titanium oxide. In patients who had adverse events, the dose could be reduced by 10 mg/day as judged necessary on a course basis. Treatment continued until tumour progression, unacceptable toxic effects, or withdrawal of consent. Patients were not allowed to crossover between groups after progression or toxic effects.

All patients were examined and tested every 2 weeks. Diagnostic imaging was undertaken 4, 8, and 12 weeks after treatment initiation, and every 8 weeks thereafter. When treatment was discontinued for any reason other than progressive disease, diagnostic imaging was done according to the planned schedule until disease progression.

The primary endpoint of this study was overall survival, defined as the time between randomisation and death from any cause or the date of last follow-up. Secondary endpoints were progression-free survival (time between randomisation and disease progression or death from any cause), objective response, disease control (a complete or partial response plus stable disease more than 6 weeks from the initiation of study treatment), duration of response (time between point when patient first achieved complete or partial response and disease progression), time to treatment failure (time between randomisation and treatment discontinuation, disease progression, or death from any cause), efficacy of TAS-102 in patients with or without *KRAS* mutations, and adverse events. Progression-free survival, type and duration of response, and time to treatment failure were assessed by an external independent radiological review committee. *KRAS* mutational status was tested by the ARMS-Scorpion method in a central laboratory.[17] Adverse events were assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (version 3.0).[18] Adverse events were deemed to be serious when they led to death, were life-threatening, led to admission or extension of hospital stay, turned into permanent or noticeable disabilities or dysfunctions, triggered congenital abnormalities, or caused other medically important disorders.

We measured dose intensity and relative dose intensity at the cutoff date. Dose intensity was defined as cumulative dose (mg/m^2) divided by the number of weeks from initial treatment to discontinuation. Relative dose intensity was defined as dose intensity (mg/m^2 per week) divided by initial dose (mg/m^2 per week).

Statistical analysis

A sample size of 162 patients with a one-sided significance level of 10% was necessary to verify superiority in overall survival with a power of 80%, with an expected hazard ratio (HR) of 0·67. Median overall survival was anticipated to be 9·0 months in the TAS-102 group and 6·0 months in the placebo group.[15] We judged a clinically relevant HR to be about 0·70. Patients continued to receive the study treatment (with group assignments remaining concealed) until the primary analysis of overall survival was done when the number of deaths reached 121 in both groups. The Kaplan-Meier method was used to estimate survival distribution. We used a stratified log-rank test, adjusted by the allocation factor, for comparisons between the two groups, and a Cox proportional hazards model to estimate HRs, the two-tailed 80% CIs corresponding to the significance level, and 95% CIs. Additionally, we did interaction tests to assess the treatment effects by the

	TAS-102 (n=112)	Placebo (n=57)
Men	64 (57%)	28 (49%)
Women	48 (43%)	29 (51%)
Age (years)	63 (28–80)	62 (39–79)
Eastern Cooperative Oncology Group performance status		
0	72 (64%)	35 (61%)
1	37 (33%)	21 (37%)
2	3 (3%)	1 (2%)
Diagnosis		
Colon cancer	63 (56%)	36 (63%)
Rectal cancer	49 (44%)	21 (37%)
Number of metastatic organs		
1	25 (22%)	11 (19%)
2	43 (38%)	20 (35%)
3	27 (24%)	12 (21%)
≥4	17 (15%)	14 (25%)
Metastatic organ		
Liver	65 (58%)	38 (67%)
Lung	87 (78%)	44 (77%)
Lymph nodes	48 (43%)	23 (40%)
Peritoneum	11 (10%)	17 (30%)
Previous treatment and reason for discontinuation		
Surgical history	103 (92%)	50 (88%)
Adjuvant chemotherapy	54 (48%)	15 (26%)
Number of palliative chemotherapies		
2	17 (15%)	13 (23%)
≥3	95 (85%)	44 (77%)
Fluoropyrimidine-based treatment	112 (100%)	57 (100%)
Refractory	109 (97%)	55 (96%)
Intolerant	3 (3%)	2 (4%)
Oxaliplatin-based treatment	112 (100%)	57 (100%)
Refractory	95 (85%)	45 (79%)
Intolerant	17 (15%)	12 (21%)
Irinotecan-based treatment	112 (100%)	57 (100%)
Refractory	106 (95%)	56 (98%)
Intolerant	6 (5%)	1 (2%)
Bevacizumab	87 (78%)	47 (82%)
Cetuximab	71 (63%)	36 (63%)
KRAS mutational status*		
Wild-type	54 (55%)	24 (48%)
Mutant	45 (45%)	26 (52%)

Data are n (%) or median (range). *KRAS mutational status assessed for 99 (88%) patients in the TAS-102 group and for 50 (88%) patients in the placebo group.

Table 1: **Demographics and baseline characteristics of the efficacy population**

allocation factor as well as baseline characteristics, including *KRAS* mutational status.

We compared progression-free survival and time to treatment failure with the log-rank test. We compared objective response, disease control, and toxic effects with Fisher's exact test. We also did interaction tests for progression-free survival and disease control to assess the differences between treatment effects by the allocation factor as well as baseline characteristics, including *KRAS* mutational status. Relative dose intensity was calculated as the ratio of the actual dose taken to the planned dose.

The efficacy analysis was done in the intention-to-treat population, and the safety analysis in the per-protocol population. We used SAS (version 8.2) for statistical analyses.

See Online for appendix

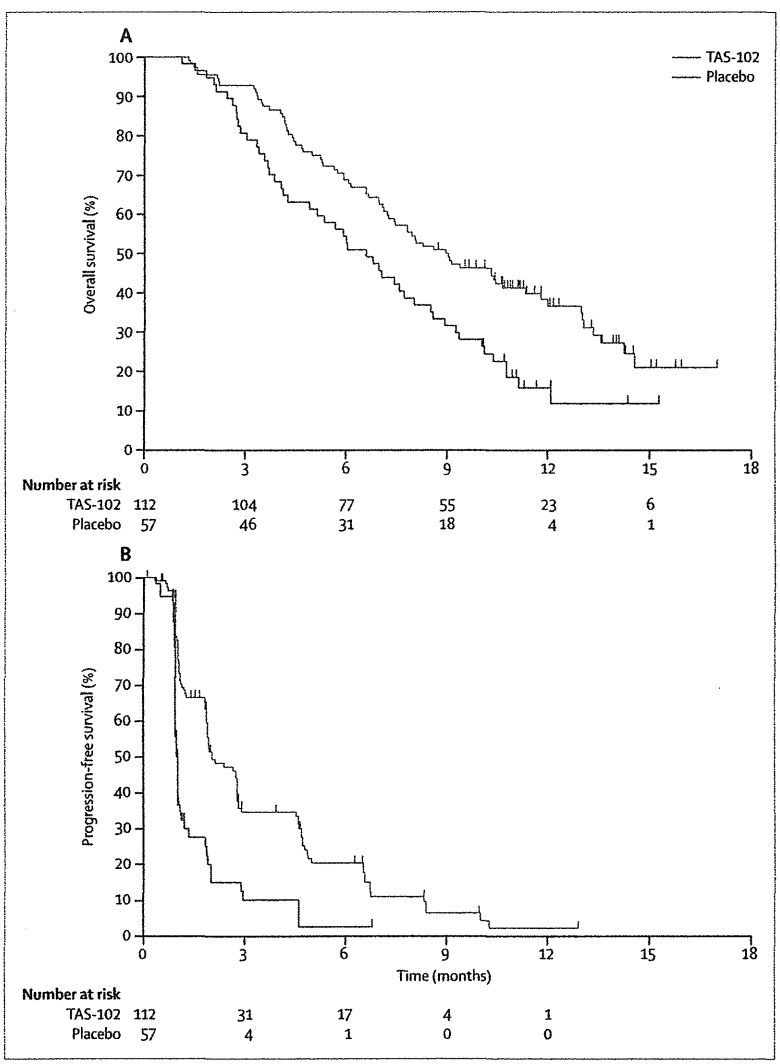

Figure 2: **Kaplan-Meier curves of overall survival (A) and progression-free survival (B) as assessed by independent review committee**

This study is registered with Japan Pharmaceutical Information Center, number JapicCTI-090880.

Role of the funding source

The study sponsor contributed to study design, data collection, and data analysis, but not to data interpretation. The corresponding author had full access to all the data and had final responsibility for the decision to submit for publication.

Results

Figure 1 shows the trial profile. Table 1 shows baseline characteristics of patients in the efficacy analysis. Most patients were judged to be refractory to all agents available for colorectal cancer treatment. Tumour tissues for central assessment of *KRAS* mutational status were available from 149 patients (88%; table 1). Baseline characteristics were much the same in the two groups, with the exception that more patients in the TAS-102 group received adjuvant chemotherapy than did those in the placebo group. Baseline characteristics in the *KRAS* population were similar to those in the efficacy population (data not shown). 49 (91%) patients with wild-type *KRAS* in the TAS-102 group and 23 (96%) in the placebo group had been given an anti-EGFR monoclonal antibody. Median follow-up was 11·3 months (IQR 10·7–14·0).

The cutoff date for overall survival was Feb 4, 2011. 123 deaths (75 in the TAS-102 group, 48 in the placebo group) had occurred by this point. Median overall survival was 9·0 months (95% CI 7·3–11·3) in the TAS-102 group and 6·6 months (4·9–8·0) in the placebo group (hazard ratio [HR] for death 0·56, 80% CI 0·44–0·71, 95% CI 0·39–0·81; p=0·0011; figure 2). In the prespecified subgroup analyses for overall survival, the effect of TAS-102 was similar in all categories, although not all improvements were significant (figure 3).

Median progression-free survival assessed by the independent review committee was 2·0 months (95% CI 1·9–2·8) in the TAS-102 group and 1·0 months (1·0–1·0) in the placebo group (HR 0·41, 95% CI 0·28–0·59; p<0·0001; figure 2). Median progression-free survival assessed by the investigators was 2·7 months (1·9–3·2) in the TAS-102 group and 1·0 months (1·0–1·0; HR 0·35, 95% CI 0·25–0·50; p<0·0001; appendix).

In both the assessment by the independent review committee and by investigators, one patient (1%) in the TAS-102 group achieved a partial response, with a duration of more than 225 days (ie, response continuing). No patients achieved an objective response in the placebo group. In the assessment by the independent review committee, 49 (43%) patients given TAS-102 achieved disease control (one [1%] patient had a partial response and 48 [43%] patients had stable disease), as did six (11%) given placebo (all six had stable disease; p<0·0001). In the investigator assessment,

61 (54%) patients given TAS-102 achieved disease control (one [1%] had a partial response and 60 [54%] had stable disease), as did eight (14%) given placebo (all eight had stable disease; p<0·0001). In the subgroup analyses and interaction tests for progression-free survival and disease control, the effect of TAS-102 was largely consistent across all categories (although not always significant; appendix).

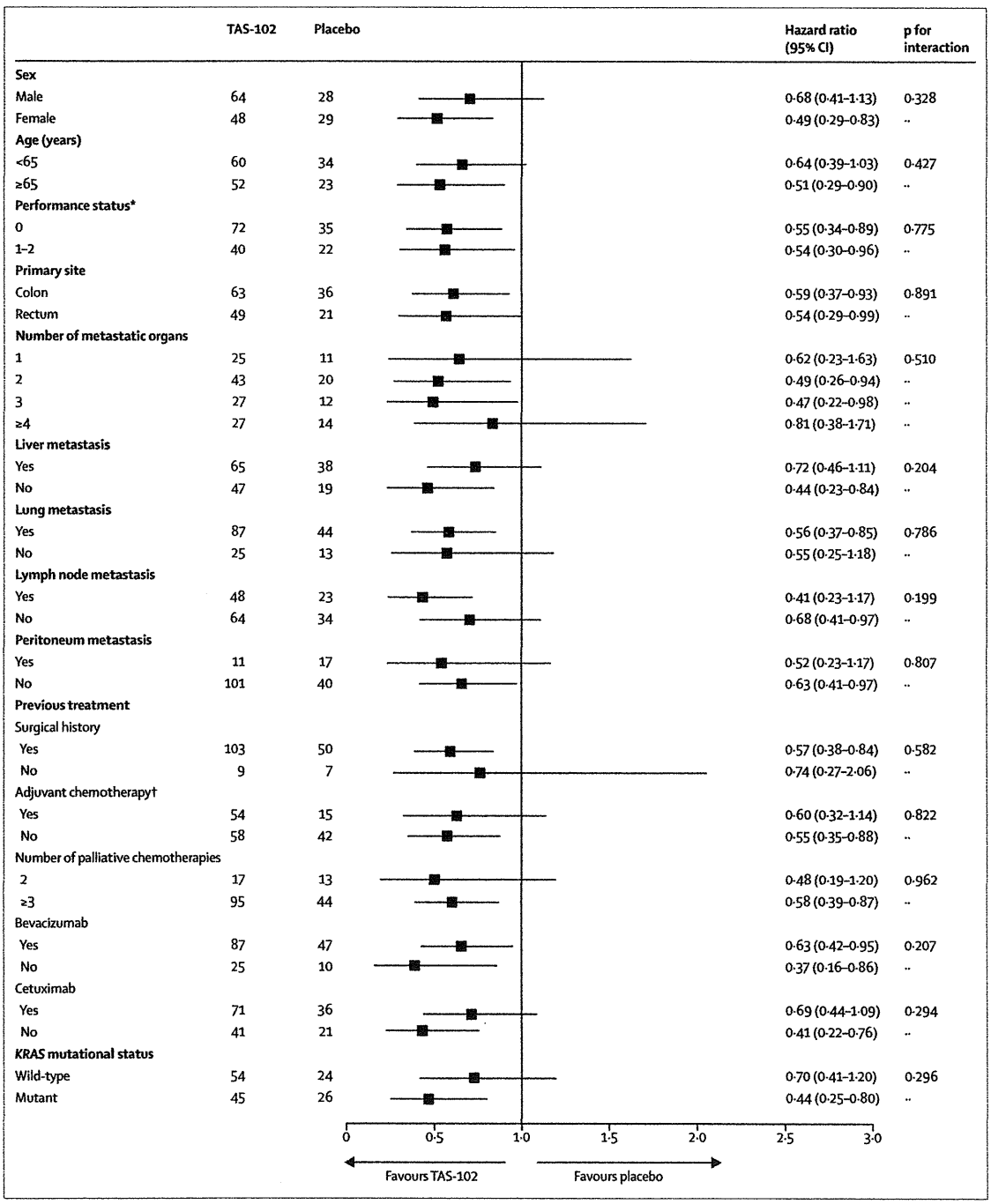

Figure 3: **Overall survival in prespecified subgroups**

*Eastern Cooperative Oncology Group criteria. †More patients received adjuvant chemotherapy in the TAS-102 group than in the placebo group, but this difference had no effect on the assessment of overall survival with the Cox proportional hazards model with one variable (p=0·605); there was no interaction (p=0·822).

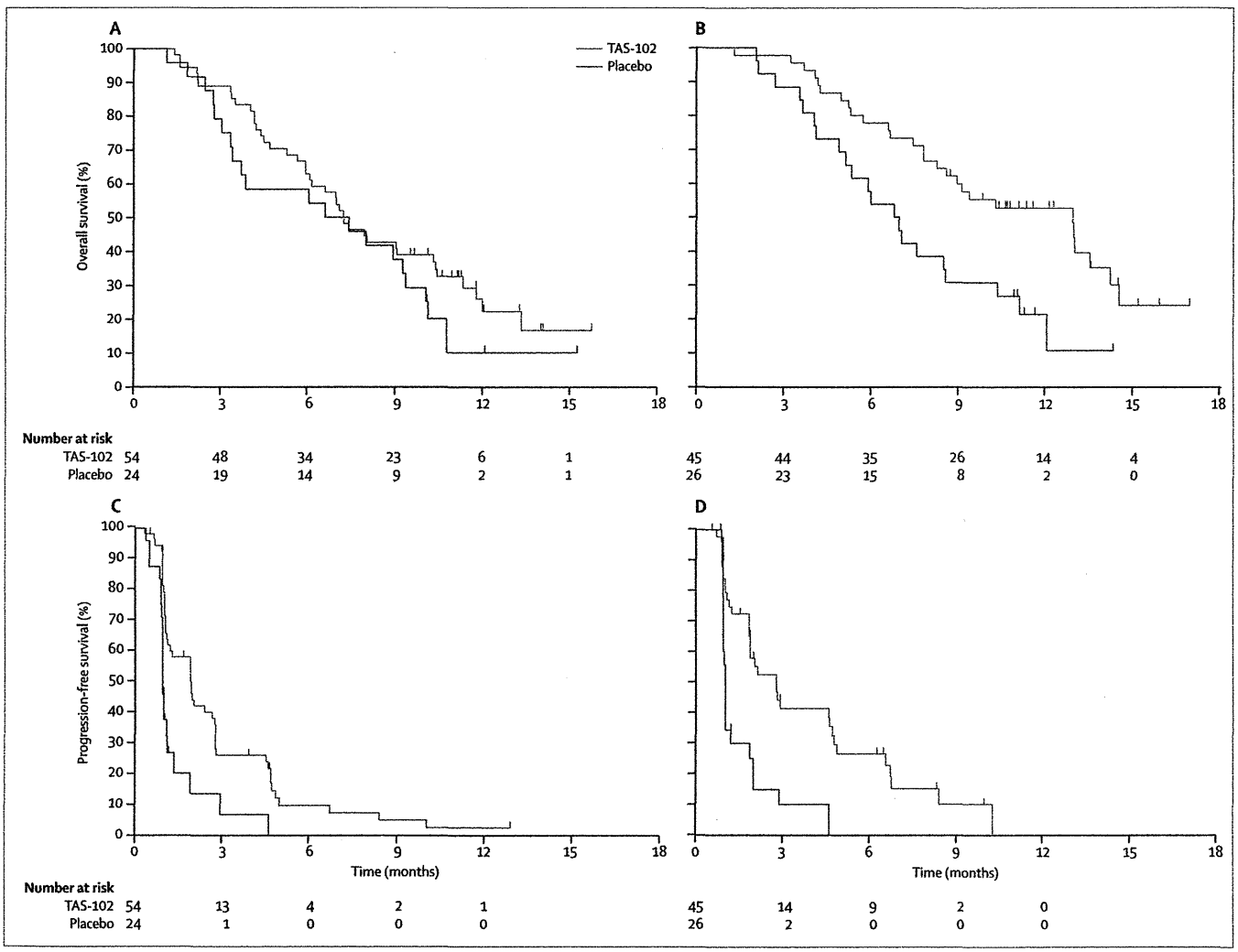

Figure 4: **Kaplan-Meier curves of overall survival and progression-free survival in patients with wild-type and mutant *KRAS***
(A) Overall survival of patients with wild-type *KRAS*. (B) Overall survival of patients with mutant *KRAS*. (C) Progression-free survival of patients with wild-type *KRAS*, as assessed by independent review committee. (D) Progression-free survival of patients with mutant *KRAS*, as assessed by independent review committee.

Median time to treatment failure assessed by the independent review committee was 1·9 months (95% CI 1·3–2·1) in the TAS-102 group and 1·0 months (1·0–1·0) in the placebo group (HR 0·40, 95% CI 0·28–0·56; p<0·0001). Median time to treatment failure assessed by the investigators was 2·7 months (95% CI 1·9–3·2) in the TAS-102 group and 1·0 months (1·0–1·0) in the placebo group (HR 0·34, 95% CI 0·24–0·49; p<0·0001).

In the TAS-102 group, 22 (20%) patients required at least one dose reduction, mainly because of neutropenia or thrombocytopenia, or both. 35 (31%) patients given TAS-102 required a treatment interruption, predominantly due to neutropenia. The median length of treatment interruption was 7 days (IQR 3·0–8·5). Toxic effects resolved sufficient to reinitiate treatment in all cases. The dose intensity of TAS-102 after the initial dose was 147 mg/m² per week and its relative dose intensity was 85·7%. At the time of data cutoff, 165 patients had discontinued treatment, 155 (94%; 99 TAS-102, 56 placebo) of whom did so because of disease progression. Four patients continued to receive TAS-102 treatment at data cutoff.

TAS-102 could be effective irrespective of *KRAS* mutational status (figure 3), although the drug seemed to have more of an effect on overall survival in patients with *KRAS* mutations. In patients with wild-type *KRAS*, median overall survival was 7·2 months (95% CI 6·1–10·3) in those given TAS-102 and 7·0 months (3·4–9·4) in those given placebo (p=0·191; figure 3). In patients with mutant *KRAS*, median overall survival was 13·0 months (8·6–14·3) in TAS-102 group and 6·9 months (5·2–8·6) in the placebo group (p=0·0056; figures 3, 4).

Median progression-free survival was 1·9 months (95% CI 1·1–2·8) in patients with wild-type *KRAS* given TAS-102 and 1·0 months (1·0–1·1) in those given placebo (HR 0·40, 95% CI 0·23–0·69; p=0·0004) as assessed by the independent review committee. It was 2·8 months (95% CI 1·9–4·7) in patients with mutant *KRAS* given TAS-102 and 1·0 month (1·0–1·2) in those given placebo (HR 0·34, 95% CI 0·19–0·61; p<0·0001; p for interaction=0·772; figure 4; appendix). 22 (41%) patients with wild-type *KRAS* in the TAS-102 group achieved disease control (one [2%] had a partial response, 21 [39%] had stable disease), as did two (8%) in the placebo group (both had stable disease; p=0·0038) as assessed by the independent review committee. 21 (47%) patients with mutant *KRAS* given TAS-102 achieved disease control (all had stable disease), as did three (12%) given placebo (all had stable disease; p=0·0037; p for interaction=0·835; appendix).

Grade 3–4 neutropenia, leucopenia, anaemia, fatigue, and diarrhoea were frequently recorded in the TAS-102 group (table 2). By contrast, grade 3 or worse adverse events were uncommon in the placebo group (table 2). No patients had hand-foot syndrome or peripheral neuropathy of grade 3 or more. Serious adverse events occurred in 21 (19%) patients in the TAS-102 group and five (9%) in the placebo group. Febrile neutropenia was the most common serious adverse event in the TAS-102 group, occurring in four (4%) patients. Eight (7%) patients in the TAS-102 group and nine (16%) in the placebo group died within 12 weeks of the start of treatment; all deaths were caused by progressive disease. Four (4%) patients in the TAS-102 group and one (2%) in the placebo group discontinued the study because of drug-related adverse events and one (1%) patient in the TAS-102 group discontinued treatment because of a non-related adverse event. No treatment-related deaths were reported during this study. The proportion of patients who received subsequent treatments in both groups was similar (table 3).

	TAS-102 (n=113)		Placebo (n=57)		p value*
	Any grade	Grade 3 or 4	Any grade	Grade 3 or 4	
Haematological					
Neutropenia	81 (72%)	57 (50%)	1 (2%)	0	<0·0001
Leucopenia	86 (76%)	32 (28%)	2 (4%)	0	<0·0001
Anaemia	82 (73%)	19 (17%)	9 (16%)	3 (5%)	<0·0001
Lymphopenia	39 (35%)	11 (10%)	7 (12%)	2 (4%)	0·0019
Thrombocytopenia	44 (39%)	5 (4%)	1 (2%)	0	<0·0001
Non-haematological					
Fatigue	66 (58%)	7 (6%)	24 (42%)	2 (4%)	0·052
Diarrhoea	43 (38%)	7 (6%)	12 (21%)	0	0·037
Nausea	73 (65%)	5 (4%)	16 (28%)	0	<0·0001
Anorexia	70 (62%)	5 (4%)	19 (33%)	2 (4%)	0·0006
Febrile neutropenia	5 (4%)	5 (4%)	0	0	0·170
Vomiting	38 (34%)	4 (4%)	14 (25%)	0	0·290

Data are n (%). The safety population included all patients who received at least one dose of the study treatment. *p values were calculated with Fisher's exact test for the difference in the incidence of adverse events of any grade.

Table 2: **Adverse events with a frequency of at least 3% in the safety population**

	TAS-102 (n=108)*	Placebo (n=57)*
Subsequent cancer treatment	46 (43%)	26 (46%)
Fluoropyrimidine-based treatment	30 (28%)	21 (37%)
Irinotecan-based treatment†	8 (7%)	12 (21%)
Oxaliplatin-based treatment	13 (12%)	10 (18%)
Bevacizumab	13 (12%)	12 (21%)
Anti-EGFR monoclonal antibody	12 (11%)	5 (9%)

Data are n (%). *Number of patients who discontinued the study treatment. †More patients in the placebo group received irinotecan-based treatment than in the TAS-102 group (p=0·022 by Fisher's exact test).

Table 3: **Cancer treatment after discontinuation of study treatment**

Discussion

Compared with placebo, TAS-102 reduces the risk of death in patients refractory or intolerant to two or more regimens of standard chemotherapy containing a fluoropyrimidine, irinotecan, and oxaliplatin. Additionally, TAS-102 significantly improves progression-free survival and increases the proportion of patients who achieve disease control, relative to placebo. Although only one patient achieved a partial response in the TAS-102 group, the proportion who achieved disease control in this group was significantly higher than in the placebo group. The increase in disease control in the TAS-102 group could have contributed to the improved progression-free survival and overall survival in patients treated with this agent.

KRAS mutations are generally thought to be a negative predictive marker for the treatment effect of an anti-EGFR monoclonal antibody.[19,20] Because the mechanism of action of TAS-102 involves direct incorporation of FTD into DNA, it seems likely that *KRAS* will not directly affect the activity of TAS-102. In an in-vivo study with COL-1 cells harbouring wild-type *KRAS* and HCT-116 cells harbouring mutant *KRAS*, TAS-102 had an antitumour effect on both types of tumour cell (unpublished data). We recorded no significant interaction between *KRAS* mutational status and activity of TAS-102. Moreover, when we did an adjusted analysis for overall survival, progression-free survival, and disease control as assessed by independent review committee, including the interaction between *KRAS* mutational status and effect of TAS-102, we obtained results similar to those of the primary analysis (data not shown). However, TAS-102 had greater efficacy in the patients with mutant *KRAS* than in those with the wild-type allele. Because this subgroup analysis was based on a small number of patients, further investigation in future clinical studies with large sample sizes are necessary. The results of our pharmacogenomic study to assess the

Panel: Research in context

Systematic review

In April, 2008, we searched PubMed, the database of the American Society of Clinical Oncology, and National Comprehensive Cancer Network clinical practice guidelines in oncology (both colon and rectal cancers) for reports published in English. We used the keywords "colorectal cancer", "standard chemotherapy and colorectal cancer", "fluoropyrimidine, irinotecan, oxaliplatin, and colorectal cancer", "cetuximab and colorectal cancer", "panitumumab and colorectal cancer", "bevacizumab and colorectal cancer", "*KRAS* and colorectal cancer", "*KRAS* and cetuximab", "*KRAS* and panitumumab", and "salvage therapy". Established standard treatments for patients with metastatic colorectal cancer are chemotherapy based on fluoropyrimidine, oxaliplatin, and irinotecan (in combination and sequentially), and monoclonal antibodies targeting VEGF (bevacizumab) and EGFR (cetuximab and panitumumab in patients with *KRAS* wild-type tumours only). For patients who have disease progression despite all available standard treatment, additional options are needed; many could maintain good performance status and be candidates for new treatment options.

Interpretation

TAS-102 has promising efficacy with an easily manageable safety profile in patients with metastatic colorectal cancer who are refractory or intolerant to standard chemotherapies with fluoropyrimidine, irinotecan, and oxaliplatin. The results of our study could further improve the outcomes of patients with unresectable colorectal cancer who have already received standard chemotherapy regimens.

value of expression of thymidine kinase 1 and thymidine phosphorylase as predictive factors of the treatment effect of TAS-102 will be reported elsewhere.

The toxic effects of TAS-102 were generally mild and the agent was well tolerated. Myelosuppression was the main adverse event caused by TAS-102, but was manageable with dose reductions or temporary interruptions in treatment. Non-haematological adverse events such as peripheral neuropathy, hand-foot syndrome, fatigue, and diarrhoea—often recorded with other cytotoxic agents[21,22]—were uncommon. Subsequent treatments that could be potential confounders of an overall survival endpoint, such as cytotoxic and molecular targeting agents, were given to similar or greater proportions of patients in the placebo group than in the TAS-102 group.

No clear definitions of refractory disease or intolerance were specified in the protocol, except that recurrence during or within 6 months after completion of adjuvant chemotherapy was defined as refractory. However, previous treatments were discussed before enrolment to ensure that all participants were eligible. Additionally, the initial imaging diagnosis was done 4 weeks after randomisation, which is earlier than is usual in similar studies (normally 8 weeks).[4,5] Because disease progression had been identified in 38 (67%) patients in the placebo group at initial imaging, median progression-free survival in the placebo group was 1 month in assessments by the independent review and the investigators, and thus is unlikely to be excessively biased.

Our double-blind, randomised, placebo-controlled phase 2 trial had a small sample size and only Japanese patients were enrolled. In view of the differences in haematological toxic effects, we believe that the investigators in charge might have been aware of the assignment for some patients, but that each patient was not aware of his or her assignment, because no patient's withdrawal because of their assignment was recorded. However, all secondary efficacy endpoints were assessed by independent review.

The issue of the different recommended doses in Japan and the USA (35 mg/m^2 *vs* 25 mg/m^2), despite similar pharmacokinetic profiles in the two populations, needs to be resolved. The recommended dose in patients from the USA is low on the basis of the high incidence of neutropenia of grade 3 or worse—one of the dose-limiting toxic effects of TAS-102—in patients with heavily pretreated metastatic breast cancer who had received several lines of previous aggressive chemotherapies and might have been particularly sensitive to TAS-102 because of poor bone-marrow reserves.[14] US investigators have done an additional trial to investigate the tolerability of the Japanese recommended dose of TAS-102 in US patients for pretreated metastatic colorectal cancer, which has been suggested to be tolerable and to have a safety profile consistent with that in Japanese patients.[23]

In conclusion, TAS-102 has promising efficacy with a manageable safety profile in patients with metastatic colorectal cancer who are refractory or intolerant to standard chemotherapy (panel). An international phase 3 trial to confirm the clinical benefits of TAS-102 in all populations is in progress (RECOURSE; NCT01607957), comparing TAS-102 monotherapy (with the same dosage and dose schedule as in our study) plus best supportive care with placebo plus best supportive care in patients with metastatic colorectal cancer who are refractory or intolerant to all approved agents including fluoropyrimidine, irinotecan, oxaliplatin, bevacizumab, and anti-EGFR monoclonal antibodies.

Contributors

All authors wrote the report and approved the final draft. TY, NM, KYamaz, TN, YK, HB, AT, KYamag, KM, NS, YT, TM, and TE collected data. TY advised on the content of the study protocol related to *KRAS* research, on doubts that arose during the study, and on measurement methods and data interpretation. HB and AO coordinated trial implementation in all sites, including coordination of the study protocol and resolution of doubts in its interpretation. CH and TT interpreted data. TT analysed data.

Conflicts of interest

TY has received consulting fees from Takeda; honoraria from Chugai, Takeda, Yakult, Bristol-Myers Squibb, and MerckSerono; and research funding from Daiichi Sankyo, Taiho, Bayer, and ImClone. YK has received consulting fees, honoraria, and research funding from Taiho.

HB owns Taiho stock, and has received honoraria, research funding, and travel grants from Taiho. AT and TE have received honoraria from Taiho. KYamag has received honoraria from Chugai, Bristol-Myers Squibb, and MerckSerono. KM has received consulting fees from Ono and Novartis; honoraria from Taiho, Chugai, Yakult, Bristol-Myers Squibb, and Takeda; and research funding from Taiho, Yakult, Daiichi Sankyo, Pfizer, AstraZeneca, Kyowa Hakko Kirin, Eizai, and MerckSerono. TM and TE have received research funding from Taiho. CH has received consulting fees from Taiho. TT is employed by Taiho, and owns Taiho stock. AO is employed by Bayer; has received consulting fees from Takeda, Daiichi Sankyo, Novartis, Chugai, and Taiho; and has received honoraria from Takeda, Daiichi Sankyo, Taiho, GlaxoSmithKline, Pfizer, Yakult, MerckSerono, and Bristol-Myers Squibb. The other authors declare that they have no conflicts of interest.

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ORIGINAL ARTICLE

Differences of the Lymphatic Distribution and Surgical Outcomes Between Remnant Gastric Cancers and Primary Proximal Gastric Cancers

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Abstract
Background Although remnant gastric cancer (RGC) following distal gastrectomy is located in the proximal stomach, little is known about the differences of the lymphatic distribution and surgical outcomes between RGC and primary proximal gastric cancer (PGC).
Methods Between 1997 and 2008, 1,149 patients underwent gastrectomy for gastric cancer. Of these, 33 (2.9%) RGC patients and 207 (18.5%) PGC patients were treated at our department. We reviewed their hospital records retrospectively.
Results Compared with the PGC patients, those with RGC had a slightly higher age at onset (p=0.09), higher incidence of undifferentiated cancer (p=0.06), higher incidence of vascular invasion (p=0.09), and higher incidence of T4 (p=0.07). Gastrectomy for RGC involved greater blood loss (p<0.005), longer surgical duration (p=0.01), combined resection, and high incidence of complications. However, the survival rate for RGC patients was similar to that for PGC patients (p=0.67). 2) Patients with RGC had a different pattern of lymph node metastasis compared with that in PGC. Particularly in advanced RGC with pT2–T4 tumors, RGC frequently demonstrated jejunal mesentery lymph node metastases (RGC vs. PGC, 35% vs. 0%) and splenic hilar lymph node metastases (RGC vs. PGC, 17% vs. 10%). The jejunal mesentery lymph node metastases were detected only following Billroth II reconstruction (Billroth I vs. Billroth II, 0% vs. 67%).
Conclusion Although the clinical behaviors of the two gastric cancers were different, the survival rates were similar. The pattern of metastasis indicates that the jejunal mesentery and splenic hilar lymph nodes should be specifically targeted for en bloc resection during complete gastrectomy in RGC.

Keywords Remnant gastric cancer · Lymph node metastasis · Prognosis · Proximal gastric cancer · Lymphatic distribution · Lymph node dissection

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Introduction

Gastric cancer is the second leading cause of cancer-related death in the world[1]. However, recent advances in diagnostic methods, less invasive treatment techniques, and perioperative management have increased the early detection of gastric cancer and decreased the mortality and morbidity rates.[2–4] Consequently, the number of successfully treated patients has been increasing, and some of these patients are at risk of developing second primary cancer in the remnant stomach. This implies that more cases of remnant gastric cancer (RGC) will be encountered in the future.

In previous studies, RGC was commonly found at an advanced stage, resulting in low rates of curative resection

(38–40%) and a consequent poor prognosis.[5,6] However, recently, the prognosis of RGC following distal gastrectomy has been improving due to diagnostic and technological advances. Indeed, at our institute, more than half of the RGC patients were treated for T1 or T2, node-negative, and early stage cancer and almost 80% of patients with RGC underwent curative resection. Therefore, it is necessary to characterize the clinical features of RGC to develop optimal surgical and treatment planning. However, there is limited information available to help guide the treatment of patients with RGC. RGC after distal gastrectomy is located in the same proximal stomach as a primary proximal gastric cancer (PGC). This study was designed to clarify the differences of the lymphatic distribution and surgical outcomes between RGC and PGC.

Patients and Methods

Patients

Between 1997 and 2008, 1,149 patients underwent gastrectomy for gastric cancer. Of these, 33 consecutive patients with primary RGC (2.9%) and 207 patients with PGC (18.5%) were treated in the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. The follow-up program after gastrectomy consisted of regular physical examinations and laboratory blood tests, chest X-rays, an upper gastrointestinal series or endoscopy, and ultrasonography or computer tomography for the first 5 years and yearly endoscopy thereafter if possible. All patients underwent gastrectomy with lymphadenectomy for RGC. The clinicopathological findings of these patients were determined retrospectively on the basis of their hospital records. Macroscopic and microscopic classifications of gastric cancers were based on the Japanese Classification of Gastric Carcinomas.[7] Consistent with the TNM staging system,[8] patients with lymph node metastases were reclassified into three groups based on the total number of positive nodes. Histological types were classified as differentiated (papillary, moderately, or well-differentiated adenocarcinoma) and undifferentiated (poorly or undifferentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous adenocarcinoma).

Comparison of associated clinical factors between RGC and PGC patients

Comparison between RGC and PGC was performed because RGC was located in the proximal stomach. To examine treatment targets in particular, several clinicopathological factors such as age, sex, histological types, lymphatic invasion, venous invasion, tumor size, depth of tumor, area and number of lymph node metastases, pathological stage, and surgical factors such as surgical duration, bleeding amount, organs with combined resection, and complications were retrospectively evaluated from the hospital records.

Statistical Analysis

Cause-specific death was recorded when the cause of death was specified as recurrent RGC. Chi-square test and Fisher's exact probability test were performed for categorical variables, while Student's t test and Mann–Whitney U test for unpaired data of continuous variables were performed to compare the clinicopathological characteristics between two groups. The cumulative cause-specific overall survival rates were calculated using the Kaplan–Meier method, and log rank test was used for assessment of differences between clinical factors. A $p<0.05$ was considered significant.

Results

Clinicopathological characteristics of patients with primary remnant gastric cancer

Table 1 shows the characteristics of the 33 RGC patients. The mean patient age was 68 years, and the male/female ratio was 2.7:1. Ten patients had symptoms and the remaining 23 patients were asymptomatic. Regarding the initial gastric disease, there were 19 patients with benign disease and 14 patients with gastric cancer. Reconstruction during the first surgery was mainly Billroth I or II. More than half of the RGC patients demonstrated T1 or T2, undifferentiated, node-negative, and early stage cancer. In 78.8% (26/33) of the patients, resections were performed with curative intent. En bloc resection of the tumor by total remnant gastrectomy was performed with jejunal mesentery and D2 lymphadenectomy and concomitant organ resection. In addition, splenectomy was performed in 18 patients, distal pancreatectomy in four, partial colon resection in two, and liver resection in two. Reconstruction was performed in 16 patients by Billroth I, in 16 patients by Billroth II, and in one by Roux-en Y procedure for all resected RGC tumors. Tumors were located at the anastomotic site in 16 (61%) patients, corpus and/or cardia in nine (34%), and throughout the entire remnant in one (4%). The median interval between the first and second surgery was 20 years. Of the 33 RGC patients, RGC was detected in 19 (58%) by routine screening in whom the follow-up periods were short (0.5–2 year). On the other hand, RGC was detected incidentally in 14 (42%) patients in whom the follow-up periods were more than 5 years. Patients with early stage RGC such as stages I and II tended to have been diagnosed every second year (data not shown). Therefore, surveillance endoscopic screening following distal gastrectomy should be performed every second year for at least 20 years.

Table 1 Clinicopathologic characteristics of patients with primary RGC

Age	Mean±SD (years)	68±10	
Sex	Male	24	(72.7%)
	Female	9	(27.3%)
Symptom	Yes	11	(33.3%)
	No	22	(66.7%)
Initial gastric disease	Benign	19	(57.6%)
	Cancer	14	(42.4%)
Interval between first and second surgeries	Median (year)	20 (2–51)	
Reconstruction of first surgery	Billroth I	16	(48.5%)
	Billroth II	16	(48.5%)
	Roux en Y	1	(3.0%)
Depth of invasion	T1	10	(30.3%)
	T2	10	(30.3%)
	T3	7	(21.2%)
	T4	6	(18.2%)
Histology	Well	11	(33.3%)
	Moderate	2	(6.1%)
	Poor	13	(39.4%)
	Sig	7	(21.2%)
Lymph node metastasis	N0	20	(60.6%)
	N1	7	(21.2%)
	N2≤	6	(18.2%)
Stage	I	17	51.5%)
	II	5	(15.2%)
	III	4	(12.1%)
	IV	7	(21.2%)
Surgery type	Total	33	(100.0%)
	Partial	0	(0.0%)
Extent of lymphadenectomy	D1	9	(27.3%)
	D2	22	(66.7%)
	D2<	2	(6.1%)
Combined resection	Spleen	18	(54.5%)
	Distal pancreas	4	(12.1%)
	Liver	2	(6.1%)
	Colon	2	(6.1%)

Primary Remnant Gastric Cancer and Upper One Third Gastric Cancers

Table 2 shows a comparison of clinicopathological factors between the 33 RGC and 207 primary PGC. RGC patients had a slightly higher age at onset (p=0.09), higher incidence of undifferentiated cancer (p=0.06), higher incidence of vascular invasion (p=0.09), and higher incidence of T4 (p=0.07) than those with PGC. Gastrectomy for RGC involved greater blood loss (p<0.005), longer surgical duration (p=0.01), combined resection, and high incidence of complications. As shown in Fig. 1, the survival curves for the two groups were similar. Figure 2 shows the metastatic region and extent of lymph node involvement between RGC and PGC. Particularly in advanced RGC with pT2–T4 tumors, compared with PGC, RGC more frequently demonstrated jejunal mesentery lymph node metastasis (RGC vs. PGC, 35% vs. 0%) and splenic hilar lymph node metastasis (RGC vs. PGC, 17% vs. 10%) because RGC had a different pattern of lymphatic flow after initial distal gastrectomy. The jejunal mesentery lymph node metastases were detected only following Billroth II reconstruction (Billroth I vs. Billroth II, 0% vs. 67%).

Discussion

RGC following distal gastrectomy has been reported to account for 1–2% of all gastric cancers in Japan.[9,10] Previously, RGC was reported to be caused by multiple factors, and the

Table 2 Comparison of clinicopathological factors between 33 RGC and 207 PGC patients

Variables		RGC (n=33)		PGC (n=207)		p value
Age	Years (mean)	68		65		0.09
Sex	Male	24	(73%)	157	(76%)	
	Female	9	(27%)	50	(24%)	0.87
Histological type	Differentiated	13	(39%)	118	(57%)	
	Undifferentiated	20	(61%)	89	(43%)	0.06
Lymphatic invasion	Negative	16	(48%)	96	(46%)	
	Positive	17	(52%)	111	(54%)	0.82
Venous invasion	Negative	16	(48%)	132	(64%)	
	Positive	17	(52%)	75	(36%)	0.09
Tumor size	mm (mean)	55		51		0.53
Depth of tumor	T1	10	(30%)	69	(33%)	
	T2	10	(30%)	75	(36%)	
	T3	7	(21%)	54	(26%)	
	T4	6	(18%)	9	(4%)	0.07
Lymph node metastasis	Negative	20	(61%)	118	(57%)	
	Positive	13	(39%)	89	(43%)	0.70
Stage	I	17	(52%)	104	(50%)	
	II	5	(15%)	24	(12%)	
	III	4	(12%)	43	(21%)	
	IV	7	(21%)	36	(17%)	0.84
Surgical duration	min (mean)	381		326		<0.05
Bleeding	g (mean)	931		604		<0.005
Combined resection	Spleen	18	(55%)	81	(39%)	0.09
	Distal pancreas	4	(12%)	9	(4%)	0.16
	Liver	2	(6%)	4	(2%)	0.42
	Colon	2	(6%)	6	(3%)	0.68
Postoperativre complication	Leakage	6	(18%)	12	(6%)	<0.05
	Pancreatic fistula	3	(9%)	6	(3%)	0.21
	Wound infection	4	(12%)	8	(4%)	0.11
	Pneumonia	3	(9%)	6	(3%)	0.21

Significant values are shown in boldface type

P values were derived from χ^2 or Fisher's exact test and were considered significant at < 0.05

incidence, pathological features, and potential mechanisms have been extensively investigated.[11–13] RGC is commonly found at an advanced stage, resulting in low rates of curative resection (38–40%) and a consequently poor prognosis.[5,6] However, recently, the incidence and etiology of RGC have been changing[14] because of the long latency periods, decreasing prevalence of gastrectomy for benign disease,[5,15] early detection, and improved outcomes in patients with gastric cancers.[16,17] Moreover, recent advances in diagnostic and treatment techniques have led to a higher detection rate of early RGC following distal gastrectomy.[18] Consequently, endoscopic therapy such as EMR or ESD is performed for treatment of early stage RGC[19,20]. Therefore, it is important to re-evaluate the clinical features of RGC in order to develop optimal surgical and treatment planning.

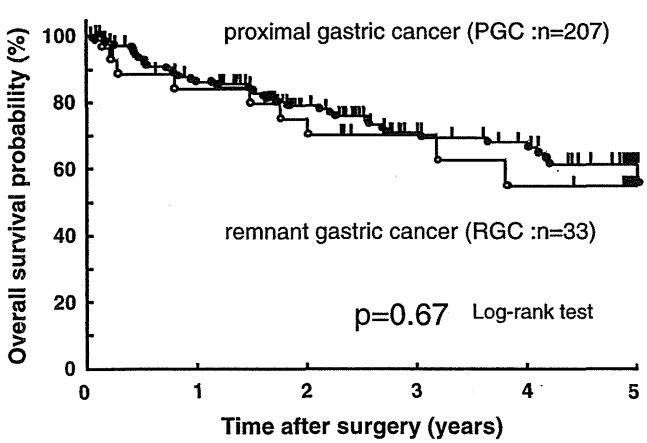

Fig. 1 Survival curves of 33 RGC and 207 PGC patients

In comparison between RGC and primary PGC, the two survival rates were similar (Fig. 1) even though RGC showed a slightly higher involvement of jejunal mesenteric or splenic hilar nodes and a higher incidence of undifferentiated cancer, vascular invasion, and T4 cancers, and gastrectomy for RGC

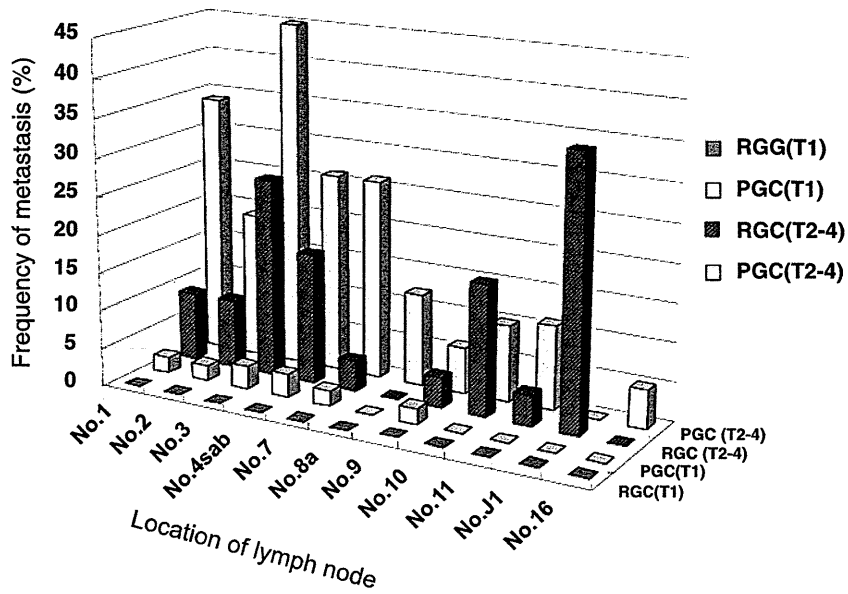

Fig. 2 Comparison of the metastatic region and extent of lymph node metastasis between RGC and PGC. Compared with that in PGC, RGC frequently showed jejunal mesentery lymph node metastasis and splenic hilar lymph node metastasis because RGC has a different pattern of lymphatic flow after the initial distal gastrectomy

involved more blood loss, longer surgical duration, combined resection, and a higher incidence of complications than PGC (Table 2). These results are consistent with several recent reports;[6,21,22] however, the reason for this similarity remains unclear. One of reasons might be that the incidence and etiology of RGC following distal gastrectomy have been changing owing to diagnostic and technological advances, although previously RGC was commonly found at more advanced stage, resulting in low rates of curative resection (38–40%) and a consequent poor prognosis. Indeed, at our department, more than half of the RGC patients were treated for T1 or T2, node-negative, and early stage cancer, in contrast to that in previous series (Table 1) and almost 80% of patients underwent curatively resection with extensive lymphadenectomy. On the contrary, in recent years, the incidence of PGC has been increasing, whereas the prognosis of PGC has not been improved in comparison with middle and lower gastric cancers (data not shown). Therefore, the prognoses of RGC and PGC might be similar at this point in time. Indeed, RGC is not always advanced at diagnosis, and if it is, extensive surgery for RGC is not necessarily associated with poor prognosis in comparison to that for primary gastric cancer.

Concerning lymph node metastasis from RGC, the main lymphatic flow from a tumor located in the upper one third of the stomach drains into lymph nodes along the celiac artery through the lymph nodes at the lesser curvature, the right side of cardia, and the left gastric artery. In the remnant stomach, these lymphatic pathways have been transected during the initial surgery, thus altering the lymphatic flow at the greater curvature, splenic artery, and splenic hilum.[5,23] Indeed, patients with RGC have a different pattern of lymph node metastasis compared with that in PGC (Fig. 2). Regarding the ligation of the left gastric artery, of the 33 RGC patients analyzed, 14 (42%) patients underwent the initial gastrectomy for gastric cancer and all left gastric arteries were ligated. On the other hand, there were no patients who underwent ligation of the left gastric artery for the initial benign disease. As a result, four (12%) patients exhibited splenic hilar lymph node metastasis out of the 33 RGC patients: Two patients had initial gastric cancer, and the remaining two patients had initial benign disease. Thus, the ligation of the left gastric artery was not the main reason for the metastases to the splenic hilar lymph node. Indeed, other interruption of lymphatic flows might also influence lymphatic flow from a tumor. In our hospital, the incidence of splenic hilar lymph node metastasis from RGC was higher than that from PGC [RGC vs. PCG, 12% (4/33) vs. 7% (14/207)]. Therefore, the interruption of lymphatic flow at the initial surgery might alter lymphatic flow from a tumor. However, the detailed mechanisms of lymphatic flow remain unclear.

In early RGC, no lymph node metastasis was detected although a low incidence of peri-gastric lymph node metastasis was noted in PGC. Namely, differences of the metastatic region and extent of lymph node involvement between RGC and PGC were small. On the other hand, in advanced RGC, the incidences of splenic hilar lymph node metastasis (RGC vs. PGC, 17% vs. 10%) and jejunal mesentery lymph node metastasis (RGC vs. PGC, 35% vs. 0%) were higher because RGC has a different pattern of lymphatic flow after initial distal gastrectomy. Concerning the initial surgery, the splenic hilar lymph node metastases occurred following every type of reconstruction. In contrast, the jejunal mesentery lymph node metastases occurred only following Billroth II reconstruction (Billroth I vs. Billroth II, 0% vs. 67%). This incidence of metastasis was higher than the previously reported incidences of 9–26%.[23–25] Therefore, the splenic hilar lymph node dissection is essential for curative gastrectomy in all RCG

patients. Additionally, the jejunal mesentery lymph node dissection should be performed in patients with RGC following Billroth II reconstruction.

Owing to recent advances in diagnostic and treatment techniques, RGC is not always advanced at diagnosis and, if it is, extensive surgery for RGC does not necessarily lead to poor prognosis in comparison to that for primary PGC. Patients with RGC have a different pattern of lymph node metastasis compared with that in PGC. Therefore, in decision making regarding the area of lymphadenectomy, the jejunal mesentery and splenic hilar lymph nodes should be specifically targeted for en bloc resection during complete gastrectomy in RGC.

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DIAGNOSTIC PATHOLOGY

RESEARCH Open Access

Stromal micropapillary component as a novel unfavorable prognostic factor of lung adenocarcinoma

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Abstract

Background: Pulmonary adenocarcinomas with a micropapillary component having small papillary tufts and lacking a central fibrovascular core are thought to result in poor prognosis. However, the component consists of tumor cells often floating within alveolar spaces (aerogenous micropapillary component [AMPC]) rather than invading fibrotic stroma observed in other organs like breast (stromal invasive micropapillary component [SMPC]). We previously observed cases of lung adenocarcinoma with predominant SMPC that was associated with micropapillary growth of tumors in fibrotic stroma observed in other organs. We evaluated the incidence and clinicopathological characteristics of SMPC in lung adenocarcinoma cases.

Patients and Methods: We investigated the clinicopathological characteristics and prognostic significance of SMPC in lung adenocarcinoma cases by reviewing 559 patients who had undergone surgical resection. We examined the SMPC by performing immunohistochemical analysis with 17 antibodies and by genetic analysis with epidermal growth factor receptor (*EGFR*) and *KRAS* mutations.

Results: SMPC-positive (SMPC(+)) tumors were observed in 19 cases (3.4%). The presence of SMPC was significantly associated with tumor size, advanced-stage disease, lymph node metastasis, pleural invasion, lymphatic invasion, and vascular invasion. Patients with SMPC(+) tumors had significantly poorer outcomes than those with SMPC-negative tumors. Multivariate analysis revealed that SMPC was a significant independent prognostic factor of lung adenocarcinoma, especially for disease-free survival of pathological stage I patients ($p = 0.035$). SMPC showed significantly higher expression of E-cadherin and lower expression of CD44 than the corresponding expression levels shown by AMPC and showed lower surfactant apoprotein A and phospho-c-Met expression level than corresponding expression levels shown by tumor cell components without a micropapillary component. Fourteen cases with SMPC(+) tumors (74%) showed *EGFR* mutations, and none of them showed *KRAS* mutations.

Conclusions: SMPC(+) tumors are rare, but they may be associated with a poor prognosis and have different phenotypic and genotypic characteristics from those of AMPC(+) tumors.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/vs/9433341526290040.

Keywords: lung adenocarcinoma, micropapillary component, stromal micropapillary component, aerogenous micropapillary component, prognostic factor

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Background

A new lung adenocarcinoma classification system has been proposed by the International Association for the Study of Lung Cancer, American Thoracic Society, and European Respiratory Society (IASLC/ATS/ERS) [1]. In this classification, the micropapillary component (MPC) was recommended as a new subtype of lung adenocarcinoma in addition to the lepidic, acinar, papillary, and solid subtypes defined in the 2004 World Health Organization (WHO) classification [2]. MPC was defined as tumor cells growing in papillary tufts lacking fibrovascular cores and may float within alveolar spaces. MPC-predominant lung adenocarcinoma shows a high incidence of nodal metastasis and a poor prognosis [3-8]. MPC-predominant carcinomas developing in various other organs, such as the breast and urinary bladder, known as invasive micropapillary carcinoma, also have a poor prognosis. However, localization of MPC in the lungs is significantly different from that in the other organs; MPC in lung adenocarcinoma is distinguished by floating tumor cells within alveolar spaces (aerogenous micropapillary component, AMPC), while MPC in other organs has been observed primarily in the stroma as invasive components (stromal invasive micropapillary component, SMPC) [3,4].

Few studies have examined lung adenocarcinoma with SMPC [9,10]. Recently, we reported 2 cases of SMPC-predominant lung adenocarcinoma [9]. The proportion of SMPC in both tumors was greater than 50% in area. We observed that SMPC had a strong association with vascular invasion, similar to the cases of SMPC-predominant carcinoma in other organs. However, a large-scale investigation on pulmonary SMPC has not been conducted.

The aims of this study included: (1) clarifying the incidence of SMPC in lung adenocarcinoma; (2) elucidating the clinicopathological characteristics of the tumor; and (3) determining the prognoses of the SMPC-positive (SMPC(+)) tumors and comparing them with those of SMPC-negative (SMPC(-)) tumors. We reviewed 559 resected lung adenocarcinomas for this study with performing immunohistochemical and genetic analysis.

Methods

Patients

We analyzed 565 consecutive cases of primary lung adenocarcinoma treated by surgical resection at the Kanagawa Cancer Center between February 2007 and December 2010. Formalin fixation of the resected lung tissue was performed within 48 hours to reduce the loss of immunohistochemical antigen expression and degeneration of DNA. Six patients who had received preoperative chemotherapy were excluded. A total of 559 cases were enrolled in the study. The median follow-up time was 634.5 days (range, 28-1512 days). All patients provided informed consent, and the studies were performed according to the requirements of the institutional review board of Kanagawa Cancer Center.

Pathological review

Excised specimens were fixed in a solution of 10% buffered formaldehyde, and the sections were embedded in paraffin. Next, 4-μm-thick sections, including the largest cut surface of the tumor, were prepared and stained using hematoxylin and eosin (HE) as well as alcian blue and elastica-van-Gieson (AB-EVG) to detect cytoplasmic mucin production and the elastic fiber framework. Lymphatic invasion and pulmonary metastasis were evaluated on HE sections. Vascular and pleural invasion was evaluated in AB-EVG sections. Sections were reviewed by 2 observers (M.O. and T.Y.) who were unaware of the clinical data. Tumor size was measured as the maximal diameter on the cut sections of the lung. Pathological stage was determined based on the criteria of the 7th TNM classification of Union of International Cancer Control [11].

Histological definition of micropapillary components

Histopathological diagnosis of lung adenocarcinoma was determined according to the IASLC/ATS/ERS international multidisciplinary classification of lung adenocarcinoma [1]. Comprehensive histological subtyping was performed on the primary tumor and divided by percentage into 5 distinctive subtypes: lepidic, acinar, papillary, micropapillary, and solid, totaling 100% per tumor. We defined the subtype as positive when it occupied at least 1% of the entire tumor. We classified a micropapillary subtype into 2 components, AMPC and SMPC, using the following criteria: AMPC is widely recognized in the lungs as tumor cells floating within alveolar spaces, and SMPC includes papillary components consisting of tufts lacking central fibrovascular cores, surrounded by lacunar spaces and identified as invasive components in the stroma as previously described [9] (Figure 1A and 1B). Additionally, a tumor area without micropapillary components was defined as a non-micropapillary component (nMPC).

Tumor tissue microarray (TMA) synthesis

TMAs were constructed using a manual tissue-arraying instrument (KIN-4; Azumaya, Tokyo, Japan) as previously described [12], and specimens were punched using a stylet 3 mm in diameter.

Immunohistochemistry

The 17 antibodies used for immunohistochemical characterization of tumor cells in TMA in this study are listed in Table 1. Immunohistochemical staining was

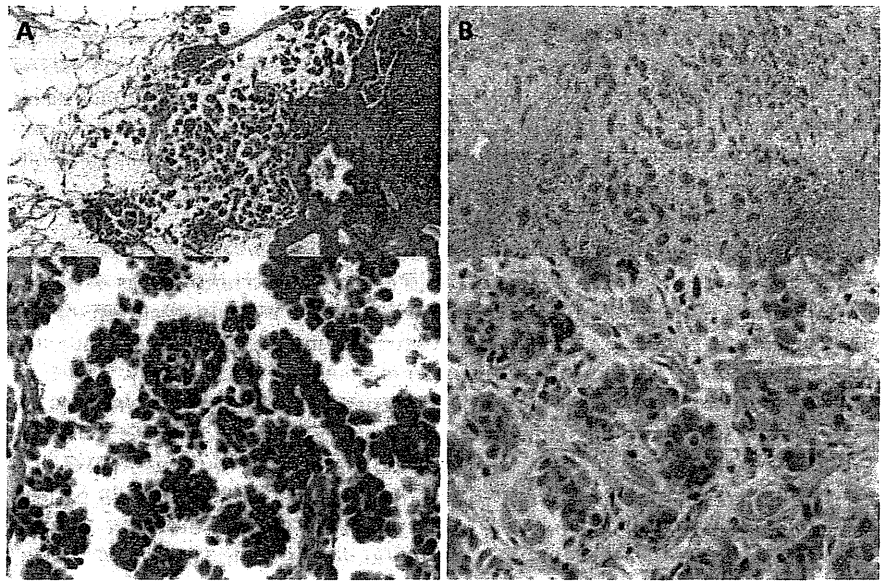

Figure 1 Microscopic features of micropapillary component in the lung adenocarcinoma stained with hematoxylin-eosin (HE). A, AMPC. AMPC is the micropapillary component in which tumor cells are floating within alveolar spaces. B, SMPC. SMPC are tumor cells observed in the stroma and consisting of a papillary component with a tuft lacking central fibrovascular cores surrounded by acinar spaces. (A, B: upper panel: magnification, ×100; lower panel: magnification, ×400) SMPC, stromal micropapillary component; AMPC, aerogenous micropapillary component.

Table 1 Antibodies

Classification/Antibody	Clone	Dilution	Source
Cellular adhesion molecules			
E-cadherin	NCH-38	1:100	DakoCytomation, Carpinteria, CA, USA
CD44	DF1485	1:400	Novocastra, Newcastle upon Tyne, UK
Laminin5γ2	4G1	1:50	DakoCytomation, Glostrup, Denmark
Growth factor			
VEGF-C	Polyclonal	1:50	Abcam, Cambridge, UK
Apoptosis-associated proteins			
bcl2	124	1:50	DakoCytomation, Glostrup, Denmark
p53	DO-7	Pre-diluted	Nichirei, Tokyo, Japan
cleaved caspase-3	Polyclonal	1:400	Cell signaling, Danvers, MA, USA
Mucin-related proteins			
MUC1	Ma695	1:100	Novocastra, Newcastle upon Tyne, UK
MUC6	CLH5	1:100	Novocastra, Newcastle upon Tyne, UK
Hypoxia induced protein			
HIF-1α	EP1215Y	1:500	Abcam, Cambridge, UK
Others			
TTF-1	8G7G3/1	1:100	DakoCytomation, Carpinteria, CA, USA
SP-A	PE10	1:100	Dako, Kyoto, Japan
Vimentin	V9	Pre-diluted	DakoCytomation, Carpinteria, CA, USA
Ki-67	MIB-1	1:50	Dako, Glostrup, Denmark
LYVE1	15A5B2	1:400	Oriental Yeast, Tokyo, Japan
c-Met	EP1454Y	1:200	Abcam, Cambridge, UK
Phospho-c-Met	Polyclonal	1:800	Stressgen, Ann Arbor, MI, USA

VEGF-C, vascular endothelial growth factor-C; HIF-1α, hypoxia induced factor 1-α; TTF-1, thyroid transcription factor-1; SP-A, surfactant apoprotein A; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1.

performed as follows. TMA recipient blocks were cut into 4-μm-thick sections and mounted on silane-coated slides. HE staining was performed on initial sections to verify histology. The remaining sections were deparaffinized in xylene and dehydrated in a graded alcohol series, and endogenous peroxidase was blocked using 3% hydrogen peroxide in absolute methyl alcohol. Heat-induced epitope retrieval was performed for 20 min at 95°C in 0.02 mol/L citrate buffer (pH 6.0) in samples fixed with 10% formalin if necessary. The slides were rinsed using deionized water and incubated with primary antibodies. They were then washed 3 times in phosphate-buffered saline and incubated with EnVision+ System-HRP (DAKO, Glostrup, Denmark). The reaction products were visualized using 3-3'-diaminobenzidine tetrahydrochloride, and sections were counterstained using hematoxylin. Additionally, a similar staining method was used for anti-podoplanin antibody (clone D2-40, pre-diluted; Ventana, Tucson, AZ, USA) to evaluate lymphatic permeation.

Calculation of staining scores

Immunostaining was scored based on staining intensity and percentage of positively stained cells, with 2 observers evaluating immunostained samples independently. When the observers gave different scores to immunostained samples, the slides were reviewed together under a multiheaded microscope until a consensus was reached. Sections were classified by staining intensity as negative (total absence of staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining). Staining scores were calculated by multiplying the percentage of positive tumor cells per section (0-100%) by the staining intensity; scores obtained ranged from 0 to 300. Expression of p53, cleaved caspase-3, and Ki-67 were determined by counting 300 tumor cells under a high power field (×400) and results are shown as the percentage of positive cells.

Mutation analysis

Mutation analyses of *EGFR* gene exons 19 and 21 and *KRAS* gene codons 12 and 13 were performed using loop-hybrid mobility shift assays and gene sequencing procedures described elsewhere [13].

Statistical analysis

All calculations were performed using SPSS software (Dr. SPSS II for Windows Standard version 11.0; SPSS Inc., Chicago, IL, USA). The Chi-square for independence or Fisher's exact probability test was performed to analyze differences in patient characteristics between the 2 groups. The Fisher's exact probability test was performed if there were 5 or fewer observations in a group. For univariate analysis, all cumulative survival was estimated using the Kaplan-Meier method, and differences in variables were calculated using the log-rank test. Multivariate regression analysis was conducted according to the Cox proportional hazard model. The Mann-Whitney *U* test was used to compare staining scores. Differences were considered significant when the *P* value was less than 0.05.

Results

Clinicopathological characteristics of patients with SMPC

Figure 2 shows a Venn diagram of the relationship between the micropapillary component sets in the 559 patients examined in this study. SMPC was observed in 19 patients (3.4%) and AMPC in 99 (17.7%) patients. A mixture of SMPC and AMPC was observed in 14 patients, pure SMPC without AMPC in 5 patients, and pure AMPC without SMPC in 85 patients. A micropapillary pattern was observed in 50-100% in 2 SMPC tumor and less than 50% in 17 SMPC tumors. No SMPC(+) tumors were completely replaced by SMPC. Clinicopathological characteristics of patients with SMPC(+) and SMPC(-) tumors are summarized in Table 2. Patients with SMPC(+) tumors were significantly found to be at a more advanced stage, larger than 30 mm in diameter, and have more frequent lymph node metastasis compared to those with SMPC(-) tumors. Pleural, lymphatic, and vascular invasion were observed more often in patients with SMPC(+) tumors than in those with SMPC(-) tumors. (68% vs. 17%, $P < 0.001$; 74% vs. 15%, $P < 0.001$; 74% vs. 22%, $P < 0.001$, respectively). No significant differences in age, gender,

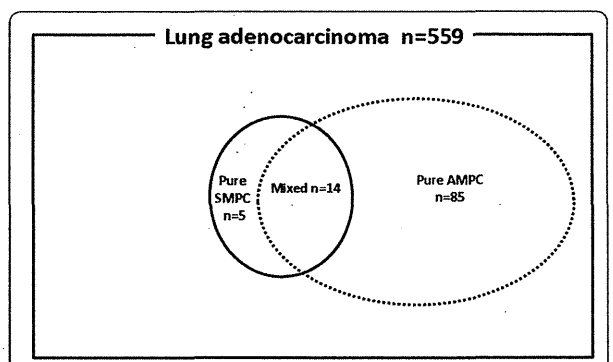

Figure 2 Venn diagram of patients included in the present study. Among the 559 cases of lung adenocarcinoma, 104 cases had MPC. Nineteen cases had SMPC (SMPC(+) tumors, the area enclosed by continuous line), and 99 had AMPC (AMPC(+) tumors, the area enclosed by dotted line). A mixture of SMPC and AMPC was observed in 14 patients, SMPC without AMPC in 5 and AMPC without SMPC in 85. MPC, micropapillary component; SMPC, stromal micropapillary component; AMPC, aerogenous micropapillary component.

Table 2 Clinicopathological characteristics of patients with SMPC

	all	%	SMPC				*P* value
			(-)	%	(+)	%	
No.	559		540	97	19	3	
Age							
Median	67		67		67		0.219*
Range	23-87		23-87		40-76		
Gender							
Female	288	52	282	52	6	32	0.077**
Male	271	48	258	48	13	68	
Smoking status							
Nonsmoker	284	51	276	51	8	42	0.596**
Smoker	275	49	264	49	11	58	
BI Average	369		364		502		
Tumor size							
Average(mm)	25		25		35		
Range(mm)	5-140		5-140		15-75		
< 30 mm	396	71	388	72	9	47	< 0.001*
≥ 30 mm	163	29	152	28	10	53	
Pathological stage							
IA	363	65	360	67	4	21	< 0.001**
IB	95	17	88	16	6	32	
IIA	36	6	31	6	5	26	
IIB	13	2	13	2	0	0	
IIIA	42	8	39	7	3	16	
≥ IIIB	10	2	9	2	1	5	
Lymph node metastasis							
NX	69	12	68	13	1	5	
N0	420	75	409	75	11	58	0.002**
≥ N1	70	13	63	12	7	21	
Pleural invasion							
Negative	452	80	446	83	6	32	< 0.001**
Positive	107	20	94	17	13	68	
Lymphatic invasion							
Negative	466	83	461	85	5	26	< 0.001**
Positive	93	17	79	15	14	74	
Vascular invasion							
Negative	427	76	422	78	5	26	< 0.001**
Positive	132	24	118	22	14	74	

* Mann-Whitney's U test

** Chi-square for independence test

No., number of patients; BI, Brinkman index = (number of cigarettes per day) × (duration of years); SMPC, stromal micropapillary component; AMPC, aerogeneous micropapillary component

or smoking status were observed between patients with SMPC(+) and SMPC(-) tumors.

Survival analysis

Among all stage patients, median follow-up time was 654 days (range, 33-1512 days) in SMPC(-) tumors, 240 days (range, 28-661 days) in SMPC(+) tumors, 664 days (range, 28-1512 days) in AMPC(-) tumors, and 467 days (range, 36-1412 days) in AMPC(+) tumors. Among the stage I patients, median follow-up time was 767 days (range, 59-1343 days) in SMPC(-) tumors, 192 days (range, 227-485 days) in SMPC(+) tumors, 767 days (range, 59-1343 days) in AMPC(-) tumors, and 836 days (range, 140-1233 days) in AMPC(+) tumors. Recurrence occurred in 28 of 559 cases. SMPC(+) tumors recurred in 4 of 19 in all stage and in 2 of 10 in p-stage I, and AMPC(+) tumors recurred in 8 of 99 cases and 4 of 69 cases, respectively. In all stage, disease-free survival (DFS) of patients with SMPC(+) tumors was significantly poorer than that in patients with SMPC(-) tumors (Figure 3A, P < 0.001); the same result was observed in patients with AMPC(+) and AMPC(-) tumors (Figure 3B, P = 0.045,). In p-stage I patients, DFS of those with SMPC(+) tumors showed significantly poorer outcome than that of patients with SMPC(-) tumors (Figure 3C, P < 0.001); the same result was observed between patients with AMPC(+) and AMPC(-) tumors (Figure 3D, P = 0.023).

In univariate analysis, high pathological stage ($P < 0.001$), pleural invasion ($P < 0.001$), lymphatic invasion ($P < 0.001$), vascular invasion ($P < 0.001$), SMPC(+) ($P < 0.001$), and AMPC(+) tumors ($P = 0.045$) showed an unfavorable influence on survival for all stage, and pleural invasion ($P < 0.001$), lymphatic invasion ($P < 0.001$), vascular invasion ($P < 0.001$), SMPC(+) ($P < 0.001$), and AMPC(+) tumors ($P = 0.023$) showed an unfavorable influence on survival for p-stage I (Table 3, 4). In multivariate analysis, pathological stage ($P = 0.028$), lymphatic invasion ($P = 0.009$), and vascular invasion ($P = 0.011$) were identified as significant independent prognostic factors for all stage (Table 3). Though not observed for all stage, the presence of SMPC(+) tumors ($P = 0.035$) was identified as a significant independent prognostic factor for p-stage I, as well as lymphatic invasion ($P = 0.020$) and vascular invasion ($P = 0.049$) (Table 4). The presence of AMPC(+) tumors was not a significant prognostic factor for all stage or p-stage I.

Immunohistochemical findings

We evaluated immunohistochemical profiles of SMPC, AMPC, and nMPC. These lesions were evaluated in TMAs for 33 cases, including 19 SMPC(+) tumors and 14 pure AMPC tumors. The latter 14 tumors were selected from 85 pure AMPC tumors according to operation date, patient age, gender, and smoking status to match clinical background factors between SMPC and AMPC. nMPC was generally included in TMA cores of SMPC and AMPC. The total number of TMA was 19 SMPC and 28 AMPC. Staining scores are summarized in Table 5.

In cellular adhesion molecules, E-cadherin staining scores in patients with SMPC, AMPC, and nMPC were

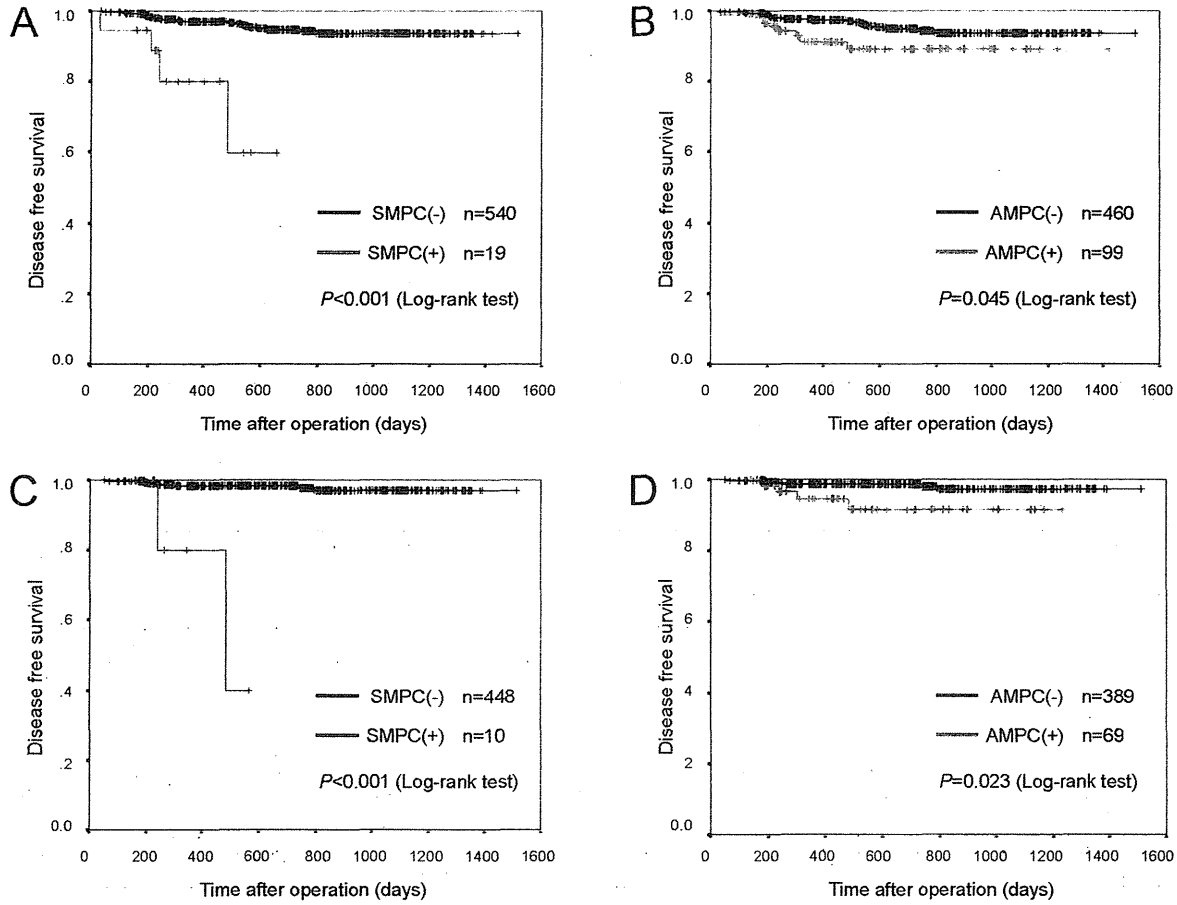

Figure 3 Cumulative disease-free survival rates of patients according to presence of SMPC and AMPC. A, B are cumulative disease-free survival rates in all stage, and C, D are that of in p-stage I. Cumulative disease-free survival rates stratified by presence of SMPC are shown in A and C; and those stratified by presence of AMPC are shown in B and D. In all stage and in p-stage I, SMPC(+) tumors and AMPC(+) tumors had significantly poorer outcomes. Outcomes of SMPC(+) tumors were more significantly negative than those of AMPC(+) tumors. SMPC, stromal micropapillary component; AMPC, aerogenous micropapillary component.

215.3, 143.9, and 187.1, respectively, and although the differences were not significant between patients with SMPC or nMPC and between patients with AMPC or nMPC (P = 0.312, 0.127, respectively), staining scores of SMPC were significantly higher than those for patients with AMPC (P = 0.020) (Figure 4A-C). CD44 staining scores in SMPC, AMPC, and nMPC were 60.8, 205.9, and 141.3, respectively. The CD44 expression level in SMPC was significantly lower than in AMPC (P < 0.001) and significantly higher than that in nMPC lesions (P = 0.015) (Figure 4D-F).

For other antibodies, staining scores of surfactant apoprotein A (SP-A) in the SMPC, AMPC, and nMPC were 45.2, 82.6, and 123.2, respectively, and although the difference was not significant between AMPC and nMPC (P = 0.203), the staining score in SMPC was significantly lower than those in nMPC (P = 0.024) (Figure 4G-I). Similarly, staining scores of phospho-c-Met in SMPC, AMPC, and nMPC were 34.2, 50.5, 88.0, respectively, and staining scores in SMPC were significantly lower than those in nMPC (Figure 4J-L).

Mutation analysis

Mutation analysis was performed in 33 patients for whom TMAs were constructed for immunohistochemical analysis. Table 6 summarizes the results of the mutation analysis. Although no cases examined possessed the *KRAS* mutations, *EGFR* mutations were detected in 20 cases (61%): 14 in patients with SMPC(+) tumors (74%) and 6 in patients with SMPC(-) tumors (43%). There was no significant association between the existence of SMPC and *EGFR* mutations. Among the 20 cases with *EGFR* mutations, 7 had deletions at exon 19, 13 had a point mutation at exon 21, and there were no

Table 3 Impact of potential prognostic factors on DFS of patients of lung adenocarcinoma in all stage by univariate and multivariate analysis

	No.	%	Univariate analysis	Multivariate analysis		
			P value	Hazard ratio	95% CI	*P* value
Total	559					
Age						
< 65	213	38	0.388	1.000		
≥ 65	346	62		1.933	0.849-4.402	0.116
Gender						
Female	288	52	0.768	1.000		
Male	271	48		0.807	0.232-2.803	0.735
Smoking status						
Non-smoker	284	49	0.560	1.000		
Smoker	275	51		1.164	0.342-3.956	0.808
Tumor size						
< 30 mm	396	71	0.059	1.000		
≥ 30 mm	163	29		0.819	0.338-1.985	0.658
Pathological stage						
I	458	82	<0.001	1.000		
II, III, IV	101	18		2.768	1.113-6.884	0.028
Pleural invasion						
Negative	452	81	<0.001	1.000		
Positive	107	19		0.848	0.345-2.083	0.719
Lymphatic invasion						
Negative	466	83	<0.001	1.000		
Positive	93	17		3.430	1.363-8.634	0.009
Vascular invasion						
Negative	427	76	<0.001	1.000		
Positive	132	24		3.309	1.312-8.350	0.011
SMPC						
Negative	540	97	<0.001	1.000		
Positive	19	3		1.871	0.528-6.630	0.332
AMPC						
Negative	460	83	0.045	1.000		
Positive	99	17		1.132	0.450-2.845	0.792

DFS, disease free survival; No., number of patients; SMPC, stromal micropapillary component; AMPC, aerogeneous micropapillary component; CI, confidence interval.

Table 4 Impact of potential prognostic factors on DFS of patients of lung adenocarcinoma in p-stage I by univariate and multivariate analysis

	No.	%	Univariate Analysis	Multivariate analysis		
			P value	Hazard ratio	95% CI	*P* value
Total	458					
Age						
< 65	172	38	0.394	1.000		
≥ 65	286	62		2.191	0.474-10.131	0.316
Gender						
Female	249	54	0.063	1.000		
Male	209	46		0.157	0.014-1.787	0.136
Smoking status						
Non-smoker	248	54	0.204	1.000		
Smoker	210	46		0.768	0.117-5.052	0.784
Tumor size						
< 30 mm	358	78	0.264	1.000		
≥ 30 mm	100	22		0.304	0.037-2.504	0.268
Pleural invasion						
Negative	402	88	< 0.001	1.000		
Positive	56	12		1.519	0.328-7.040	0.593
Lymphatic invasion						
Negative	415	91	< 0.001	1.000		
Positive	43	9		5.016	1.295-19.434	0.020
Vascular invasion						
Negative	390	85	< 0.001	1.000		
Positive	68	15		4.494	1.006-20.081	0.049
SMPC						
Negative	448	98	< 0.001	1.000		
Positive	10	2		9.028	1.164-70.031	0.035
AMPC						
Negative	389	98	0.023	1.000		
Positive	69	2		1.825	0.378-8.808	0.454

DFS, disease free survival; No., number of patients; SMPC, stromal micropapillary component; AMPC, aerogeneous micropapillary component; CI, confidence interval.

multiple mutations. Among the 13 cases with a point mutation at exon 21, 12 had an L858R mutation and one had an L861Q mutation.

Discussion

The present study revealed the incidence of SMPC(+) lung adenocarcinoma in consecutive surgical cases to be 3.4%, which is lower than that of AMPC(+) lung adenocarcinoma (17.7%). In non-pulmonary organs, the incidence of invasive micropapillary carcinoma was reported to be 7% in breast carcinoma [14], 0.9% in urinary bladder cancer [15], and 9.4% in colon cancer [16]. Generally, invasive micropapillary carcinomas occur infrequently in any organ.

Prognosis of lung adenocarcinoma with MPC has been reported to be worse and have the potential for high malignancy [17,18], but no studies have separately evaluated SMPC and AMPC. We showed that SMPC(+)