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.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P: Global cancer statistics, 2002. CA Cancer J Clin 2005;55:74–108.
2. Cunningham D, Starling N: Adjuvant chemotherapy of colorectal cancer. Lancet 2007;370:1980–1981.
3. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pitot HC, Alberts SR: A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004;22:23–30. Epub 2003 Dec 2009.
4. Kuebler JP, Wieand HS, O'Connell MJ, Smith RE, Colangelo LH, Yothers G, Petrelli NJ, Findlay MP, Seay TE, Atkins JN, Zapas JL, Goodwin JW, Fehrenbacher L, Ramanathan RK, Conley BA, Flynn PJ, Soori G, Colman LK, Levine EA, Lanier KS, Wolmark N: Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage II and III colon cancer: results from NSABP C-07. J Clin Oncol 2007;25:2198–2204.
5. Andre T, Sargent D, Tabernero J, O'Connell M, Buyse M, Sobrero A, Misset JL, Boni C, de Gramont A: Current issues in adjuvant treatment of stage II colon cancer. Ann Surg Oncol 2006;13:887–898. Epub 2006 Apr 2014.
6. Benson AB, 3 rd, Schrag D, Somerfield MR, Cohen AM, Figueredo AT, Flynn PJ, Krzyzanowska MK, Maroun J, McAllister P, Van Cutsem E, Brouwers M, Charette M, Haller DG: American Society of Clinical Oncology recommendations on adjuvant chemotherapy for stage II colon cancer. J Clin Oncol 2004;22:3408–3419. Epub 2004 Jun 3415.
7. Diep CB, Thorstensen L, Meling GI, Skovlund E, Rognum TO, Lothe RA: Genetic tumor markers with prognostic impact in Dukes' stages B and C colorectal cancer patients. J Clin Oncol 2003;21:820–829.
8. Wang Y, Jatkoe T, Zhang Y, Mutch MG, Talantov D, Jiang J, McLeod HL, Atkins D: Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. J Clin Oncol 2004;22:1564–1571.
9. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, Hamilton SR, Laurent-Puig P, Gryfe R, Shepherd LE, Tu D, Redston M, Gallinger S: Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med 2003;349:247–257.
10. Branagan G, Finnis D: Prognosis after anastomotic leakage in colorectal surgery. Dis Colon Rectum 2005;48:1021–1026.
11. Fujita S, Teramoto T, Watanabe M, Kodaira S, Kitajima M: Anastomotic leakage after colorectal cancer surgery: a risk factor for recurrence and poor prognosis. Jpn J Clin Oncol 1993;23:299–302.
12. Ptok H, Marusch F, Meyer F, Schubert D, Gastinger I, Lippert H: Impact of anastomotic leakage on oncological outcome after rectal cancer resection. Br J Surg 2007;94:1548–1554.
13. McArdle CS, McMillan DC, Hole DJ: Impact of anastomotic leakage on long-term survival of patients undergoing curative resection for colorectal cancer. Br J Surg 2005;92:1150–1154.
14. Walker KG, Bell SW, Rickard MJ, Mehanna D, Dent OF, Chapuis PH, Bokey E L: Anastomotic leakage is predictive of diminished survival after potentially curative resection for colorectal cancer. Ann Surg 2004;240:255–259.
15. Yamashita K, Sakuramoto S, Kikuchi S, Katada N, Kobayashi N, Watanabe M: Transfusion alert for patients with curable cancer. World J Surg 2007;31:2315–2322.
16. Morris M, Platell C, de Boer B, McCaul K, Iacopetta B: Population-based study of prognostic factors in stage II colonic cancer. Br J Surg 2006;93:866–871.
17. Rubin DB: Estimating causal effects from large data sets using propensity scores. Ann Intern Med 1997;127:757–763.
18. McMillan DC, Canna K, McArdle CS: Systemic inflammatory response predicts survival following curative resection of colorectal cancer. Br J Surg 2003;90:215–219.
19. Law WL, Choi HK, Lee YM, Ho JW, Seto CL: Anastomotic leakage is associated with poor long-term outcome in patients after curative colorectal resection for malignancy. J Gastrointest Surg 2007;11:8–15.
20. Alves A, Panis Y, Trancart D, Regimbeau JM, Pocard M, Valleur P: Factors associated with clinically significant anastomotic leakage after large bowel resection: multivariate analysis of 707 patients. World J Surg 2002;26:499–502.
21. Lipska MA, Bissett IP, Parry BR, Merrie AE: Anastomotic leakage after lower gastrointestinal anastomosis: men are at a higher risk. ANZ J Surg 2006;76:579–585.
22. Peeters KC, Tollenaar RA, Marijnen CA, Klein Kranenbarg E, Steup WH, Wiggers T, Rutten HJ, van de Velde CJ: Risk factors for anastomotic failure after total mesorectal excision of rectal cancer. Br J Surg 2005;92:211–216.
23. Yeh CY, Changchien CR, Wang JY, Chen JS, Chen HH, Chiang JM, Tang R: Pelvic drainage and other risk factors for leakage after elective anterior resection in rectal cancer patients: a prospective study of 978 patients. Ann Surg 2005;241:9–13.
24. Figueredo A, Charette ML, Maroun J, Brouwers MC, Zuraw L: Adjuvant therapy for stage II colon cancer: a systematic review from the Cancer Care Ontario Program in evidence-based care's gastrointestinal cancer disease site group. J Clin Oncol 2004;22:3395–3407.
25. Quasar Collaborative G, Gray R, Barnwell J, McConkey C, Hills RK, Williams NS, Kerr DJ (2007) Adjuvant chemotherapy versus observation in patients with colorectal cancer: a randomised study. Lancet;370:2020–2029.
26. Andre T, Boni C, Mounedji-Boudiaf L, Navarro M, Tabernero J, Hickish T, Topham C, Zaninelli M, Clingan P, Bridgewater J, Tabah-Fisch I, de Gramont A: Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343–2351.
27. Caplin S, Cerottini JP, Bosman FT, Constanda MT, Givel JC: For patients with Dukes' B (TNM Stage II) colorectal carcinoma, examination of six or fewer lymph nodes is related to poor prognosis. Cancer 1998;83:666–672.
28. Petersen VC, Baxter KJ, Love SB, Shepherd NA: Identification of objective pathological prognostic determinants and models of prognosis in Dukes' B colon cancer. Gut 2002;51:65–69.
29. Nakamura T, Mitomi H, Kanazawa H, Ohkura Y, Watanabe M: Tumor budding as an index to identify high-risk patients with stage II colon cancer. Dis Colon Rectum 2008;51:568–572.
30. Garrity MM, Burgart LJ, Mahoney MR, Windschitl HE, Salim M, Wiesenfeld M, Krook JE, Michalak JC, Goldberg RM, O'Connell MJ, Furth AF, Sargent DJ, Murphy LM, Hill E, Riehle DL, Meyers CH, Witzig TE: Prognostic value of proliferation, apoptosis, defective DNA mismatch repair, and p53 overexpression in patients with resected Dukes' B2 or C colon cancer: a North Central Cancer Treatment Group Study. J Clin Oncol 2004;22:1572–1582.
31. Noura S, Yamamoto H, Ohnishi T, Masuda N, Matsumoto T, Takayama O, Fukunaga H, Miyake Y Ikenaga M, Ikeda M, Sekimoto M, Matsuura N, Monden M: Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. J Clin Oncol 2002;20:4232–4241.
32. Koch M, Kienle P, Kastrati D, Antolovic D, Schmidt J, Herfarth C, von Knebel Doeberitz M, Weitz J: Prognostic impact of hematogenous tumor cell dissemination in patients with stage II colorectal cancer. Int J Cancer 2006;118:3072–3077.

33. Benson AB, 3rd: New approaches to assessing and treating early-stage colon and rectal cancers: cooperative group strategies for assessing optimal approaches in early-stage disease. Clin Cancer Res 2007;13:6913 s–6920 s.
34. Gertsch P, Baer HU, Kraft R, Maddern GJ, Altermatt HJ: Malignant cells are collected on circular staplers. Dis Colon Rectum 1992;35:238–241.
35. Umpleby HC, Fermor B, Symes MO, Williamson RC: Viability of exfoliated colorectal carcinoma cells. Br J Surg 1984;71:659–663.
36. Gabay C, Kushner I: Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 1999;340:448–454.
37. Nozoe T, Matsumata T, Sugimachi K: Preoperative elevation of serum C-reactive protein is related to impaired immunity in patients with colorectal cancer. Am J Clin Oncol 2000;23:263–266.
38. Oka M, Hirazawa K, Yamamoto K, Iizuka N, Hazama S, Suzuki T, Kobayashi N: Induction of Fas-mediated apoptosis on circulating lymphocytes by surgical stress. Ann Surg 1996;223:434–440.
39. Eilber FR, Morton DL: Impaired immunologic reactivity and recurrence following cancer surgery. Cancer 1970;25:362–367.
40. Waymack JP, Rapien J, Garnett D, Tweddell JS, Alexander JW: Effect of transfusion on immune function in a traumatized animal model. Arch Surg 1986;121:50–55.
41. Jensen LS, Andersen AJ, Christiansen PM, Hokland P, Juhl CO, Madsen G, Mortensen J, Moller-Nielsen C, Hanberg-Sorensen F, Hokland M: Postoperative infection and natural killer cell function following blood transfusion in patients undergoing elective colorectal surgery. Br J Surg 1992;79:513–516.
42. Barbul A, Breslin RJ, Woodyard JP, Wasserkrug HL, Efron G: The effect of in vivo T helper and T suppressor lymphocyte depletion on wound healing. Ann Surg 1989;209:479–483.
43. Tadros T, Wobbes T, Hendriks T: Opposite effects of interleukin-2 on normal and transfusion-suppressed healing of experimental intestinal anastomoses. Ann Surg 1993;218:800–808.
44. Kaplan RN, Rafii S, Lyden D: Preparing the "soil": the premetastatic niche. Cancer Res 2006;66:11089–11093.
45. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S: Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–1201.
46. Rafii S, Lyden D: S100 chemokines mediate bookmarking of premetastatic niches. Nat Cell Biol 2006;8:1321–1323.
47. Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z, Bergers G: HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 2008;13:206–220.
48. Katoh H, Hosono K, Ito Y, Suzuki T, Ogawa Y, Kubo H, Kamata H, Mishima T, Tamaki H, Sakagami H, Sugimoto Y, Narumiya S, Watanabe M, Majima M: COX-2 and prostaglandin EP3/EP4 signaling regulate the tumor stromal proangiogenic microenvironment via CXCL12-CXCR4 chemokine systems. Am J Pathol 2010;176:1469–1483.
49. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC: Expansion of myeloid immune suppressor Gr+CD11b+cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 2004;6:409–421.
50. Li MO, Flavell RA: TGF-beta: a master of all T cell trades. Cell 2008;134:392–404.
51. Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC, Moses HL: Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+myeloid cells that promote metastasis. Cancer Cell 2008;13:23–35.

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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, ≥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 ($\geq$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.

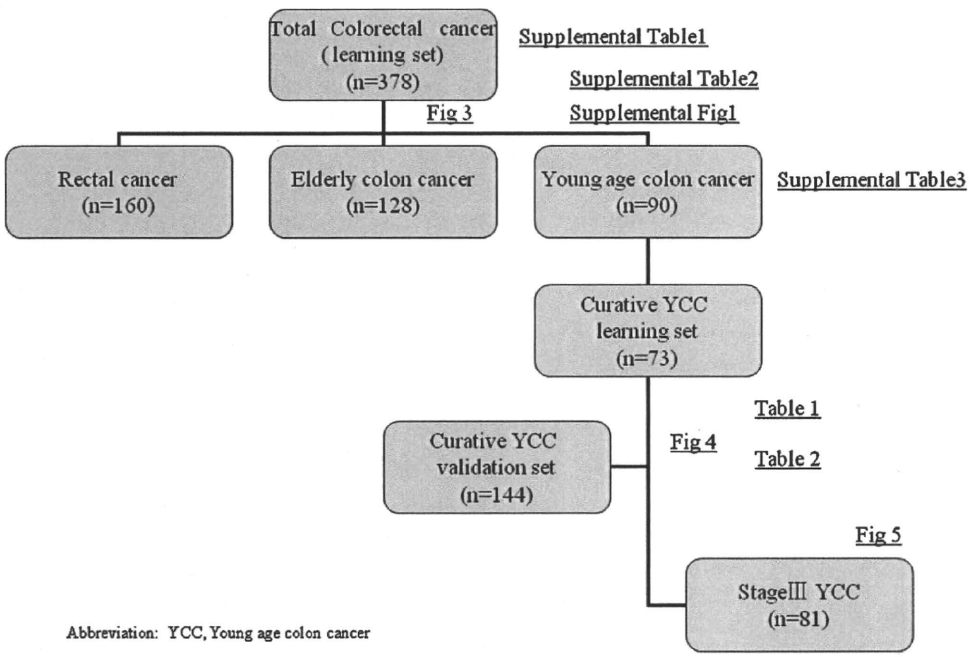

Fig. 1. Flow chart of our analytical process. [Color figure can be viewed in the online issue, available at wileyonlinelibrary.com.]

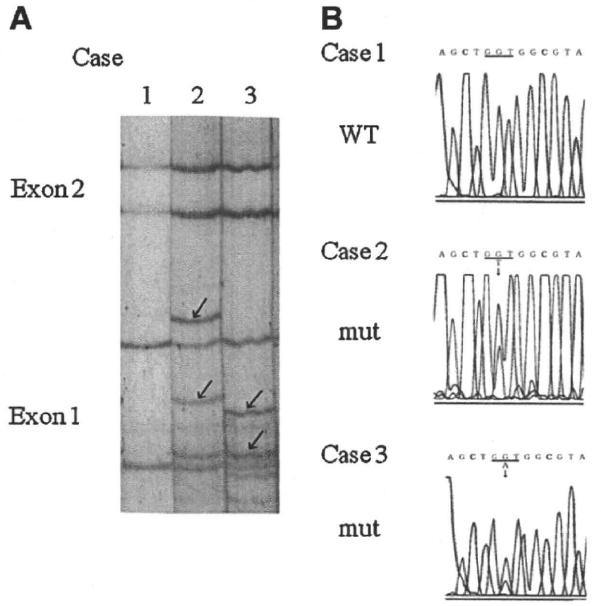

Fig. 2. Detection of K-*ras* mutation in colorectal cancer (CRC) tissues. **A**: Non-RI-SSCP analysis of amplified products of exons 1 and 2 of the K-*ras* gene in CRC. Lane 1, wild-type case; Lane 2, mutant case: Lane 3, mutant case. Arrows indicate mutant alleles. **B**: Direct sequencing of the corresponding 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.

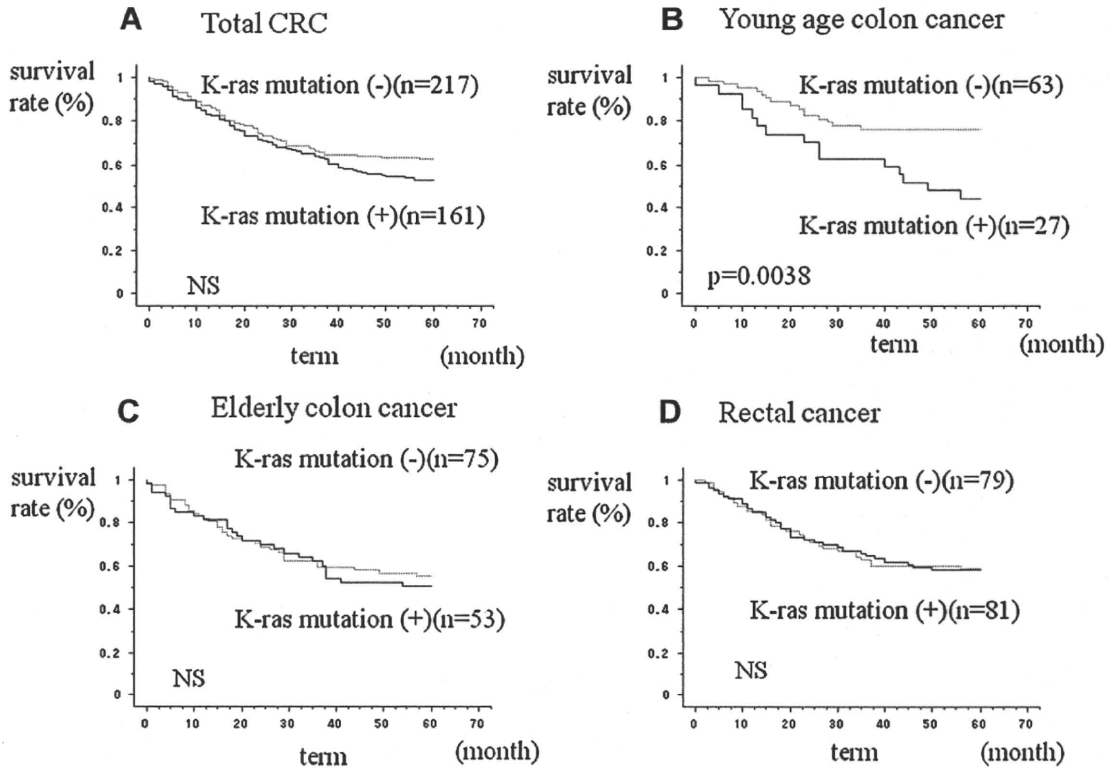

Fig. 3. K-*ras* mutation and prognosis in CRC. **A**: No significant difference in survival between the presence and absence of K-*ras* mutation in 378 CRC cases. **B**: Survival comparison according to K-*ras* mutations revealed a significant difference in young colon cancer patients (YCC; $P = 0.0038$).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

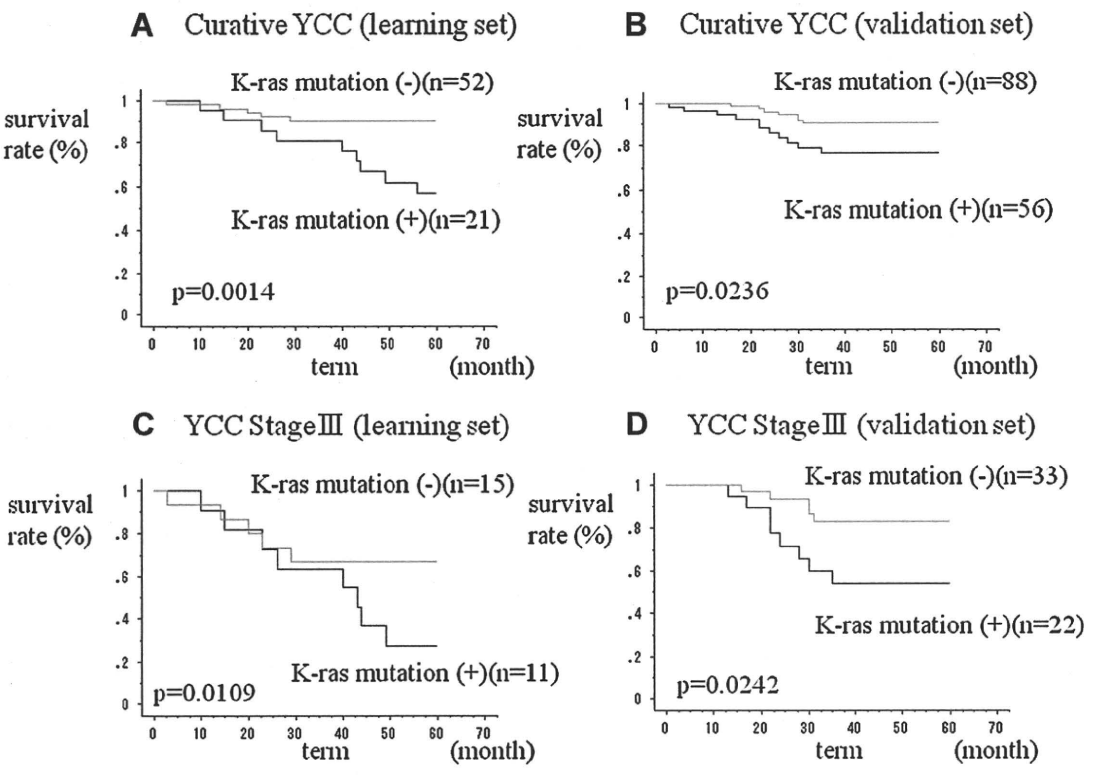

Fig. 4. K-*ras* mutation and prognosis in young colon cancer (YCC). **A**: Significant difference in survival between presence and absence of K-*ras* mutation in 73 curative YCC (learning set; $P = 0.0014$). **B**: Significant difference in survival according to K-*ras* mutation in curative YCC (validation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

		Univariate analysis		Multivariate analysis		
Variables		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

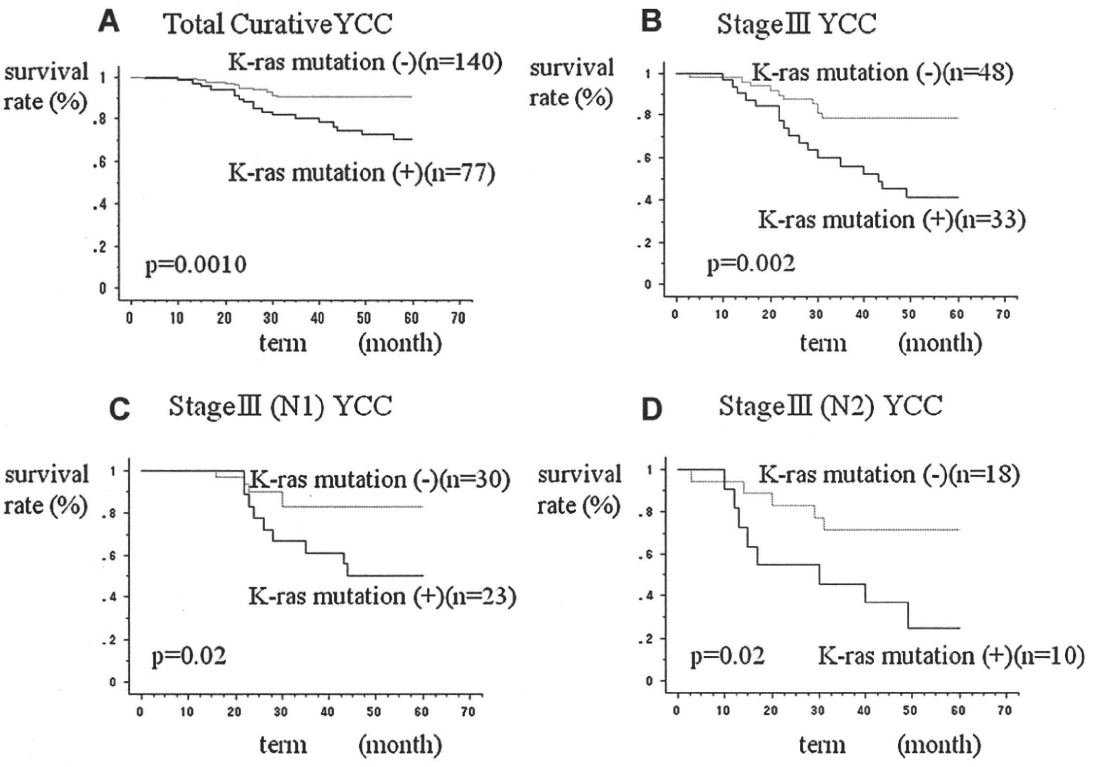

Fig. 5. Prognostic significance of K-*ras* mutation in stage III YCC in curable cases. **A**: Validation of significant difference in survival comparison between presence and absence of K-ras mutation in 217 YCC cases ($P = 0.0010$). **B**: Significant difference in survival according to K-ras mutation 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.

REFERENCES

1. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al.: Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 2005;353:1659–1672.
2. Romond EH, Perez EA, Bryant J, et al.: Trastuzumab plus adjuvant chemotherapy for operable HER-2 positive breast cancer. N Engl J Med 2005;353:1673–1684.
3. Denetru GD, von Mehren M, Blanke CD, et al.: Efficiency and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 2002;347:472–480.
4. Heinrich MC, Corless CL, Demetri GD, et al.: Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol 2003;21:4342–4349.
5. Paez JG, Janne PS, Lee JC, et al.: EGFR mutations in lung cancer: Correlation with clinical response to gefinitib therapy. Science 2004;304:1497–1500.
6. Lynch TJ, Bell DW, Sordella R, et al.: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 2004; 350:2129–2139.
7. Slamon DJ, Godolphin W, Jones LA, et al.: Studies of the HER-2/ neu proto-oncogene in human breast and ovarian cancer. Science 1989;244:707–712.
8. Pterson MC, Dietrich KD, Danyluk J, et al.: Correlation between c-erb-B2 amplification and risk of recurrent disease in node-negative breast cancer. Cancer Res 1991;51:556–567.
9. Taniguchi M, Nishida T, Hirota S, et al.: Effect of c-kit mutation on prognosis of gastrointestinal stromal tumors. Cancer Res 1999;59:4297–4300.
10. Singer S, Rubin BP, Lux ML, et al.: Prognostic value of KIT mutation type, mitotic activity, and histologic subtype in gastrointestinal stromal tumors. J Clin Oncol 2002;20:3898–3905.
11. Schubbert S, Shannou K, G B: Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007;7:295–308.
12. Shirasawa S, Furuse M, Yokoyama N, et al.: Altered growth of human colon cancer cell lines disrupted at activated K-ras. Science 1993;260:85–88.
13. Okada F, Rak JW, Croix BS: Impact of oncogenes in tumor angiogenesis: Mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cell. Proc Natl Acad Sci USA 1998;95:3609–3614.
14. Vogelstein B, Fearon ER, Hamilton SR, et al.: Genetic alterations during colorectal-tumor development. N Engl J Med 1988;2:532–535.
15. Benhattar J, Losi L, Chaubert P, et al.: Prognostic significance of K-ras mutations in colorectal carcinoma. Gastroenterology 1993;104:1044–1048.
16. Tanaka M, Omura K, Watanabe Y, et al.: Prognostic factors of colorectal cancer: K-ras mutation, overexpression of the p53 protein, and cell proliferative activity. J Surg Oncol 1994;57:57–64.
17. Span M, Moerkerk PT, De Goeij AF, et al.: A detailed analysis of K-ras point mutations in relation to tumor progression and survival in colorectal cancer patients. Int J Cancer 1996;69:241–245.
18. Cerottini JP, Caplin S, Saraga E, et al.: The type of K-ras mutation determines prognosis in colorectal cancer. Am J Surg 1998; 175:198–202.
19. Pajkos G, Kiss I, Sandor J, et al.: The prognostic value of the presence of mutations at the codon 12, 13, 61 of K-ras oncogene in colorectal cancer. Anticancer Res 2000;20:1695–1701.
20. Andersen SN, Lovig T, Breivik J, et al.: K-ras mutations and prognosis in large-bowel carcinomas. Scand J Gastroenterol 1997; 32:62–69.
21. Hardingham JE, Butler WJ, Roder D, et al.: Somatic mutations, acetylator status, and prognosis in colorectal cancer. Gut 1998; 42:669–672.
22. Tortola S, Marcuello E, Gonzalez I, et al.: p53 and K-ras gene mutations correlate with tumor aggressiveness but are not of routine prognostic value in colorectal cancer. J Clin Oncol 1999; 17:1375–1381.
23. Bouzourence H, Gervaz P, Cerottini JP, et al.: p53 and K-ras as prognostic factors for Dukes' stage B colorectal cancer. Eur J Cancer 2000;36:1008–1015.
24. Esteller M, Gonzalez S, Risques RA, et al.: K-ras and p16 aberrations confer poor prognosis in human colorectal cancer. J Clin Oncol 2001;19:299–304.
25. Pan ZZ, Wan DS, Chen G, et al.: Co-mutation of p53, K-ras genes and accumulation of p53 protein and its correlation to clinicopathological features in rectal cancer. World J Gastroenterol 2004; 10:3688–3690.
26. Hsieh JS, Lin SR, Chang MY, et al.: APC, K-ras, and p53 gene mutations in colorectal cancer patients: correlation to clinicopathologic features and postoperative surveillance. Am Surg 2005; 71:336–343.
27. Locker GY, Hammilton S, Harris J, et al.: ASCO 2006 update of recommendation for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006;24:5313–5327.
28. Allegra CJ, Jessup JM, Somerfield MR, et al.: American Society of Clinical Oncology provisional clinical opinion: Testing for K-RAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009;27: 2091–2096.
29. Yamashita K, Tatebayashi T, Shinoda H: Simplified rapid non-radioactive PCR-SSCP method applied to K-ras mutation analysis. Pathol Int 1996;46:801–804.
30. Yamashita K, Kuba T, Shinoda H, et al.: Detection of K-ras point mutations in the supernatants of peritoneal and pleural effusions for diagnosis complementary to cytologic examination. Am J Clin Pathol 1998;109:704–711.
31. Yamashita K, Kida Y, Shinoda H, et al.: K-ras point mutations in the supernatants of pancreatic juice and bile are reliable for diagnosis of pancreas and biliary tract carcinomas complementary to cytologic examination. Jpn J Cancer Res 1999;90:240–248.
32. Yamashita K, Yoshida T, Shinoda H: Novel method for simultaneous analysis of p53 and k-ras mutations and p53 protein expression in single histologic sections. Arch Pathol Lab Med 2001;125:347–352.
33. Yasui W, Yokozaki H, Shimamoto F, et al.: Molecular–pathological diagnosis of gastrointestinal tissues and its contribution to cancer histopathology. Pathol Int 1999;49:763–774.

34. Araujo SE, Bernardo WM, Habr-Gama A: DNA ploidy status and prognosis in colorectal cancer: A meta-analysis of published data. Dis Colon Rectum 2007;50:1800–1810.
35. Zhou W, Goodman SN, Galizia G: Counting alleles to predict recurrence of early-stage colorectal cancers. Lancet 2002;359: 219–225.
36. Ribic CM, Sargent DJ, Moore MJ: Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy. N Engl J Med 2003;349: 247–257.
37. Andre T, Boni C, Mounedji-Boudiaf L: Oxaliplatin, fluorouracil, and leucovorin as adjuvant treatment for colon cancer. N Engl J Med 2004;350:2343–2351.
38. Kuebler JP, Wieand HS, O'Connell MJ: Oxaliplatin combined with weekly bolus fluorouracil and leucovorin as surgical adjuvant chemotherapy for stage 2 and 3 colon cancer: Results from NSABP C-07. J Clin Oncol 2007;25:2198–2204.
39. Andreyev HJ, Norman AR, Cunningham D: Kirsten ras mutations in patients with colorectal cancer: The multicenter "RASCAL" study. J Natl Cancer Inst 1998;90:675–684.
40. Andreyev HJNA, Cunningham D, Oates J, et al.: Kirsten ras mutations in patients with colorectal cancer: The 'RASCAL2' study. Br J Cancer 2001;85:692–696.
41. Goel A, Nagasaka T, Arnold CN, et al.: The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology 2007; 32:127–138.
42. Young J, Simms LA, Biden KG, et al.: Features of colorectal canecrs with high-level microsatellite instability occurring in familial and sporadic settings: Parallel pathways of tumorigenesis. Am J Pathol 2001;159:2107–2116.
43. Liu B, Farrington SM, Petersen GM, et al.: Genetic instability occurs in the majority of young patients with colorectal cancer. Nat Med 1995;1:348–352.
44. Whitehall VL, Walsh MD, Young J, et al.: Methylation of O6-methylguanine DNA methyltransferase characterizes a subset of colorectal cancer with low-level DNA microsattelite instability. Cancer Res 2001;61:827–830.
45. Halling KC, French AJ, McDonnell SK: Microsatellite instability and 8p allelic imbalance in stage B2 and C colorectal cancer. J Natl Cancer Inst 1999;91:1295–1303.
46. Lengauer C, Kinzler KW, B V: Genetic instability in colorectal cancers. Nature 1997;386:623–627.
47. Samowitz WS, Sweeney C, Herrick J: Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancer. Cancer Res 2005;65:6063–6069.
48. Barber TD, Vogelstein B, Kinzler KW, et al.: Somatic mutations of EGFR in colorectal cancers and glioblastomas. N Engl J Med 2004;351:2883.
49. Samuels Y, Wang Z, Bardelli A, et al.: High frequency of mutations of the PIK3CA gene in human cancers. Science 2004; 304:554.
50. Rajagopalan H, Bardelli A, Lengauer C, et al.: Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature 2002;418:934.
51. Benvenuti S, Sartore-Bianchi A, Di Nicolantonio F, et al.: Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. Cancer Res 2007;67: 2643–2648.
52. Khambata-Ford SGC, Meropol NJ, Basik M, et al.: Expression of epiregulin and amphiregulin and K-ras mutation status predict disease control in matastatic colorectal cancer patients treated with cetuximab. J Clin Oncol 2007;25:3230–3237.
53. Helena L, Issa JD, Dimitra K, et al.: Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: A systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. Lancet oncol 2008;9:962–972.
54. Zlobec I, Bihl MP, Schwarb H, et al.: Clinico-pathological and protein characterization of BRAF and K-RAS mutated colorectal cancer and implications for prognosis. Int J Cancer 2009; 11.
55. Sato T, Kokuba Y, Koizumi W, et al.: Phase I trial of neoadjuvant preoperative chemotherapy with S-1 and irinotecan plus radiation in patients with locally advanced rectal cancer. Int J Radiat Oncol Biol Phys 2007;69:1442–1447.
56. Sato T, Kokuba Y, Koizumi W, et al.: Irinotecan and S-1 neoadjuvant chemoradiation therapy in patients with advanced rectal cancer. Hepatogastroenterology 2007;54:1391–1393.
57. Winder T, Mundlein A, Rhomberg S, et al.: Different type of K-Ras mutations are conversely associated with overall survival in patients with colorectal cancer. Oncol Rep 2009;21:1283–1287.

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ORIGINAL PAPER

Clinical outcomes of advanced non-small cell lung cancer patients screened for epidermal growth factor receptor gene mutations

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Abstract
Purpose To evaluate the relationship between the epidermal growth factor receptor (EGFR) mutation status and the effectiveness of gefitinib monotherapy or chemotherapy in patients with advanced non-small cell lung cancer (NSCLC).
Methods We retrospectively analyzed a cohort of 100 patients with stage IIIB/IV NSCLC screened for two major EGFR mutations (exon 19 deletions and L858R mutation).
Results Forty-six out of 48 EGFR mutation-positive patients (96%) received gefitinib, whereas only 3 out of 52 EGFR mutation-negative patients (6%) received gefitinib. Favorable objective response rates to gefitinib as first- and second-line treatment (87 and 80%, respectively) were observed in EGFR mutation-positive patients. Overall response rate to chemotherapy as first-line treatment did not differ significantly between patients with EGFR mutations and those without mutation (32 vs. 28%, respectively; $P = 0.7198$). As to first-line treatment, EGFR mutation-positive patients treated with gefitinib experienced significantly longer progression-free survival (PFS) than did patients who received chemotherapy (median survival, 7.8 months vs. 5.1 months, respectively; $P = 0.0323$). Similarly, as to second-line treatment, EGFR mutation-positive patients treated with gefitinib had significantly longer PFS than did patients who received chemotherapy (median survival, 6.5 months vs. 4.0 months, respectively; $P = 0.0048$). Patients with EGFR mutations survived longer than those without EGFR mutations after first-line treatment (median, 24.3 vs. 12.6 months, respectively; $P = 0.0029$).
Conclusion EGFR mutation-positive patients benefit from either first- or second-line gefitinib monotherapy. Further large-scale prospective studies to confirm this finding are needed.

Keywords Epidermal growth factor receptor · Mutation screening · Gefitinib · Non-small cell lung cancer · Cytotoxic chemotherapy · Clinical outcomes

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Introduction

Gefitinib, an orally bioavailable, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), was the first targeted drug for non-small cell lung cancer (NSCLC). Phase II trials of gefitinib monotherapy in unselected NSCLC patients showed antitumor activity, but demonstrated objective response rates of only 8–18% (Fukuoka et al. 2003; Kris et al. 2003). However, subset analyses of these trials and a retrospective study showed that favorable response to gefitinib was observed in certain

patient subgroups, such as females, patients with adenocarcinoma, Asian patients, and nonsmokers (Fukuoka et al. 2003; Kris et al. 2003; Miller et al. 2004). These results suggest that identifying predictive molecular or genetic biomarkers for gefitinib sensitivity may help to select patients who are most likely to benefit from treatment.

In 2004, three independent groups of investigators reported that somatic EGFR mutations correlate with sensitivity of NSCLC to the EGFR TKIs, gefitinib or erlotinib (Lynch et al. 2004; Paez et al. 2004; Pao et al. 2004). Subsequently, multiple groups of researchers confirmed and extended this striking correlation between EGFR mutations and gefitinib sensitivity, reporting response rates ranging from approximately 60 to 94% in retrospective analyses (Cortes-Funes et al. 2005; Han et al. 2005; Huang et al. 2004; Kim et al. 2005; Mitsudomi et al. 2005; Takano et al. 2005; Taron et al. 2005; Tokumo et al. 2005). Recently, several prospective phase II studies also confirmed the correlation (Asahina et al. 2006; Inoue et al. 2006; Sequist et al. 2008; Sugio et al. 2009; Sunaga et al. 2007; Sutani et al. 2006; Tamura et al. 2008; Yang et al. 2008; Yoshida et al. 2007), and a combined analysis from seven phase II trials in Japan (I-CAMP; Iressa Combined Analysis of Mutation Positives) demonstrated a total response rate of 76.4% (Morita et al. 2009).

To date, many types of mutations in NSCLC patients have been reported, but only four types of TKI-sensitive mutations, including exon 18 and 21 point mutation (G719A/C, L858R and L861Q) and exon 19 in-frame deletion, have been elucidated (Greulich et al. 2005). Of these mutations, the two most common, representing approximately 90% of all EGFR mutations, are the exon 19 deletions and L858R point mutation (Uramoto and Mitsudomi 2007). In a previous study on prospective validation for prediction of gefitinib sensitivity by these two common hot spots for EGFR mutations, we reported a promising overall response rate of 90.5% (Yoshida et al. 2007). Therefore, in order to select patients who might benefit from gefitinib treatment, we continued to screen patients for the two hot spot mutations.

To clarify the relationship between EGFR mutation status and the effectiveness of gefitinib monotherapy or cytotoxic chemotherapy in patients with NSCLC, we performed a retrospective analysis of clinical outcomes of consecutive patients who were screened for two major EGFR mutations.

Patients and methods

Patients

A cohort of 100 patients with inoperable stage IIIB/IV NSCLC were screened for EGFR mutations prior to selection for gefitinib treatment or cytotoxic chemotherapy at Aichi Cancer Center Hospital in Nagoya, Japan, between November 2004 and December 2006. Eligibility criteria were adults (defined as $\geq$20 years of age) with cytological or histological confirmation of locally advanced (stage IIIB for which thoracic irradiation was not indicated) or metastatic (stage IV) NSCLC who underwent prospective screening of EGFR mutations; $\geq$1 measurable or assessable lesion, according to the Response Evaluation Criteria in Solid Tumors (Therasse et al. 2000); and written informed consent, in accordance with institutional regulations. Eligible patients were admitted to the study regardless of prior chemotherapy, performance status (PS), or functions of main organs. Exclusion criteria were pulmonary fibrosis, interstitial pneumonia, or prior treatment with an EGFR TKI or antibody. This study was approved by the institutional review board of Aichi Cancer Center Hospital.

EGFR mutation analysis

Mutational analysis of the exon 19 deletion and the L858R mutation in the EGFR gene was performed as described previously (Yatabe et al. 2006). Briefly genomic DNA was extracted from tumors embedded in paraffin blocks or from aspirated tumors obtained from pleural effusions, superficial lymph nodes, or subcutaneous metastases. One reference pathologist (Y.Y.) reviewed all specimens and marked grossly near the tumor-rich lesion on an unstained slide in order to enrich the tumor cell population as much as possible. The exon 19 deletion mutation was determined by common fragment analysis using polymerase chain reaction (PCR) with an FAM-labeled primer set; the PCR products were subjected to electrophoresis on an ABI PRISM 310 instrument (Applied Biosystems, Foster City, CA, USA). The shorter segment of DNA amplified by PCR showed a deletion mutation in a new peak in the electropherogram. The L858R mutation was detected by the Cycleave real-time quantitative PCR technique, using the Cycleave PCR core kit (Takara Co. Ltd., Ohtsu, Japan) with an L858R-specific cycling probe and a probe specific for the wild-type gene. Fluorescence intensity was measured with a Smart Cycler system (SC-100, Cepheid, Sunnyvale, CA, USA).

Statistical analysis

Data were analysed using the chi-square test; $P < 0.05$ was regarded as statistically significant. Confidence intervals (CIs) were calculated using binomial values. Progression-free survival (PFS) and overall survival (OS) were calculated using the Kaplan–Meier method; survival differences were analysed by log-rank test. All analyses were performed with Stat View version 5 software (SAS institute Inc, Cary, NC, USA) on a Macintosh computer.

Results

Patients characteristics

From November 2004 through December 2006, 100 consecutive patients with NSCLC at Aichi Cancer Center hospital were examined to detect EGFR mutations. Patient characteristics are shown in Table 1. All patients were Japanese. EGFR mutations were detected in 48% (48/100) of the patients. Of the patients with EGFR mutations, 23 had the exon 19 deletions, and 25 had the L858R mutation. EGFR mutations were detected more frequently in women and patients who never smoked, whereas fewer EGFR mutations were detected in stage IIIB patients.

Figure 1 depicts the treatment of EGFR mutation-positive patients. Of the 48 EGFR mutation-positive patients, 96% (46/48) received gefitinib monotherapy; 47.9% (23/48), 31.3% (15/48), and 25% (12/48) of the EGFR mutation-positive patients received gefitinib as first-, second- and third-line treatment, respectively. Of the 12 patients with EGFR mutation who were treated with gefitinib as third-line treatment, two patients received gefitinib monotherapy as first-line, two patients received gefitinib monotherapy as second-line, and eight patients received cytotoxic chemotherapy as both first- and second-line.

Only 6% (3/52) of the 52 patients without EGFR mutations received gefitinib monotherapy as first- (two patients) or second-line (one patient) treatment, whereas 96.2% (50/52) of the patients without EGFR mutations received cytotoxic chemotherapy as first-line treatment.

In this study, all patients received first-line treatment, 65% (65/100) of the patients received second-line treatment and median follow-up time for the survivors was 20.2 months (ranging from 9.5 months to 74.6 months).

Table 1 Patient characteristics according to EGFR mutation status

	EGFR mutation status		*P*
	Mutation	Wild-type	
All cases	48	52	
Sex			<0.0001
Male	15	38	
Female	33	14	
Age, years			0.4942
≤60	18	23	
>60	30	29	
Histology			0.1985
Adenocarcinoma	47	48	
Non-adenocarcinoma	1	4	
Smoking status			<0.0001
Never smoker	32	11	
Smoker	16	41	
Stage at initial diagnosis			0.0341
IIIB	7	17	
IV	41	35	
ECOG PS at initial diagnosis			0.169
0/1	42	40	*P* (0/1 vs. ≥2)
2	2	7	
3	3	3	
4	1	2	
Timing of mutation screening			0.4803
Pre-treatment	31	30	
After first-line treatment	11	16	
After second-line treatment	6	5	
After third-line treatment	0	1	
Mutation genotype			
Exon 19 deletion	23	–	
L858R	25	–	

EGFR epidermal growth factor receptor, *PS* performance status

EGFR mutations and response to gefitinib

Objective response rate (complete response rate + partial response rate) to first-line gefitinib therapy was 87% in patients with EGFR mutations. Disease control rate (complete response rate + partial response rate + stable disease rate) in response to first-line gefitinib therapy was 87% in patients with EGFR mutations. Objective response rate for second-line gefitinib therapy was 80% in patients with EGFR mutations. Disease control rate in response to second-line gefitinib therapy was 86.7% in patients with EGFR mutations (Table 2). No objective responses were observed in patients with wild-type EGFR treated with first- or second-line gefitinib.

No statistically significant differences in rates of objective response and disease control between first- and second-line gefitinib treatments were observed. Furthermore, response rate to gefitinib monotherapy in patients with exon 19 deletions, compared with that in patients with the L858R mutation, did not differ significantly in either first- or second-line treatment (data not shown).

EGFR mutations and response to cytotoxic chemotherapy

Objective response to first- and second-line cytotoxic chemotherapy was not influenced by EGFR mutation status (Table 3). Objective response rate to first-line cytotoxic chemotherapy was 32% in patients with EGFR mutations and 28% in patients with wild-type EGFR ($P = 0.7198$).

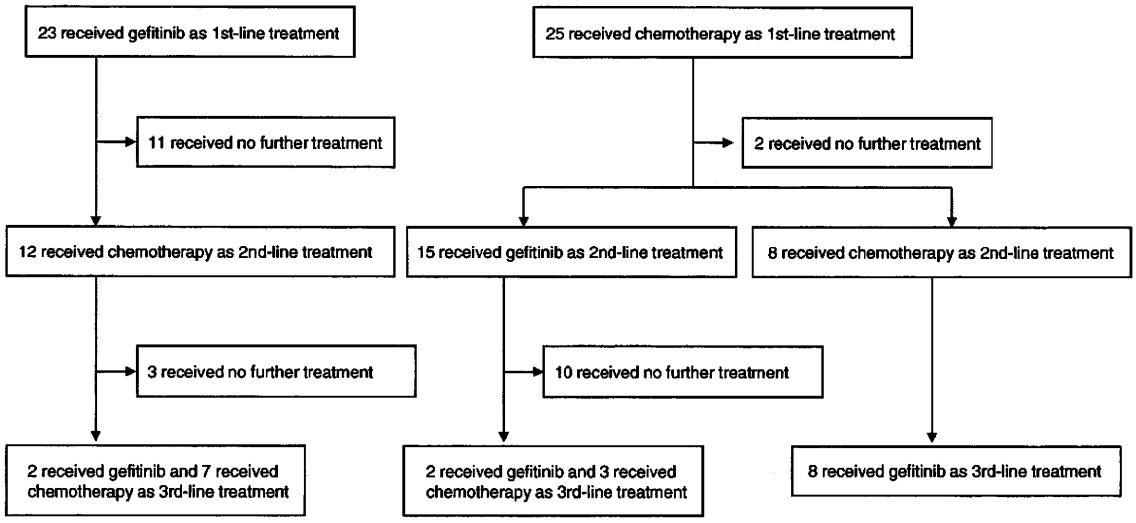

Fig. 1 Treatment flow chart for 48 EGFR mutation-positive patients

Table 2 Response to gefitinib monotherapy in EGFR mutation-positive patients (%)

	First-line ($n = 23$)	Second-line ($n = 15$)
CR	1 (4.3)	1 (6.7)
PR	19 (82.6)	11 (73.3)
SD	0 (0)	1 (6.7)
PD	3 (13.0)	2 (13.3)
OR	20 (87.0)	12 (80.0)
DC	20 (87.0)	13 (86.7)

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *OR* objective response (CR + PR), *DC* disease control (CR + PR + SD)

Objective response rate to second-line cytotoxic chemotherapy was 20% in patients with EGFR mutations and 6.9% in patients with EGFR wild-type ($P = 0.1690$).

EGFR mutation status significantly affected the disease control rate to first-line cytotoxic chemotherapy, but not to second-line cytotoxic chemotherapy. The disease control rate to first-line cytotoxic chemotherapy was 88% in patients with EGFR mutations and 60% in patients with wild-type EGFR ($P = 0.0132$). The disease control rate to second-line cytotoxic chemotherapy was 60% in patients with EGFR mutations and 48.3% in patients with EGFR wild-type ($P = 0.4190$).

PFS in EGFR mutation-positive patients

As illustrated by the Kaplan–Meier curves in Fig. 2a and b, EGFR mutation-positive patients treated with gefitinib monotherapy as first-line treatment experienced significantly longer PFS than did patients who received first-line cytotoxic chemotherapy (median survival, 7.8 months vs. 5.1 months, respectively; $P = 0.0323$). Similarly, EGFR mutation-positive patients treated with gefitinib monotherapy as second-line treatment had significantly longer PFS than did patients who received cytotoxic chemotherapy as second-line treatment (median survival, 6.5 months vs. 4.0 months, respectively; $P = 0.0048$). All 15 patients who received gefitinib monotherapy as second-line treatment had previously received cytotoxic chemotherapy as first-line treatment.

Of the 20 patients who received cytotoxic chemotherapy as second-line treatment, 12 of the patients had received gefitinib as first-line treatment and 8 of the patients had received cytotoxic chemotherapy as first-line treatment previously; no statistically significant difference in PFS was observed between these two groups that had been treated with gefitinib monotherapy as first-line vs. cytotoxic chemotherapy as first-line (data not shown).

In patients treated with gefitinib as first- or second-line treatment, no statistically significant difference in PFS was observed in patients with exon 19 deletions, as compared with patients with the L858R mutation (data not shown).

PFS after cytotoxic chemotherapy, according to EGFR mutation status

In patients treated with cytotoxic chemotherapy as first-line treatment, no significant difference in PFS was observed in patients with EGFR mutations vs. patients who were EGFR wild-type (median survival, 5.1 months vs. 4.4 months, respectively; $P = 0.7184$) (Fig. 3a). Similarly, in patients treated with cytotoxic chemotherapy as second-line treatment, no significant difference in PFS was observed in

Table 3 Response to chemotherapy according to EGFR mutation status (%)

	Chemotherapy as first-line treatment			Chemotherapy as second-line treatment		
	Mutation ($n = 25$)	Wild-type ($n = 50$)	P	Mutation ($n = 20$)	Wild-type ($n = 29$)	P
Type of chemotherapy regimen						
Platinum plus newer agents[a]	22 (88.0)	41 (82.0)		17 (85.0)	18 (62.1)	
Single newer agent	3 (12.0)	9 (8.0)		3 (15.0)	11 (37.9)	
Response						
CR	0 (0)	0 (0)		0 (0)	0 (0)	
PR	8 (32.0)	14 (28.0)		4 (20.0)	2 (6.9)	
SD	14 (56.0)	16 (32.0)		8 (40.0)	12 (41.4)	
PD	3 (12.0)	17 (34.0)		6 (30.0)	14 (48.3)	
NE	0 (0)	3 (6)		2 (10.0)	1 (3.4)	
OR	8 (32.0)	14 (28.0)	0.7198	4 (20.0)	2 (6.9)	0.1690
DC	22 (88.0)	30 (60.0)	0.0132	12 (60.0)	14 (48.3)	0.4190

CR complete response, *PR* partial response, *SD* stable disease, *PD* progressive disease, *OR* objective response (CR + PR), *DC* disease control (CR + PR + SD

[a] Newer agents were consisted of paclitaxel, docetaxel, vinorelbine, gemcitabine, irrinotecan, amurubicin, and TS-1

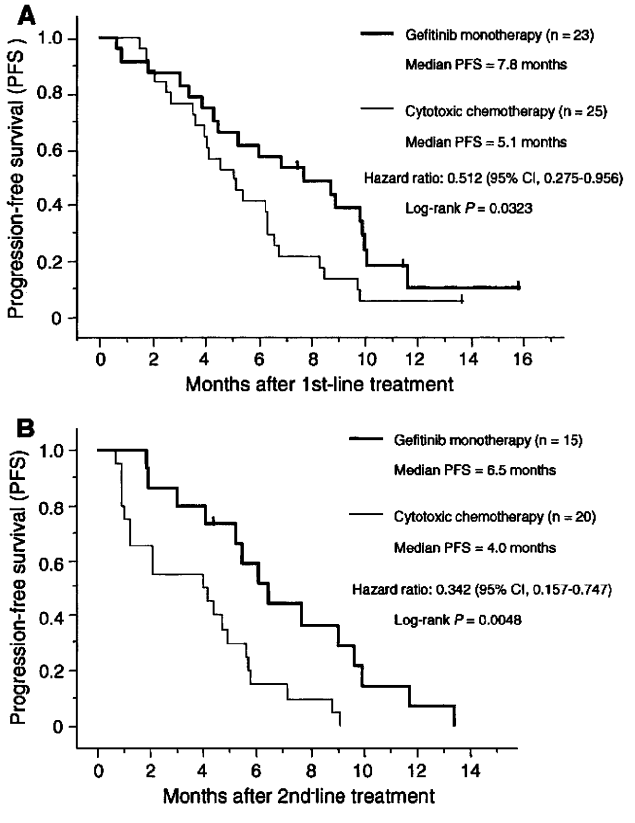

Fig. 2 **a** Kaplan–Meier estimates of progression-free survival of patients with EGFR mutations treated with first-line gefitinib or cytotoxic chemotherapy. **b** Kaplan–Meier estimates of progression-free survival of patients with EGFR mutations treated with second-line gefitinib or cytotoxic chemotherapy

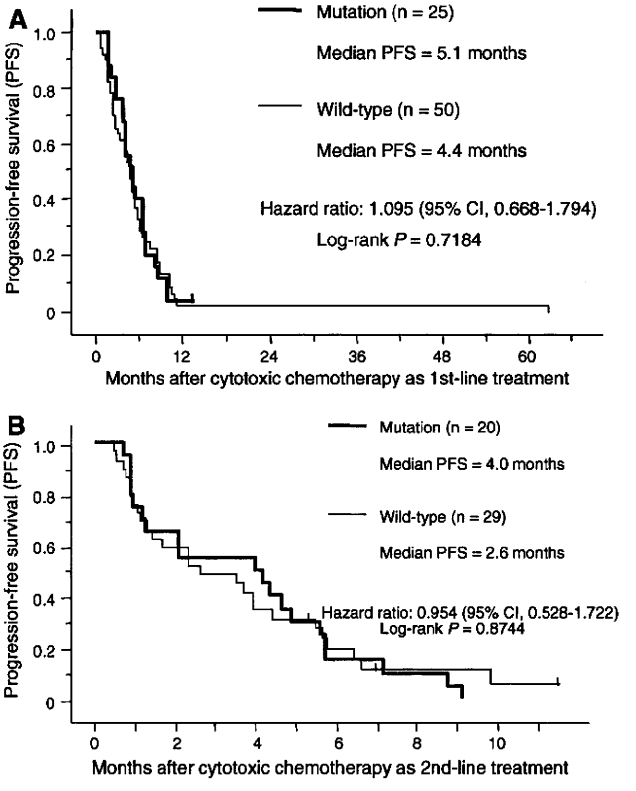

Fig. 3 Kaplan–Meier estimates of progression-free survival of patients grouped by EGFR mutation status who were treated with either first-line (**a**) or second-line (**b**) cytotoxic chemotherapy

patients with EGFR mutations vs. patients with EGFR wild-type (median survival, 4.0 months vs. 2.6 months, respectively; $P = 0.8744$) (Fig. 3b).

Overall survival and multivariate analysis

Patients with EGFR mutations survived for a significantly longer time, as calculated from the initial day of first-line treatment, than did patients who were EGFR wild-type

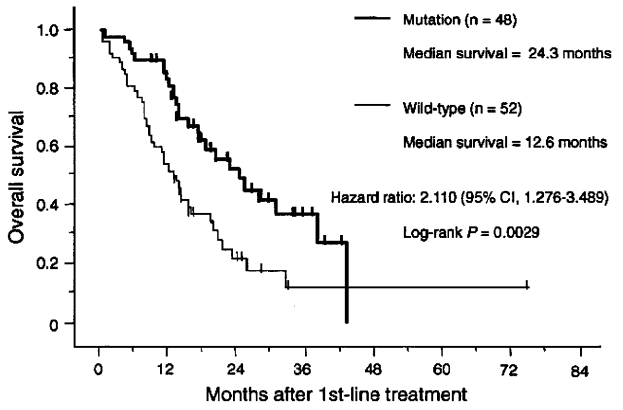

Fig. 4 Kaplan–Meier estimates of overall survival for patients, according to EGFR mutation status

Table 4 Multivariate analysis for overall survival after first-line treatment

Variables	Hazard ratio	95% CI	*P*
EGFR mutation (yes/no)	1.928	1.048–3.545	0.0347
Stage (IIIB/IV)	0.663	0.337–1.306	0.2348
Age (>60/≤60)	1.250	0.741–2.107	0.4028
Gender (male/female)	1.093	0.482–2.481	0.8312
Smoking history (yes/no)	1.268	0.551–2.916	0.5769
Performance status (0–1/2–4)	0.148	0.078–0.282	<0.0001

(median survival, 24.3 months vs. 12.6 months, respectively; $P = 0.0029$; Fig. 4).

Multivariate analysis revealed that EGFR mutations and PS significantly and independently affected overall survival (Table 4).

Discussion

Various cytotoxic chemotherapy agents are utilized in the treatment of advanced or metastatic NSCLC. In the first-line setting, combination chemotherapy such as platinum-based regimens are given empirically to most stage IIIB or IV NSCLC patients, resulting in objective response rates of 30–40%, median survival times of 8–10 months, and 1-year survival rates of 30–40% (Kelly et al. 2001; Schiller et al. 2002). Recently, novel, small molecule therapeutic agents that specifically target certain molecular pathways, including the EGFR TKIs, gefitinib and erlotinib, have been developed. A new approach for selecting patients by the presence of molecular or genetic biomarkers, such as EGFR mutations and gene copy number, is evolving (Cappuzzo et al. 2005, 2007; Han et al. 2006).

Cappuzzo et al. demonstrated that, in NSCLC patients treated with gefitinib, a high gene copy number, rather than EGFR mutations, was a better predictor of survival (Cappuzzo et al. 2005). Furthermore, molecular analyses from large placebo-controlled phase III trials of TKIs also showed that EGFR gene copy number was superior to mutations as a predictor of clinical benefit (Hirsch et al. 2006; Tsao et al. 2005). These studies included mostly Caucasian patients with NSCLC. On the other hand, studies in Japan and Korea demonstrated that EGFR mutation was the most important biomarker to identify NSCLC patients for treatment with gefitinib (Han et al. 2006; Ichihara et al. 2007; Sone et al. 2007; Takano et al. 2005).

In the INTACT and TRIBUTE studies, which were conducted to compare TKIs (gefitinib in the INTACT trial, and erlotinib in the TRIBUTE trial) with placebo in combination with cytotoxic chemotherapy, patients with EGFR mutations exhibited better PFS after cytotoxic chemotherapy than did patients without mutations (Bell et al. 2005; Eberhard et al. 2005). Similarly, Hotta et al. reported that EGFR mutation-positive patients treated with first-line cytotoxic chemotherapy yielded better PFS than did EGFR mutation-negative patients, and furthermore, no significant difference in PFS in patients (with and without mutations) who were treated with cytotoxic chemotherapy following gefitinib monotherapy. Therefore, they suggested that early use of cytotoxic chemotherapy prior to gefitinib treatment was advantageous for EGFR mutation-positive patients (Hotta et al. 2007).

This study assessed whether EGFR mutation-positive status of NSCLC patients influenced clinical outcome of first- and second-line treatment with cytotoxic chemotherapy or gefitinib monotherapy. In contrast to the findings of Hotta et al. (2007), we observed that PFS following first- and second-line cytotoxic chemotherapy was not associated with EGFR mutation status (Fig. 3a, b). Moreover, in our study, EGFR mutation-positive patients treated with first- or second-line gefitinib exhibited better PFS than did patients treated with first- or second-line cytotoxic chemotherapy (Fig. 2a, b). Thus, our findings suggest that patients with EGFR mutations might benefit from either first- or second-line gefitinib monotherapy. The reason for different clinical outcomes in our study and previous studies by other investigators (Bell et al. 2005; Eberhard et al. 2005; Hotta et al. 2007) is unclear. However, possible explanations include differences in ethnicity of study participants and eligibility criteria (e.g., stage of disease and prior treatment) in the various studies. Most of the study participants in the INTACT and TRIBUTE trials were non-Asian patients. In our study, which was conducted in Japan, the EGFR mutation-positive patients had stage IIIB and IV disease. In the study conducted by Hotta et al. (2007), 82% of the EGFR mutation-positive patients had recurrent disease after surgery. Previous research by other investigators has not elucidated how EGFR mutations affect clinical outcomes in