–2.75)	0.710	n/d	n/d
Perioperative transfusion	0.86 (0.26–2.84)	0.803	n/d	n/d
Propensity score	n/d	n/d	1.50 (0.16–13.88)	0.724

DFS disease-free survival, *HR* hazard ratio, *CI* confidence interval, *n/d* not determined, post-CRP (2w), *CRP* level at 2 week after operation

[a] End-point: date of death or April 30, 2007, no patient was lost to follow-up

[b] Significance based on Cox's proportional hazard model

[c] Poor consists of poorly differentiated, mucinous, and undifferentiated types

Variables with no event were excluded from multivariate analysis

Multivariable model 2 indicates the adjusted effect of Lk by applying propensity score which is a conditional probability of presenting Lk given by other clinicopathological factors and CRP level

been reported to be a risk factor of local recurrences in curatively operated CRC patients[10–12,19] which included several stage CRCs. However, to our knowledge, our study is the first report concerning Lk with high risk of recurrence limited in stage II disease. Interestingly, in our study, Lk was significantly implicated in systemic recurrence (P=0.003) rather than local recurrence in stage II.

In our study, there was no prognostic difference between colon cancer and rectal cancer. Although tumor position did not affect Lk and long-term prognosis in this study, anastomosis and prognosis in rectal cancer is thought to be affected by various factors compared with that in colon cancer.[10,20–23] However, even when separately analyzed on tumor positions, Lk was still significant prognostic factor (Fig. 1b, c).

Adjuvant chemotherapy for stage II CRC has been controversial at present because stage II patients show good prognosis and only a part of high-risk stage II patients may benefit in prognosis from previous studies.[6,24,25] Nevertheless, at present, standard chemotherapy is not recommended for stage II CRC patients because of excellent prognosis. Our current study included many such patients even with Lk who actually underwent adjuvant chemotherapy, but which did not include the most active agents such as oxaliplatin, CPT-11, bevacizumab, or cetuximab, suggesting that Lk anyway showed high risk for stage II CRC irrespective of adjuvant therapy. Therefore, our current result is worthy of further study on high-risk patient selection in stage II CRC and also on more powerful adjuvant chemotherapy such as FOLFOX in stage II patients with Lk in order to elucidate the benefit of adjuvant chemotherapy for these patients. In addition, neoadjuvant chemo-radiotherapy for locally advanced rectal cancer is now becoming standard. However, during the terms of this current study, we did not think that neoadjuvant treatment is really effective for such patients from a prognostic point of view. Thus, Lk in patients with neoadjuvant treatment should be also studied in the future.

Several parameters have been reported as independent prognostic factor or chemosensitive marker for patient selection allowing for the application of adjuvant chemotherapy in stage II CRC.[6,24,26] The number of evaluated lymph nodes,[27] T4 factor (direct invasion into adjacent structure),[16,28] tumor budding/infiltrating,[29] vascular involvement,[16,28] or perforation through the tumor[28] were such high-risk potential markers. In the present study, vascular involvement tended to be a prognostic factor, however, it was not insufficient to select high-risk patients. On the other hand, the number of evaluated lymph nodes and T4 factor did not indicate any prognostic significance in our current cohort of stage II CRC. Several molecular and genetic markers have also been reported to indicate poor prognosis of stage II CRC such as the DNA aneuploid,[30] 17p or 18q allelic imbalance,[7] gene expression profiling by cDNA microarray,[8] and micrometastasis detected by reverse transcriptase-polymerase chain reaction of CEA[31] or CK20.[32] In addition, microsatellite instability (MSI) has been reported as chemoresistant marker.[9] Actually, the largest stage II colon cancer trial (ECOG 5202, the US Gastrointestinal Intergroup including the National Cancer Institute of Canada) is ongoing, in which patients are now selected prospectively for adjuvant chemotherapy based on 18q loss of heterozygosity and MSI status.[33] Nevertheless, all such genetic and molecular tools are unsuitable for routine application at present because they are costly and time-consuming methods and have not been validated yet. In this meaning, Lk is easily available for patient selection at any minute.

Viable cancer cells in the lumen may be present at the site of the anastomosis at the time of surgery, which can be detected on suture or staple lines of anastomosis,[34] and on the occasion of Lk, those may be capable of implantation and subsequent local recurrence.[35] However, this theory alone did not explain the association of Lk with systemic recurrence in the present study. Systemic inflammatory response, as evidenced by raised circulating concentrations of CRP, has been reported to predict recurrence and disease-specific survival in curatively operated CRC patients.[18] Consistently, the sustained CRP elevation at either 1 or 2 weeks after operation was significantly associated with Lk, and especially, CRP at 2 weeks after operation per se predicted poor prognosis (P=0.022) in the present study. CRP may reflect the inflammatory response promoted by various cytokines which are presumably released from leukocytes in the malignant process.[36] On the other hand, a raised CRP level was thought to be related to the reduction of circulating lymphocytes.[37] In addition, the reduction of lymphocytes in the peripheral blood was shown to reflect the immune suppression in patients with malignant tumor,[38] and tumor-induced immune suppression adversely affects their prognosis.[39]

Perioperative allogeneic blood transfusion was reported to be an independent risk factor for Lk in a dose-dependent manner.[23] Also in the present study, perioperative blood transfusion affected Lk most robustly even when CRP was included in the multivariable logistic analysis (data not shown). Allogenic blood transfusion impairs the cell-mediated immune response[40] and predisposes to postoperative infectious complication,[41] and cell-mediated immune responses, which include mainly macrophage and T-lymphocyte, has been thought to affect the healing process.[42] Tadros T. et al. reported that perioperative blood transfusion impaired the healing of experimental intestinal anastomosis in an animal model using bursting pressure of anastomosis, in addition, cell-mediated immune response, as evidenced by exogenous IL-2, reversed the negative effects of blood transfusion on anastomotic repair.[43] Taken together, Lk may lead to systemic recurrences partly through cancer immune suppression together with sustained CRP elevation and perioperative blood transfusion. Conversely, we could also say that Lk is favored by a local depression of the immune system for the presence of undetected micrometastasis.

Recently, it has been suggested that tumor progression such as invasion and metastasis is coordinated by both cancer cells and host stromal cells, which consist tumor microenvironment.[44–46] A variety of host bone marrow-derived cells, which include inflammatory cells, cancer-associated fibroblasts, and endothelial progenitor cells compose of a tumor microenvironment.[47–49] Host inflammatory cells produce much more TGF-β than tumor cells, leading to inhibition of host tumor immune surveillance,[50,51] which may lead to cancer cell escape and intravasate into circulation. Local inflammation caused by Lk may additionally affect the above mechanism and may result in metastasis-prone phenotype. However, in order to answer the reason why Lk was associated with systemic recurrence, further experimental studies, such as comparison of circulating cancer cells or cytokines in both patients and experimental model, may be needed.

In conclusion, we showed that Lk was the most robust independent prognostic factor among the clinicopathological factors in stage II CRC. These results suggest that Lk may be appropriate for the selection of high-risk patients. And, Lk was associated with systemic recurrence in both colon and rectal cancer. Because Lk necessarily occurs at a given rate in spite of perioperative treatment with maximal attention and it is immediately available for clinical use from cost and technical point of view, Lk could be a factor for selecting high-risk patients. As only 12 patients (out of 207) had an Lk in this study, the prognostic impact of Lk should be validated in a larger study. On the other hand, because the DFS of patients without Lk was still 88.7%, further molecular tools would be necessary.

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Genetic Alterations of K-*ras* May Reflect Prognosis in Stage III Colon Cancer Patients Below 60 Years of age

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Purpose: Genetic alterations that are closely associated with patient prognosis can be landmarks of definitive therapeutic targets as well as useful biomarkers in human cancer clinics.
Methods: Three hundred seventy-eight colorectal cancer (CRC) patients were examined for K-*ras* mutations by single-strand conformation polymorphism (SSCP), with a subsequent 144 young colon cancer (YCC) patients added to validate its prognostic significance.
Results: K-*ras* mutations were identified in 161 (43%) of the 378 CRC patients and were significantly associated with tumor location (colon vs. rectum; 80/218 = 37% vs. 81/160 = 51%; $P = 0.0068$) and age (≥60 vs. <60; 103/220 = 47% vs. 58/158 = 37%; $P = 0.049$). The incidence of K-*ras* mutations was 30% in YCC patients as compared to 55% in elderly rectal cancer patients ($P = 0.0004$). K-*ras* mutations significantly correlated with a worse prognosis ($P = 0.0014$) only in 73 curatively resected YCC with stages I–III, but not in other CRCs, which was further validated in the independent set of the corresponding 144 YCC patients ($P = 0.024$). Both univariate and multivariate analyses identified K-*ras* mutations as an independent prognostic factor (HR = 5.5, $P = 0.029$; HR = 3.6, $P = 0.011$) in both learning and validation sets of the curatively resected YCC with stages I–III, respectively, and the prognostic relevance was marked in stage III YCC patients ($P = 0.002$), but not in stages I, II, and IV.
Conclusion: In curative YCC, K-*ras* mutations could have excellent prognostic value. Hence, the K-*ras* mutation status could be a good indicator to predict the clinical outcome in curatively resected stage III YCC patients, and K-*ras* pathway inhibition may be a relevant therapeutic target in CRC, excluding YCC patients with no K-*ras* mutation.
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KEY WORDS: **colorectal cancer; k-ras mutation; prognosis**

INTRODUCTION

Cancer, especially solid tumor, is a dismal disease that can ultimately lead to death. As the optimal strategy for solid tumors, attention has recently been focused on molecular therapies, such as the targeting of c-erbB2/HER2/neu for breast cancer [1,2], c-kit for gastrointestinal stromal tumors (GIST) [3,4], and epidermal growth factor receptor (EGFR) for non-small cell lung carcinoma [5,6]. Genetic alterations of such genes have been occasionally reported to be of prognostic significance [7–10]. As a result, cancer researchers have reached the consensus that the DNA status of therapeutic targets has a prognostic value.

In colorectal cancer (CRC), one of the most frequent causes of cancer-related deaths world-wide, K-*ras* is a critical oncogene with a prevalent mutation. K-*ras* persistently activates diverse onco-pathways, such as Raf/MEK (mitogen-activated protein/extracellular signal-regulated kinase)/ERK (extracellular signal-regulated kinase), PI3K (phosphatidylinositol 3-kinase)/PDK1 (3-phosphoinositide-dependent protein kinase-1)/Akt, and TIAM1 (T-cell lymphoma invasion and metastasis-inducing protein 1)/Rac (a Rho family GTPase) [11]. In CRC, somatic knockout of a mutant K-*ras* gene led to defective tumorigenesis accompanied by reduced expression of vascular endothelial growth factor (VEGF) [12,13], indicating that K-ras pathway activation plays a critical role in tumor progression in CRC.

K-*ras* mutations are an early event in adenoma, a precancerous form of CRC [14], but its prognostic value remains controversial, with both supporters [15–19] and detractors [20–26]. As a result, the American Society of Clinical Oncology (ASCO) 2008 update of recommendations addresses the utility of KRAS gene mutation testing in patients with metastatic colorectal carcinoma to predict response to anti-EGFR

Additional Supporting Information may be found in the online version of this article.

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monoclonal antibody (MoAb) therapy with cetuximab or panitumumab but did not acknowledge K-*ras* mutation as having any clinical usefulness as a prognostic marker at present [27,28]. We believe that mutations relevant to CRC should be evaluated for their clinical and prognostic significance, not only for predicting outcome but also in the search for a therapeutic target in CRC. In this study, detailed clinicopathological analysis was performed with a larger number of CRC patients than previously evaluated to reach accurate conclusions regarding the clinical significance of K-*ras* mutations.

MATERIALS AND METHODS

Three hundred seventy-eight patients with CRC were used to identify a subgroup with definite prognosis in terms of K-*ras* mutations and definition of clinicopathological factors.

From among CRC patients surgically resected at Kitasato University East Hospital between 1995 and 2004, 378 cases were investigated. Data on the CRC patients are shown in Supplemental Table I, in which the 6th Japanese Classification of Colorectal Cancer (JCCC), equivalent to the Dukes' stage, was applied.

Patients were divided into two groups, categorized as either elderly, $\geq$60 years old or young, <60 years old. If 40, 50, 60, and 70 years old were used to define young age, patient numbers below the cut-off were 8 (2.1%), 48 (12.6%), 158 (42.0%), and 281 (74.0%) in 378 CRC patients, respectively (Supplemental Table II). K-ras mutation exhibited the most intense association with age at a cut-off value of 50 (relative ratio = 2.2, $P = 0.02$), followed by 60 (RR 1.5, $P = 0.049$), when significant associations were found, but patients younger than 50 years of age were too few (13% of all CRC patients). We thus used 60 years old as the cut-off. Moreover, CRC was divided into either colon or rectal cancer, with colon cancer further divided into cecal, ascending, transverse, descending, and sigmoid.

According to the JCCC, pT was designated as follows: pT0 (mucosal invasion, M), pT1 (submucosal invasion, SM), pT2 (muscularis propria invasion, MP), pT3 (subserosal invasion or serosal exposure, SS/SE or A1/A2), and pT4 (invasion to the surrounding organs, SI or AI). Factors pN, H, LM, and P represented lymph node metastasis, hepatic metastasis, lung metastasis, and peritoneal dissemination, respectively. pN was defined as pN1/N2, the first/second tiers of lymph node metastasis, respectively. pN1 was defined as the first tier (Pericolic lymph nodes), and pN2 was defined as the second tier (Intermediate lymph nodes). CRC was classified into JCGC stages 0, I, II, III, and IV, based on pT, pN, and pM. Stages 0 and I were equivalent to pT0N0M0 and pT1/T2N0M0, respectively. Stage II was characterized by pT3N0M0. Stage III was defined by the presence of lymph node metastasis without distant metastasis (M0). Finally, stage IV featured distant metastases.

All cases were informative regarding the preoperative values of tumor markers CEA and CA19-9. The cut-off value determined by BRL Laboratory (Tokyo, Japan) was 2.5 ng/ml and 37.0 U/ml, respectively. Patients were followed up for at least 5 years, or until death. Follow-up was at least every 3 months during the first year, and then every 6 months. Assessment included medical history-taking, physical examination, biological tests, determination of serum CEA and CA19-9 levels (evaluated at every visit), colonoscopy, chest radiography, and chest computed tomography (CT; once yearly), abdominal ultrasonography, and abdominal CT (every 6 months). Recurrence was diagnosed on the basis of imaging and, if necessary, either cytological analysis or biopsy findings. Treatment of recurrence or metastasis included surgical resection (if possible), or 5-FU-based chemotherapy or radiotherapy.

All 378 cases were further analyzed for K-*ras* gene mutations and clinicopathological factors, including patient survival. The observation period ranged from 1 to 60 months, with a mean follow-up period of 42.7 months.

Validation Set for Prognostic Significance of K-*ras* Mutations in 144 Patients With Curatively Resected Young Colon Cancer (YCC) With Stages I–III

An additional and independent set of 144 young colon cancer (YCC) patients, who had undergone curative resection of the tumors with stages I–III at the Kitasato University Main Hospital between 1995 and 2006, was prospectively registered for further validation of the prognostic significance of K-*ras* mutations. They were further analyzed in terms of K-*ras* gene mutations and clinicopathological factors, including patient survival. The 144 patients were observed for 1–60 months, with a mean follow-up period of 42.0 months, and the 5-year disease-specific survival (DSS) rate was calculated.

Adjuvant chemotherapy was recommended largely for curatively resected stage III patients, although it was heterogeneous as standard therapy had not been developed, but administration was carried out for patients who agreed to the anti-cancer drug administration protocols approved by the authors' institution, which were 5-FU-based regimens +/− leucovorin (isovorin) or CPT-11, orally or intravenously. None of the rectal patients in the current study underwent adjuvant radiotherapy either pre- or post-operatively.

The current study was performed in accordance with the clinical research guidelines of the ethics committee of the Kitasato University School of Medicine. All patients gave written informed consent.

DNA Extraction

After taking fresh samples, surgically resected materials were fixed in 20% buffered formalin for 24–48 hr, routinely processed, embedded in paraffin wax, and cut into 4-μm thick sections. Histological sections were stained with hematoxylin–eosin for histological typing and staging. For simultaneous DNA analysis, the procedures summarized in previous articles were conducted [29–32], as shown below. (1) Sampling of specimens from surgical materials: fresh non-neoplastic colonic mucosa and colorectal/gastric tumors were scraped with disposable bamboo combs (rods made of bamboo with a spatula-like end, 3 mm × 3 mm × 120 mm) to prevent cross-contamination. (2) Extraction of DNA: tissue samples were transferred from the disposable bamboo combs into 400-μl aliquots of lysis buffer, containing 35 mmol/L Tris–HCl (pH 8.8), 175 mmol/L KCL, 300 μg/mL proteinase K, 0.45% Nonidet P-40, and 0.45% Tween 20 (PNT buffer), in 1.5-ml Eppendorf tubes, which were then incubated for 1 hr at 55°C. To inactivate proteinase K, each sample was then incubated for 10 min at 95°C, and 1 ml distilled water was added. After centrifugation (12,000 rpm × 1 min), 5-μl aliquots of supernatant were used for PCR.

Search for Mutated K-*ras* Genes Using Single-Strand Conformation Polymorphism (SSCP)

Mutations in K-*ras* gene exon 1 (including both codon 12 and 13) and exon 2 (codon 61) were initially screened by non-radioactive single-strand conformation polymorphism (SSCP) analysis [33]: PCR product samples of 10 μl were diluted threefold with gel-loading buffer (95% deionized formamide, 20 mmol/L EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol) and heated to 95°C for 10 min, followed by quenching on ice. Aliquots of 3 μl were applied to modified polyacrylamide gels [PAFG: 18% polyacrylamide-bis (49:1), 0.5× TBE, 10% glycerol, 10% formamide, 0.05% ammonium persulfate, and 30 μl TEMED] of 120 mm × 150 mm × 0.35 mm. Electrophoresis was performed with 1.5× TBE running buffer at 500 V and 30 mA for 1 hr at room temperature. Detection: Gels were stained using a silver stain plus kit (Bio-Rad, Hercules, CA), with fixation, rinsing, development, and stopping of the reaction. In this

analysis, mutated bands with PCR-SSCP were evident at 1:64 dilution of mutated alleles [30].

Direct Sequencing

Direct sequencing of 50 DNA samples, 30 with likely mutations and 20 with a likely wild-type, was performed to confirm the K-*ras* mutational status, as previously described [32]. Briefly, amplified DNA was purified from a 4% agarose gel using a QIA Quick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced using a dRhodamine dye terminator cycle sequence kit and 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA).

Statistical Analysis

Clinicopathological characteristics across CRC groups were analyzed using the χ^2 test, and logistic regression was used for multivariate analysis, with $P < 0.05$ indicating a significant difference. The Kaplan–Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed using the log-rank test. All patient deaths were cancer-related, and DSS was measured from the date of surgery to the date of death or the last follow-up. On 5-year DSS, patients who survived for more than 60 months were analyzed as survivors.

RESULTS

A flow chart of our current research, including the learning and validation sets of prognostic relevance in terms of K-*ras* mutation, is shown in Figure 1.

K-*ras* Mutations Identified in CRC

K-*ras* mutations were identified in 161 of 378 CRC patients (43%) by SSCP analysis (Fig. 2A), consistent with previous reports on CRC [24]. From among the DNA samples examined, 30 CRC cases of presumed K-*ras* mutation and 20 putative cases of no K-*ras* mutation by SSCP analysis were randomly selected to assess the actual mutation using direct sequencing, which confirmed an actual K-*ras* mutation (Fig. 2B). Clinicopathological analysis was performed in the 378 CRC patients to identify basic clinical factors according to the K-*ras* mutational status (Supplemental Table I), which revealed that K-*ras* mutation was significantly associated with tumor location (colon vs. rectum; 80/218 = 37% vs. 81/160 = 51%; $P = 0.0068$), age (≥60 vs. <60; 103/220 = 47% vs. 58/158 = 37%; $P = 0.049$), and histology (degree of differentiation; well/moderate differentiation vs. poor differentiation; 155/353 = 44% vs. 6/25 = 24%; $P = 0.05$). On the other hand, K-*ras* mutation was not associated with parameters such as TNM factors or tumor markers predicting patient prognosis (Supplement Table I). K-ras mutation was found 90.1% in exon 1 (codon 12 or 13) among the 378 cases, and this tendency was preserved in subpopulations such as 90 YCC learning sets (96.3%) and 27 stage III YCC leaning sets (90%).

Univariate Prognostic Analysis Including K-*ras* Mutational Status in CRC

Univariate prognostic analysis was performed using the log-rank test and revealed that the poor prognosis of CRC patients was significantly associated with pT factor ($P < 0.0001$), pN factor ($P < 0.0001$), histology ($P = 0.019$), H (hepatic metastasis) factor ($P < 0.0001$), LM (lung metastasis) factor ($P < 0.0001$), P (peritoneal dissemination) factor ($P < 0.0001$), vascular invasion ($P < 0.0001$), preoperative serum CEA value ($P < 0.0001$), preoperative serum CA19-9 value ($P < 0.0001$), and operative curability ($P < 0.0001$). Prognostic relevance according to lymphatic invasion could not be assessed using StatView 5.0 software, because there was no excluded case with an absence of lymphatic invasion. The presence of K-*ras* mutations did not have any prognostic significance (Fig. 3A) and therefore more detailed sub-analysis was performed to elucidate the relationship between K-*ras* mutations and clinicopathological factors, including patient prognosis.

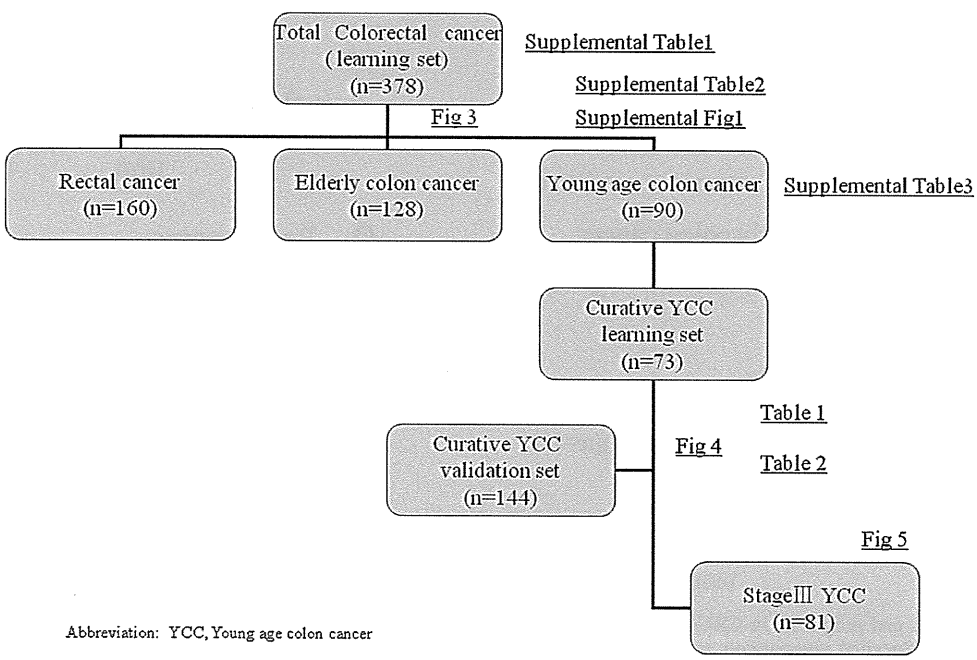

Fig. 1. Flow chart of our analytical process. [Color figure can be viewed in the online issue, available at wileyonlinelibrary.com.]

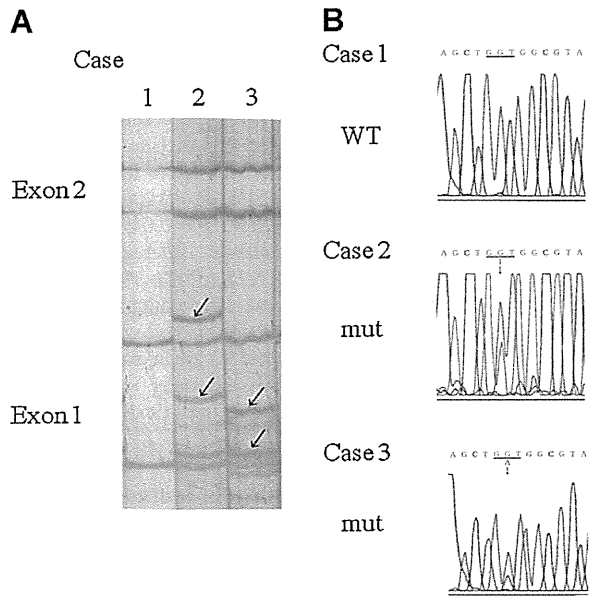

Fig. 2. Detection of K-*ras* mutation in colorectal cancer (CRC) tissues. **A**: Non-RI-SSCP analysis of amplified products of exons 1 and 2 of the K-*ras* gene in CRC. Lane 1, wild-type case; Lane 2, mutant case: Lane 3, mutant case. Arrows indicate mutant alleles. **B**: Direct sequencing of the corresponding cases in Figure 1A. Case 1 shows the wild-type sequence (GGT) of the K-*ras* gene (WT), while cases 2 and 3 have a mutant K-*ras* gene (mut), GTT and GAT, respectively. [Color figure can be viewed in the online issue, available at wileyonlinelibrary.com.]

K-*ras* Mutation Frequency According to Tumor Location and Age

K-*ras* mutation was significantly associated with tumor location and patient age (Supplemental Table I), suggesting gradual separation of CRC pathogenesis, which could be defined based on these clinical factors. The relationship of K-*ras* mutations with clinical characteristics determined by both location and age revealed that K-*ras* mutations are found significantly less often in YCC (27/90, 30%) than in other CRCs, especially elderly rectal cancer patients (50/89, 55%; $P = 0.0004$).

Univariate and multivariate Prognostic Analysis, Including K-*ras* Mutations in Curatively Resected YCC With Stages I–III in Both Learning and Validation Sets

The presence of a K-*ras* mutation had a significant predictive value for the 90 YCC patients ($P = 0.0038$; Fig. 3B), while it was not associated with patient prognosis in the other cases of CRC (Fig. 3C,D). Both univariate and multivariate prognostic analysis revealed that K-*ras* mutation was an independent prognostic factor in the 90 YCC cases (Supplemental Table III). Such prognostic relevance was confirmed ($P = 0.0014$), especially in the 73 YCC patients curatively resected with stages I–III (no significant difference in stage IV YCC; Fig. 4A). The presence of a K-*ras* mutation was not associated with any prognostic factors in the 73 YCC (Table I), suggesting that mutated K-*ras* is an independent prognostic factor in curatively resected YCC with stages I–III.

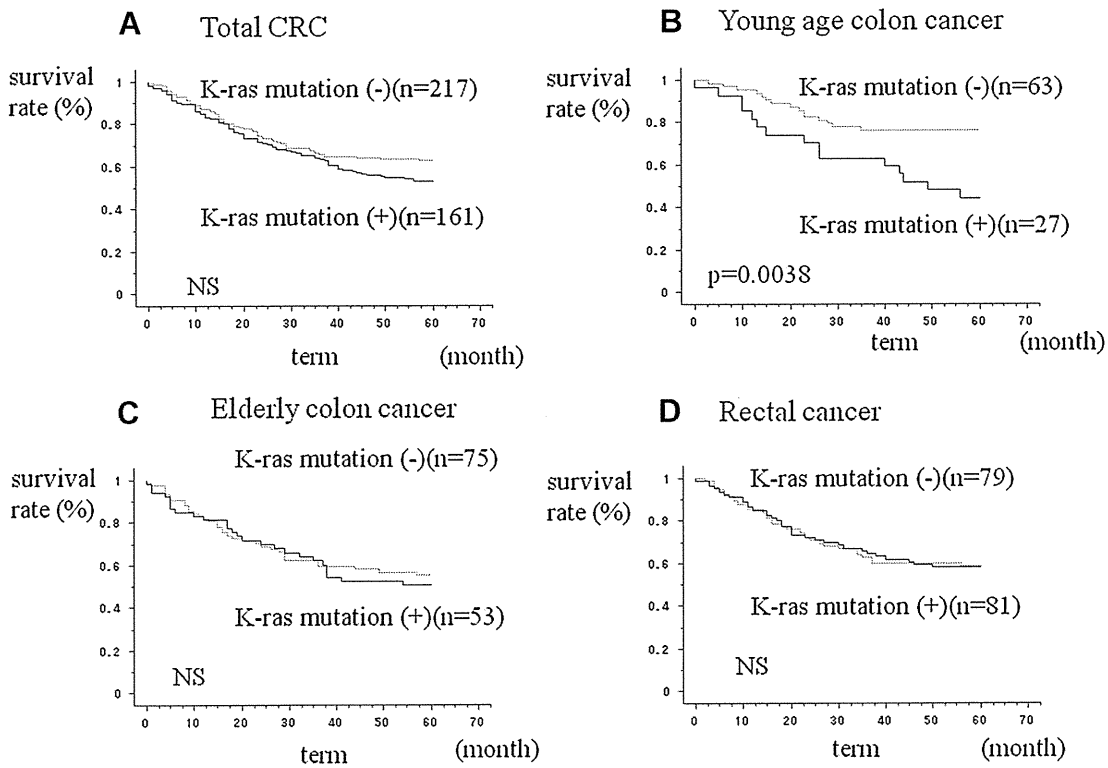

Fig. 3. K-*ras* mutation and prognosis in CRC. **A**: No significant difference in survival between the presence and absence of K-*ras* mutation in 378 CRC cases. **B**: Survival comparison according to K-*ras* mutations revealed a significant difference in young colon cancer patients (YCC; $P = 0.0038$). **C**: No significant difference in survival between the presence and absence of K-*ras* mutation in elderly colon cancer patients, and (**D**) rectal cancer irrespective of age.

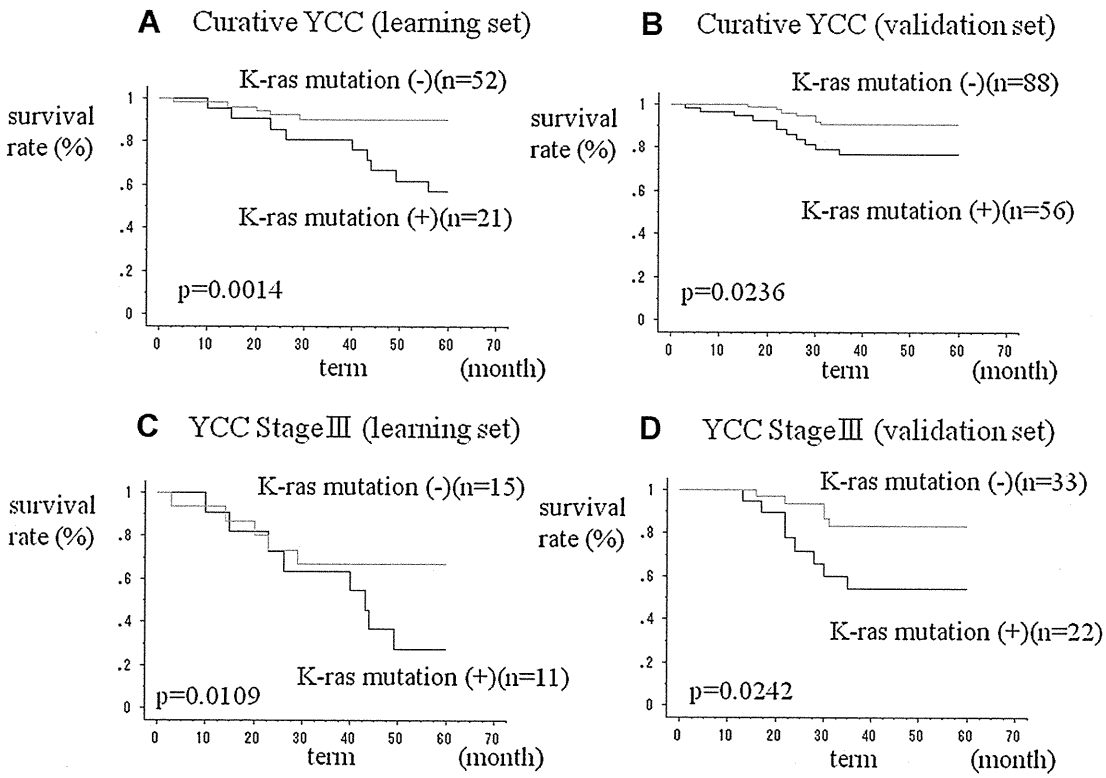

Fig. 4. K-*ras* mutation and prognosis in young colon cancer (YCC). **A**: Significant difference in survival between presence and absence of K-*ras* mutation in 73 curative YCC (learning set; $P = 0.0014$). **B**: Significant difference in survival according to K-*ras* mutation in curative YCC (validation set; $P = 0.0236$). **C**: Significant difference in survival according to K-*ras* mutation in stage III YCC (learning set; $P = 0.0109$). **D**: Significant difference in survival according to K-*ras* mutation in stage III YCC (validation set; $P = 0.0242$).

To confirm these results, an additional 144 cases (validation sets) of curatively resected YCC with stages I–III were newly analyzed as independent cases. The results again confirmed that the presence of a K-*ras* mutation still had significant prognostic value for YCC patients ($P = 0.0236$; Fig. 4B). K-*ras* mutations were not associated with any other parameters predicting outcome (Table I), suggesting that they are not related to carcinoma progression but rather represent definite pathways in YCC. Univariate and multivariate prognostic analyses of the 73 learning sets and 144 validation sets revealed that K-*ras* mutation could be a potent prognostic factor (HR = 5.5; $P = 0.0289$

TABLE I. K-ras Mutation and Its Clinicopathological and Prognostic Relation YCC

			K-ras mutational state (%)		
		Number (%)	Mutation (−) (n = 163)	Mutation (+) (n = 87)	*P*-value
Learning set (73 curative YCC)					
Sex	M/F	42 (58)/31 (42)	32 (76)/20(65)	10 (24)/11 (35	NS
pT factor	pT0, 1, 2/pT3, 4	18 (25)/55 (75)	15(83)/37(67)	3 (17)/18 (33)	NS
pN factor	Absence/presence	47 (64)/26 (36)	37 (79)/15 (58)	10 (21)/11 (42)	NS (0.057)
Histology	Differentiated/poorly differentiated	69 (95)/4 (5)	48 (70)/4 (100)	21 (30)/0 (0)	NS
Lymphatic permeation	Absence/presence	12 (16)/61 (84)	10 (83)/42 (69)	2 (17)/19 (31)	NS
Vascular permeation	Absence/presence	12 (16)/61 (84)	9 (75)/43(70)	3 (25)/18 (30)	NS
Preoperative CEA value	Low/high	52 (71)/21 (29)	38 (73)/4 (67)	14 (27)/7 (33)	NS
Preoperative CA19-9 value	Low/high	65 (89)/8 (11)	47 (72)/5 (63)	18 (28)/3 (37)	NS
Validation set (144 curative YCC)					
Sex	M/F	81 (56)/63 (34)	54 (67)/34 (54)	27 (33)/29 (46)	NS
pT factor	pT0, 1, 2/pT3, 4	50 (35)/94 (65)	28 (56)/60 (64)	22 (44)/34 (36)	NS
pN factor	Absence/presence	89 (62)/55 (38)	56 (63)/32 (58)	33 (37)/23 (42)	NS
Histology	Differentiated/poorly differentiated	141 (98)/3 (2)	85 (60)/3 (100)	56 (40)/0 (0)	NS
Lymphatic permeation	Absence/presence	43 (30)/101 (70)	27 (63)/61 (60)	16 (37)/40 (40)	NS
Vascular permeation	Absence/presence	47 (33)/97 (67)	27 (57)/61 (63)	20 (43)/36 (37)	NS
Preoperative CEA value	Low/high	117 (81)/27 (19)	74 (63)/14 (52)	43 (37)/13 (48)	NS
Preoperative CA19-9 value	Low/high	133 (92)/11 (8)	84 (63)/4 (36)	49 (37)/7 (64)	NS (0.079)
Family history	Absence/presence	124 (86)/20 (14)	74 (60)/14 (70)	50 (40)/6 (30)	NS

DSS, disease-specific survival; NS, not significant; NA, not assessible.

TABLE II. Univariate and Multivariate Prognostic Analysis in (A) 73 Curative YCC (Learning Set) and (B) 144 Curative YCC (Validation Set)

Variables		Univariate analysis		Multivariate analysis		
		DSS (5 years)	*P*-value	HR	95% CI	*P*-value
(A)						
Histology	Differentiated/poorly differentiated	83%/50%	0.0247	71.8	6.372–810.327	0.0005
pN factor	Absence/presence	98%/50%	<0.0001	60.3	5.658–643.017	0.0007
K-ras mutation	Absence/presence	90%/57%	0.0014	5.5	1.192–25.480	0.0289
PreoperativeCA19-9 value	Low/high	83%/62%	NS	1.1	0.210–6.113	NS
Preoperative CEA value	Low/high	83%/76%	NS	0.9	0.244–4.036	NS
Sex	M/F	79%/84%	NS	0.7	0.196–2.646	NS
Vascular permeation	Absence/presence	92%/79%	NS	0.4	0.041–4.431	NS
pT factor	pT0, 1, 2/pT3, 4	100%/75%	NA			
Lymphatic permeation	Absence/presence	100%/77%	NA			
(B)						
pN factor	Absence/presence	96%/69%	<0.0001	4.3	1.090–17.131	0.0373
Preoperative CA19-9 value	Low/high	87%/62%	0.0041	3.9	1.119–13.720	0.0326
Vascular permeation	Absence/presence	97%/80%	0.0144	3.7	0.453–31.022	NS
K-ras mutation	Absence/presence	90%/76%	0.0236	3.6	1.339–9.948	0.0114
Sex	M/F	84%/87%	NS	1.3	0.559–4.291	NS
Preoperative CEA value	Low/high	87%/79%	NS	0.7	0.204–2.409	NS
pT factor	pT0, 1, 2/pT3, 4	98%/79%	0.0064	0.3	0.032–3.370	NS
Family history	Absence/presence	87%/86%	NS			
Histology	Differentiated/poorly differentiated	85%/100%	NA			
Lymphatic permeation	Absence/presence	100%/79%	NA			

DSS, disease-specific survival; NS, not significant; NA, not assessible.

and HR = 3.6; $P = 0.0114$, respectively) independently of TNM factors and/or tumor markers, respectively (Table II).

Curatively Resected Stage III YCC Patients With K-*ras* Mutations Included More Patients With Metachronous Distant Metastasis of CRC

Since K-*ras* mutations were identified as a prognostic factor independent of TNM stage-determining factors, sub-analysis was performed by stage. As a result, K-*ras* mutations had prognostic relevance only in stage III in both learning sets (n = 26, $P = 0.011$, Fig. 4C) and validation sets (n = 55, $P = 0.024$, Fig. 4D). In the 81 stage III YCC patients who were curatively operated (learning plus validation sets), the presence of a K-*ras* mutation had significant predictive value in prognosis ($P = 0.002$; Fig. 5B). Even when stage III YCC patients were subdivided into JCCC N1 and N2 cases, patients with no K-*ras* mutation showed ~80% survival rate (Fig. 5C,D), a result much better than expected for ordinary stage III CRC.

In the 81 stage III YCC cases, K-*ras* mutation was not associated with the administration of adjuvant chemotherapy; 75 patients (93%) underwent 5-FU-based adjuvant chemotherapy (concomitant administration of leucovorin/isovorin, n = 16 or CPT-11, n = 1), orally (n = 59), or intravenously (n = 16). Twenty-nine of the 75 patients had a K-*ras* mutation (39%), while six patients who did not undergo adjuvant chemotherapy included four patients with K-*ras* mutation (67%; no statistical difference), and there was no significant difference in prognosis between the patients with adjuvant chemotherapy and without it (the follow-up periods ranged from 2 to 60 months).

K-*ras* mutations did not have any predictive value in stage 0/I/II/IV patients examined in the current study. Among the 66 stage 0/I YCC patients, only one with a K-*ras* mutation died due to recurrence. Of the 70 stage II YCC patients, 3 died due to recurrence, in which 20 (10%) had a K-*ras* mutation, and 1 of 49 (2%) did not (not statistically significant). In the 19 stage IV YCC patients, K-*ras* mutation was not associated with the survival status (data not shown).

DISCUSSION

The current study separated YCC patients without a K-*ras* mutation from other CRC patients from a prognostic viewpoint, and found that they showed the best prognosis among all CRC. This finding was unlikely to have resulted from the different distribution of stages within each group that were separated in terms of age and tumor location, because the prognostic relevance of K-*ras* mutation was proven even after adjusting for stage in multivariate analysis (Table II). In particular, stage III YCC patients without K-*ras* mutations clearly showed the best prognosis (~80%) as compared to other stage III CRC patients (50–60%; Figs. 2 and 3). On the other hand, in stage II YCC, a mutated K-*ras* indicated a poorer prognosis (90%) than wild-type K-ras (98%), with very rare recurrence (only 3 patients) among the 69 cases. For stage II YCC patients, we could not find a significant difference in the prognostic value, putatively due to the small number tested and small number of events included, and this should be confirmed in the future. Prognostic markers of stage II CRC, such as DNA ploidy [34], genomic imbalance [35], and microsatellite instability (MSI) [36], have been recognized as vital indicators in patient selection for post-operative adjuvant chemotherapy.

Stage III YCC patients without K-*ras* mutations had a 5-year survival rate of about 80% after surgery, comparable to that of stage II CRC patients [35]. This finding suggested that stage III YCC without a K-*ras* mutation can be recognized as stage II CRC from a prognostic viewpoint, and treated similarly, including adjuvant chemotherapy. For stage III CRC, oxaliplatin-including regimens (FOLFOX or FLOX) were demonstrated to be more effective than surgery alone in the MOSAIC trial [37] and the NSABP C-07 trial [38]; however, an adjuvant effect was achieved in only 6–7% of stage III patients or possibly in high-risk stage II patients [37]. As FOLFOX is expensive and labor-intensive, and also has serious complications, the selection of patients who truly need potent adjuvant chemotherapy is eagerly anticipated. The present study indicates that K-*ras* mutations could be a biomarker for patient selection in stage III CRC. RASCAL-2 is a larger version of RASCAL [39], the largest survey (at that time) of K-*ras* mutations in primary tumor tissues, which included data collected

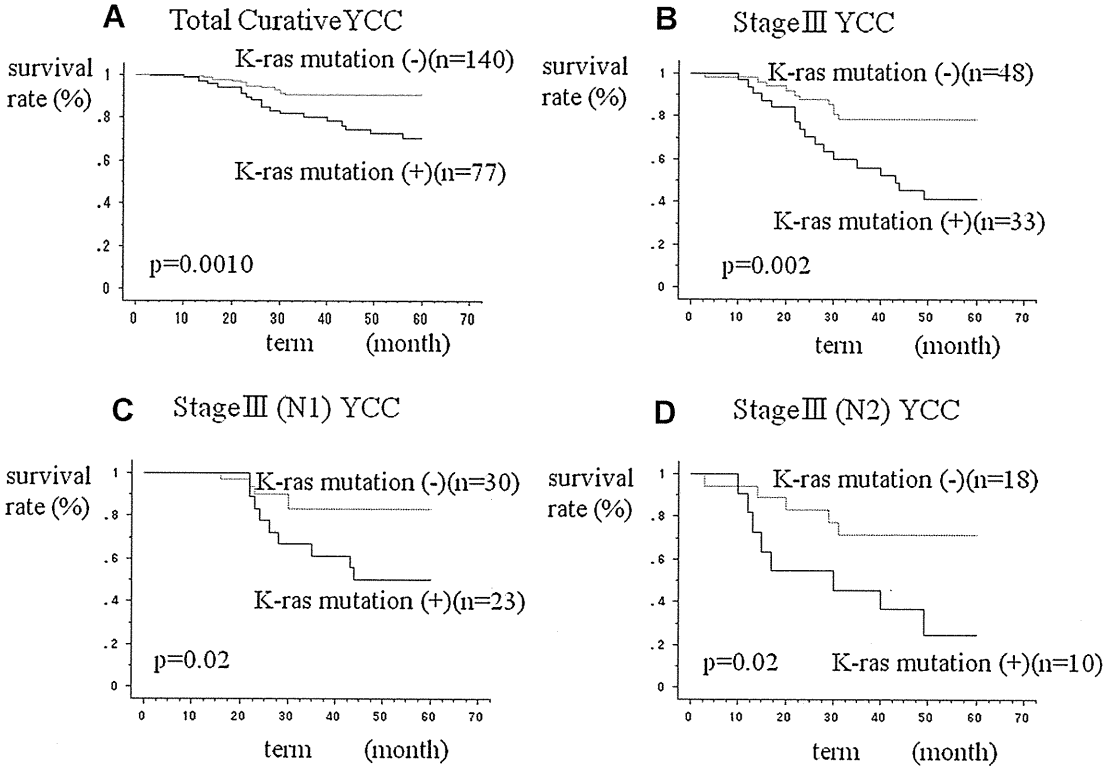

Fig. 5. Prognostic significance of K-*ras* mutation in stage III YCC in curable cases. **A**: Validation of significant difference in survival comparison between presence and absence of K-ras mutation in 217 YCC cases ($P = 0.0010$). **B**: Significant difference in survival according to K-ras mutation in stage III (Dukes C) YCC ($P = 0.002$). **C**: Significant difference in survival according to K-ras mutation in stage IIIA (N1) YCC ($P = 0.02$). Note that stage IIIA (N1) YCC patients without a K-ras mutation had more than an 80% survival rate. **D**: Significant difference in survival according to K-ras mutation in Stage IIIB (N2) YCC ($P = 0.02$). Note that Stage IIIB (N2) YCC patients without K-ras mutation had ~70% survival rate.

by groups from 13 countries on the prognostic importance of K-*ras* mutations. RASCAL-2 examined over 4,000 CRC patients and revealed that K-ras mutations had prognostic significance in stage III CRC [40]. RASCAL-2 may be so huge that K-*ras* mutations would have a prognostic impact even if patients were not limited to YCC; however, our results revealed that K-ras mutations did not have any significant impact on prognosis in CRC other than YCC (data not shown). RASCAL-2 showed that only one mutation on codon 12, glycine to valine, found in 8.6% of all patients, had a statistically significant impact on failure-free survival ($P = 0.004$, HR 1.3) and overall survival ($P = 0.008$, HR 1.29), suggesting that this mutation appeared to have a greater impact on outcome in stage III CRC cancers (failure-free survival, $P = 0.008$, HR 1.5; overall survival $P = 0.02$, HR 1.45) than in stage II tumors (failure-free survival, $P = 0.46$, HR 1.12; overall survival $P = 0.36$, HR 1.15). Our SSCP analysis did not reveal the full profile of each mutation, and we would like to elucidate such associations in the near future.

CRC has been recently proposed to originate in two pathways, MSI and chromosomal instability (CIN) [41]. MSI shows a diploid pattern of DNA content, while CIN has an aneuploid pattern. MSI is more characteristic of proximal colon cancer [42] and young CRC [43], which made us speculate that YCC includes more MSI cases than other CRC. Moreover, a K-*ras* mutation was found in only 13% of MSI CRCs [44], indicating that the mutation is more characteristic of CIN than MSI. Hence, we suppose that YCC without a K-*ras* mutation and with a good prognosis largely reflects MSI, consistent with a report that MSI showed a better prognosis than non-MSI [45]. Nevertheless, CRC sometimes harbors both phenotypes (MSI and CIN), and CIN is the dominant phenotype for aneuploidy [46], which is why K-ras mutation, due to its phenotypic dormancy, clearly showed a poor prognosis in YCC in the current study. We are interested in the relationship of both K-ras mutation and the MSI status with patient prognosis in YCC. On the other hand, even in YCC without a K-ras mutation, several patients had a poor prognosis. This may have been caused by B-*raf* mutation, which has a dismal prognosis in microsatellite-stable CRC [47], and such cases can be included in YCC without K-ras mutation. K-ras mutation might be a marker for MSI and not a prognostic indicator itself. Allowing for these findings, we are planning to profile MSI/B-*raf* mutations in combination with the K-*ras* mutational status in order to clearly explain the prognostic status of YCC in stage III.

We interpreted our results to mean that YCC without a K-*ras* mutation represents patients with a normal K-*ras* pathway. K-*ras* pathway activation may be closely associated with prognosis in CRC, and could be a therapeutic target for most CRC cases (except YCC without K-*ras* mutation). Patients with an abnormal K-*ras* pathway through the activation of either upstream or downstream oncogenes, such as EGFR [48], PI3K [49], and B-raf [50], are similar to those with K-*ras* mutations from a biological viewpoint because the K-*ras* pathway is similarly activated. On the other hand, patients with a normal K-*ras* pathway may show biologically different behavior from those with K-*ras* mutations because the K-*ras* pathway is not activated.

As an optimal strategy for solid tumors, attention has recently focused on molecular therapies by identifying genetic alterations that have been of prognostic value [7–10]. On this basis, the authors suggest the K-*ras* pathway as a therapeutic target for CRC. On the other hand, the K-*ras* mutational status was recently demonstrated to

have predictive value for sensitivity against EGFR inhibition, a newly developed CRC molecular target [51–54]. As neutralizing EGFR antibody is effective even against far-advanced CRC without K-*ras* mutation, the development of new treatments, including adjuvant chemotherapy, is eagerly anticipated. On the other hand, CRC with K-*ras* mutation proved ineffective by EGFR inhibition [53]. About 75% CRC cases with K-ras mutation had co-mutated PI3K [49] and, in such cases, downstream inhibition of both B-raf and PI3K may efficiently regulate CRC cells.

None of the rectal patients in the current study underwent radiotherapy either pre- or post-operatively, which may not represent the standard of care of rectal cancer worldwide, and perhaps would effect the outcome of the analysis. In rectal cancer, we would thus examine the K-ras mutation status and prognosis in such patients who undertake the standard therapy in the near future. Actually, we recently adopted neoadjuvant chemoradiotherapy for localized advanced rectal cancer before surgery [55,56]. Even if molecular target therapy such as anti-EGFR MoAb is used, CRC at stage IV has a dismal prognosis [51,52,57] and almost all patients will die of disease progression. That is why improving the prognosis of CRC depends upon improving treatment for curable cases, which includes adjuvant chemotherapy. The most promising treatment strategy for CRC is therefore to develop tailor-made adjuvant chemotherapy using novel indicators on the basis of oncogenic mutational profiles as in the present study.

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Analysis of *ERBB4* Mutations and Expression in Japanese Patients with Lung Cancer

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Abstract: Only the kinase domain of *ERBB4* has been analyzed in East Asian populations, but a recent large-scale mutation analysis has indicated a higher incidence of mutations in the extracellular domain. Mutations in the extracellular and kinase domains of *ERBB4* were examined by direct sequencing in 72 patients with primary lung cancer and 8 cell lines. In addition, *ERBB4* expression was determined in 60 patients by quantitative real-time polymerase chain reaction. We investigated the relationship between *ERBB4* expression and clinicopathologic characteristics including prognosis. One patient possessed Q793Q polymorphism in the kinase domain. However, we detected no mutations in extracellular or kinase domains of *ERBB4*. There was no significant difference in the clinicopathologic characteristics including prognosis of patients with high or low expression of *ERBB4*. The clinical significance of *ERBB4* in lung cancers is negligible.

Key Words: ERBB4 mutation, Extracellular domain, Lung cancer, Expression, Prognosis.

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The ERBB family of tyrosine kinase receptors consists of four members: epidermal growth factor receptor (*EGFR*), *ERBB2*, *ERBB3*, and *ERBB4*.[1] According to the NCBI database, the sequences of the kinase domains of *EGFR* and *ERBB4* are 79% identical, and *EGFR* and *ERBB4* have common ligands: heparin-binding epidermal growth factor, betacellulin, and epiregulin.[1] *EGFR* mutation has been revealed to play an important role in non-small cell lung cancer (NSCLC).

Two mutation analyses of the *ERBB4* kinase domain in East Asian patients with NSCLC have been reported: 5 of 217 (2.3%) in Korean patients[2] and none of 105 in Japanese patients.[3] Conversely, a large-scale mutation analysis in 188 patients with lung adenocarcinoma detected nine *ERBB4* mutations: two in the kinase domain, one in the transmembrane domain, and six in the extracellular domains (Figure 1*A*).[4]

The extracellular domains of the *ERBB* family are consisted of four distinct protein domains. There are two homologous large (L) domains and two cysteine-rich (CR) domains, which occur in the order L1–CR1–L2–CR2 (Figure 1*A*).[5] The L1 and L2 domains form the ligand binding pocket and the CR1 and CR2 domains are deeply involved in receptor dimerization.[5] Mutations in the *ERBB4* gene are more frequently present in the extracellular domain, especially the CR1 domain, than in the kinase domain.[4] However, only the kinase domain has been analyzed in East Asian populations.[2,3] We considered that analysis of mutations in the extracellular domains and the kinase domain of *ERBB4* would be of value.

In this study, we searched for mutations in the CR1 domain and kinase domain of *ERBB4*. We also analyzed *ERBB4* mRNA expression by real-time polymerase chain reaction and examined the relationship between the expression and clinicopathologic characteristics including prognosis.

PATIENTS AND METHODS

Cell Lines

Eight lung cancer cell lines were available. These comprised six adenocarcinomas (NCI-H23, NCI-H358, NCI-H3255, HCC78, A549, and ACC-LC-319), one adenosquamous cell carcinoma (NCI-H596), and one large cell carcinoma (ACC-LC-91). ACC-LC-319 and ACC-LC-91 were established in our institution. A549 and NCI-H596 were purchased from the American Type Culture Collection (Manassas, VA). The others were gifts from Dr. Adi F. Gazdar.

Patients

We studied 72 Japanese patients with lung cancer who underwent pulmonary resection at our institution. Tumor samples were rapidly frozen, and total RNA were extracted and genomic DNA was extracted from the blood sample using the Gene Tapping by Liquid Extraction kit (TAKARA BIO Inc., Otsu, Japan) after obtaining appropriate approval from the review board and written informed consent from the patients. The clinicopathologic characteristics of the patients were as follows: 44 were men and 28 were women, and the median age was 67 years at diagnosis (range, 38–85 years). Thirty-nine patients had pathologic stage I disease, 7 had stage II, 23 had stage III, and 3 had stage IV (TNM Classification of Malignant tumor, 6th Edition). There were 55 adenocarcinomas, 10 squamous cell

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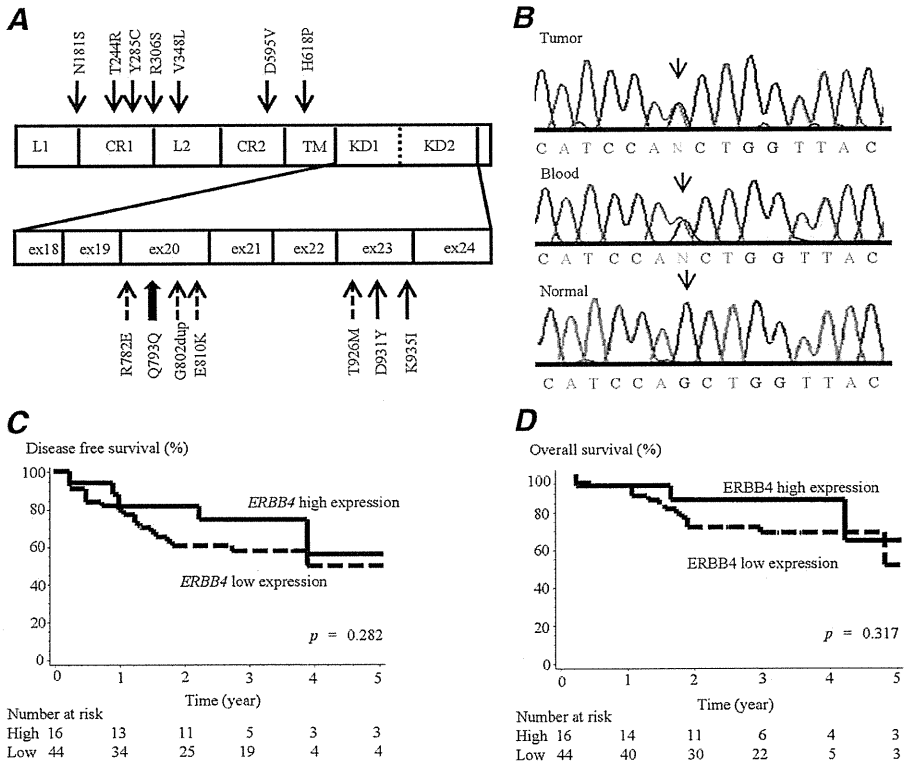

FIGURE 1. *A, ERBB4* mutations from the previous reports of Ding et al.[4] (arrow) and Soung et al.[2] (dotted arrow) and the Q793Q polymorphism identified in this study (thick arrow) are illustrated. *ERBB4* consists of four extracellular domains, a transmembrane domain (TM), and a kinase domain. The kinase domain is divided into two regions. L1, 2: large domain 1, 2 CR1: 2; CR domain 1, 2. *B,* Sequencing chromatograms for the synonymous mutation identified in exon 20 of *ERBB4* in the tumor and blood from the same patient. The nucleotide change was c.2379G>A, which did not lead to substitution of glutamine at position 793. *C* and *D,* Kaplan–Meier estimates of disease-free survival and overall survival in patients with high and low *ERBB4* expression. The median disease-free survival time and overall survival time were not significantly different among the two groups.

carcinomas, 1 adenosquamous cell carcinomas, 5 large cell carcinomas, and 1 small cell carcinoma. Twenty-four patients had never smoked, and 48 were current or former smokers.

Analysis of *ERBB4* Mutations

The *ERBB4* extracellular region and kinase domain, which was divided into *C*-terminal side (KD1) and *N*-terminal side (KD2; Figure 1*A*), were analyzed for mutations. By using total RNA or genomic DNA, *ERBB4* was analyzed by direct. Primer sequences were as follows: extracellular region, 5′-TCCTTTGTTATGCAGACACCAT-3′ and 5′-TTGTAAGGGTCCCCATGAATAC-3′; KD1, 5′-GGTGGAACCATTAACTCCCAGT-3′ and 5′-CAATGCTGATGGAGGAAAGATG-3′; and KD2, 5′-CAATGCTGATGGAGGAAAGATG-3′ and 5′-TGATCGTATGAAGCTTCCCAG-3′.

Analysis of ERBB4 Expression

Total RNA from 60 patients and 2 normal lung tissue samples were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). We analyzed *ERBB4* expressions according to the protocol of the TaqMan Gene Expression assay, using 18S rRNA as the internal reference gene. The primer IDs for *ERBB4* and 18S rRNA were Hs00171783_m1 and Hs99999901_s1 (Applied Biosystems), respectively. The 60 patients were divided into 2 groups on the basis of the average *ERBB4* expression value of 2 normal lung tissue samples.

Statistical Analysis

For comparison of proportions, a χ^2 test or Fisher's exact test was applied. For quantitative variables, Student *t* test was used. Disease-free survival (DFS) was measured from the date of first operation until the date of radiologic recurrence or death. Overall survival (OS) was measured from the date of first operation until the date of death. The Kaplan–Meier method was used to estimate the probability of survival as a function of time, and survival differences were analyzed by the log-rank test. We defined the significance level at $p < 0.05$.

RESULTS

In the *ERBB4* mutation analysis, one patient with adenocarcinoma had a c.2379G>A synonymous genetic change resulting in Q793Q in the *ERBB4* kinase domain (exon 20). The synonymous change was confirmed as a polymorphism by DNA sequencing a matched blood sample (Figure 1*B*). However, somatic mutation of *ERBB4* was not detected in this study.

The average *ERBB4* expression level was not significantly different between tumor samples and normal lung tissue samples ($p = 0.384$). The high- and low-expression groups were 27% (16 of 60 patients) and 73% (44 of 60 patients), respectively. The two groups were compared for clinicopathologic characteristics (Table 1), but we did not identify any significant difference. In the survival analysis, the median DFS and OS time was not significantly different between the patients with high and low *ERBB4* expression (Figures 1*C*, *D*).

DISCUSSION

We have previously analyzed *EGFR*, *KRAS*, *MET*, and *ERBB2* mutations and *MET* amplification, which are mutually