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.

Acknowledgments

This investigation was supported by Taiho Pharmaceutical. We thank the patients; their families; all the investigators who participated in the study (appendix); all of the committee members (Keisuke Aiba, Toshihiko Doi, Ichinosuke Hyodo, Atsushi Sato, Akira Tsuburaya, and Hiroyuki Uetake); and Masanobu Ito, Kenya Kosaka, and Mirai Sugiyama for their contributions to this report.

References

1 Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69–90.

2 Van Cutsem E, Nordlinger B, Cervantes A. Advanced colorectal cancer: ESMO clinical practice guidelines for treatment. *Ann Oncol* 2010; **21** (suppl 2): v93–97.

3 National Comprehensive Cancer Network. NCCN guidelines for: colon cancer (version 3.2012). 2012. http://www.nccn.org/professionals/physician_gls/f_guidelines.asp (accessed April 26, 2012).

4 Jonker DJ, O'Callaghan CJ, Karapetis CS, et al. Cetuximab for the treatment of colorectal cancer. *N Engl J Med* 2007; **357**: 2040–48.

5 Van Cutsem E, Peeters M, Siena S, et al. Open-label phase III trial of panitumumab plus best supportive care compared with best supportive care alone in patients with chemotherapy-refractory metastatic colorectal cancer. *J Clin Oncol* 2007; **25**: 1658–64.

6 Tanaka N, Oguchi K, Fujita R, et al. Trifluorothymidine incorporation into DNA strongly enforces the potential of TAS-102 and leads to remarkably prolonged survival of cancer patients. American Association for Cancer Research; Chicago, IL, USA; April 15, 2012. 1783.

7 Dexter DL, Wolberg WH, Ansfield FJ, et al. The clinical pharmacology of 5-trifluoromethyl-2'-deoxyuridine. *Cancer Res* 1972; **32**: 247–53.

8 Fukushima M, Suzuki N, Emura T, et al. Structure and activity of specific inhibitors of thymidine phosphorylase to potentiate the function of antitumor 2'-deoxyribonucleosides. *Biochem Pharmacol* 2000; **59**: 1227–36.

9 Murakami Y, Kazuno H, Emura T, Tsujimoto H, Suzuki N, Fukushima M. Different mechanisms of acquired resistance to fluorinated pyrimidines in human colorectal cancer cells. *Int J Oncol* 2000; **17**: 277–83.

10 Emura T, Murakami Y, Nakagawa F, Fukushima M, Kitazato K. A novel antimetabolite, TAS-102 retains its effect on FU-related resistant cancer cells. *Int J Mol Med* 2004; **13**: 545–49.

11 Hong DS, Abbruzzese JL, Bogaard K, et al. Phase I study to determine the safety and pharmacokinetics of oral administration of TAS-102 in patients with solid tumors. *Cancer* 2006; **107**: 1383–90.

12 Overman MJ, Varadhachary G, Kopetz S, et al. Phase 1 study of TAS-102 administered once daily on a 5-day-per-week schedule in patients with solid tumors. *Invest New Drugs* 2008; **26**: 445–54.

13 Overman MJ, Kopetz S, Varadhachary G, et al. Phase I clinical study of three times a day oral administration of TAS-102 in patients with solid tumors. *Cancer Invest* 2008; **26**: 794–99.

14 Green MC, Pusztai L, Theriault LR, et al. Phase I study to determine the safety of oral administration of TAS-102 on a twice daily (BID) schedule for five days a week (wk) followed by two days rest for two wks, every (Q) four wks in patients (pts) with metastatic breast cancer (MBC). *Proc Am Soc Clin Oncol* 2006; **24**: abstr 10576.

15 Doi T, Ohtsu A, Yoshino T, et al. Phase I study of TAS-102 treatment in Japanese patients with advanced solid tumours. *Br J Cancer* 2012; **107**: 429–34.

16 Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 2000; **92**: 205–16.

17 Sheridan C. EGFR inhibitors embrace *KRAS*. *Nat Biotechnol* 2008; **26**: 839–40.

18 Cancer Therapy Evaluation Program. Common Terminology Criteria for Adverse Events v3.0. Aug 9, 2006. http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf (accessed April 20, 2009).

19 Lievre A, Bachet JB, Boige V, et al. *KRAS* mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J Clin Oncol* 2008; **26**: 374–79.

20 Karapetis CS, Khambata-Ford S, Jonker DJ, et al. *K-ras* mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359**: 1757–65.

21 Cassidy J, Clarke S, Díaz-Rubio E, et al. XELOX vs FOLFOX-4 as first-line therapy for metastatic colorectal cancer: NO16966 updated results. *Br J Cancer* 2011; **105**: 58–64.

22 Tournigand C, André T, Achille E, et al. FOLFIRI followed by FOLFOX6 or the reverse sequence in advanced colorectal cancer: a randomized GERCOR study. *J Clin Oncol* 2004; **22**: 229–37.

23 Patel M, Bendell J, Mayer RJ, et al. A phase 1 dose-escalation study of TAS-102 in patients (pts) with refractory metastatic colorectal cancer (mCRC). *Proc Am Soc Clin Oncol* 2012; **30**: abstr 3631.

J Gastrointest Surg (2012) 16:503–508
DOI 10.1007/s11605-011-1804-3

ORIGINAL ARTICLE

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

Shuhei Komatsu · Daisuke Ichikawa · Kazuma Okamoto · Daito Ikoma · Masahiro Tsujiura · Atsushi Shiozaki · Hitoshi Fujiwara · Yasutoshi Murayama · Yoshiaki Kuriu · Hisashi Ikoma · Masayoshi Nakanishi · Toshiya Ochiai · Yukihito Kokuba · Eigo Otsuji

Received: 1 May 2011 / Accepted: 14 December 2011 / Published online: 4 January 2012

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

S. Komatsu (✉) · D. Ichikawa (✉) · K. Okamoto · D. Ikoma · M. Tsujiura · A. Shiozaki · H. Fujiwara · Y. Murayama · Y. Kuriu · H. Ikoma · M. Nakanishi · T. Ochiai · Y. Kokuba · E. Otsuji
Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine,
465 Kajii-cho, Kawaramachihirokoji, Kamigyo-ku,
Kyoto, Japan
e-mail: skomatsu@koto.kpu-m.ac.jp
e-mail: ichikawa@koto.kpu-m.ac.jp

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.

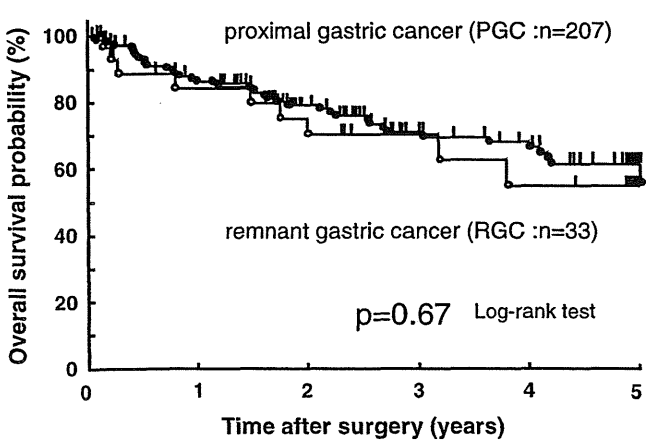

Fig. 1 Survival curves of 33 RGC and 207 PGC patients

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

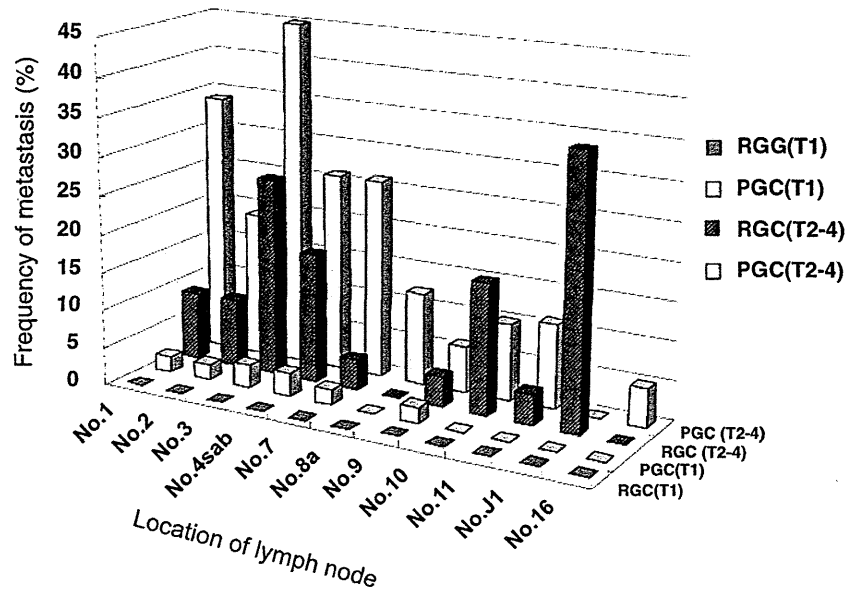

Fig. 2 Comparison of the metastatic region and extent of lymph node metastasis between RGC and PGC. Compared with that in PGC, RGC frequently showed jejunal mesentery lymph node metastasis and splenic hilar lymph node metastasis because RGC has a different pattern of lymphatic flow after the initial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

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74–108.
2. Gotoda T. Endoscopic resection of early gastric cancer. Gastric Cancer. 2007; 10:1–11.
3. Degiuli M, Sasako M, Ponti A; Italian Gastric Cancer Study Group. Morbidity and mortality in the Italian Gastric Cancer Study Group randomized clinical trial of D1 versus D2 resection for gastric cancer. Br J Surg. 2010; 97:643–649.
4. Katai H, Sasako M, Fukuda H, Nakamura K, Hiki N, Saka M, et al. JCOG Gastric Cancer Surgical Study Group. Safety and feasibility of laparoscopy-assisted distal gastrectomy with suprapancreatic nodal dissection for clinical stage I gastric cancer: a multicenter phase II trial (JCOG 0703). Gastric Cancer. 2010; 13:238–244.
5. Sasako M, Maruyama K, Kinoshita T, Okabayashi K. Surgical treatment of carcinoma of the gastric stump. Br J Surg. 1991; 78:822–824.
6. Newman E, Brennan MF, Hochwald SN, Harrison LE, Jr. Karpeh MS. Gastric remnant carcinoma: just another proximal gastric cancer or a unique entity? Am J Surg 1997; 173:292–297.
7. Japanese Gastric Cancer Association. Japanese classification of gastric carcinoma, Fourteenth Edition. (in Japanese). Tokyo: Kanehara, 2010.
8. Sobin L, Gospodarowicz M, Wittekind C, editors. International union against cancer. TNM classification of malignant tumours, Seventh Edition. New York: Wiley-Blackwell; 2010.
9. Ohashi M, Katai H, Fukagawa T, et al. Cancer of the gastric stump following distal gastrectomy for cancer. Br J Surg 2007; 94:92–95.
10. Kaneko K, Kondo H, Saito D, Shirao K, Yamaguchi H, Yokota T, et al. Early gastric stump cancer following distal gastrectomy. Gut 1998; 43:342–344.
11. Kaminishi M, Shimizu N, Shiomoyama S, Yamaguchi H, Ogawa T, Sakai S, et al. Etiology of gastric remnant cancer with special reference to the effects of denervation of the gastric mucosa. Cancer 1995; 75:1490–1496.
12. Tersmette AC, Offerhaus GJ, Tersmette KW, Giardiello FM, Moore GW, Tytgat GN, et al. Meta-analysis of the risk of gastric stump cancer: detection of high risk patient subsets for stomach cancer after remote partial gastrectomy for benign conditions. Cancer Res 1990; 50:6486–6489.
13. Ahn HS, Kim JW, Yoo MW, Park do J, Lee HJ, Lee KU, et al. Clinicopathological features and surgical outcomes of patients with remnant gastric cancer after a distal gastrectomy. Ann Surg Oncol 2008; 15:1632–1639.
14. Tanigawa N, Nomura E, Lee SW, Kaminishi M, Sugiyama M, Aikou T, et al. Society for the Study of Postoperative Morbidity after Gastrectomy. Current state of gastric stump carcinoma in Japan: based on the results of a nationwide survey. World J Surg 2010; 34:1540–1547.
15. Kodera Y, Yamamura Y, Torii A, Uesaka K, Hirai T, Yasui K, et al. Gastric stump carcinoma after partial gastrectomy for benign gastric lesion: what is feasible as standard surgical treatment? J Surg Oncol 1996; 63:119–124.
16. Maruyama K, Kaminishi M, Hayashi K, Isobe Y, Honda I, Katai H, et al. Gastric cancer treated in 1991 in Japan: data analysis of nationwide registry. Gastric Cancer 2006; 9:51–66
17. Kitano S, Shiraishi N, Uyama I, Sugihara K, Tanigawa N. Japanese Laparoscopic Surgery Study Group. A multicenter study on oncologic outcome of laparoscopic gastrectomy for early cancer in Japan. Ann Surg 2007; 245:68–72.
18. Nakayoshi T, Tajiri H, Matsuda K, Kaise M, Ikegami M, Sasaki H. Magnifying endoscopy combined with narrow band imaging system for early gastric cancer: correlation of vascular pattern with histopathology (including video). Endoscopy 2004; 36:1080–1084.
19. Takenaka R, Kawahara Y, Okada H, Tsuzuki T, Yagi S, Kato J, et al. Endoscopic submucosal dissection for cancers of the remnant stomach after distal gastrectomy. Gastrointest Endosc 2008; 67:359–363.
20. Hirasaki S, Kanzaki H, Matsubara M, Fujita K, Matsumura S, Suzuki S. Treatment of gastric remnant cancer post distal gastrectomy by endoscopic submucosal dissection using an insulation-tipped diathermic knife. World J Gastroenterol 2008; 14:2550–2555.
21. An JY, Choi MG, Noh JH, Sohn TS, Kim S. The outcome of patients with remnant primary gastric cancer compared with those having upper one-third gastric cancer. Am J Surg 2007; 194:143–147.
22. Schaefer N, Sinning C, Standop J, Overhaus M, Hirner A, Wolff M. Treatment and prognosis of gastric stump carcinoma in comparison with primary proximal gastric cancer. Am J Surg 2007; 194:63–67
23. Han SL, Hua YW, Wang CH, Ji SQ, Zhuang J. Metastatic pattern of lymph node and surgery for gastric stump cancer. J Surg Oncol 2003; 82:241–246.
24. Kunisaki C, Shimada H, Nomura M, Hosaka N, Akiyama H, Ookubo K, et al. Lymph node dissection in surgical treatment for remnant stomach cancer. Hepatogastroenterology 2002; 49:580–584.
25. Yonemura Y, Ninomiya I, Tsugawa K, Masumoto H, Takamura H, Fushida S, et al. Lymph node metastases from carcinoma of the gastric stump. Hepatogastroenterology 1994; 41:248–252.

DIAGNOSTIC PATHOLOGY

RESEARCH Open Access

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

Miki Ohe[1,2,5], Tomoyuki Yokose[1*], Yuji Sakuma[3], Yohei Miyagi[3], Naoyuki Okamoto[4], Sachie Osanai[1], Chikako Hasegawa[1], Haruhiko Nakayama[2], Yoichi Kameda[1], Kouzo Yamada[2] and Takeshi Isobe[5]

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

* Correspondence: yokose-t@kcch.jp
[1]Department of Pathology, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa, 241-0815, Japan
Full list of author information is available at the end of the article

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

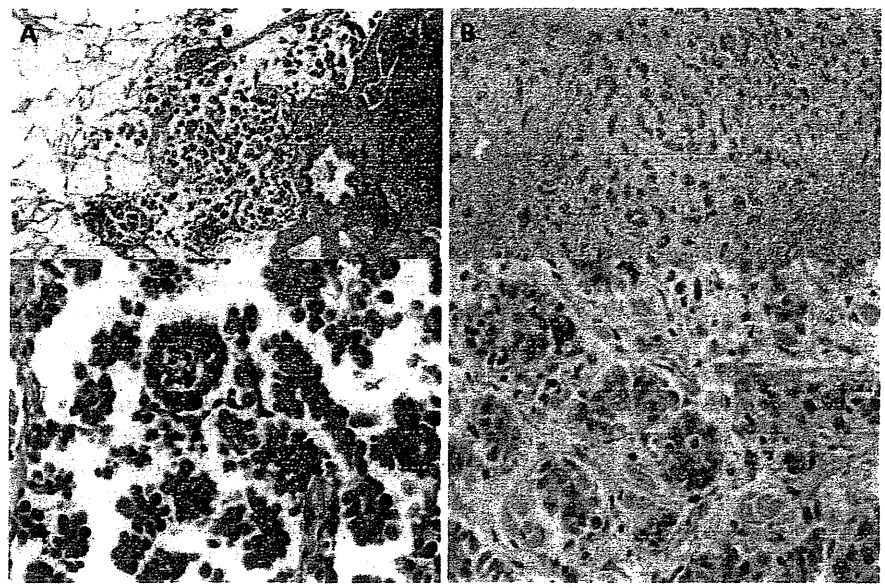

Figure 1 Microscopic features of micropapillary component in the lung adenocarcinoma stained with hematoxylin-eosin (HE). A, AMPC. AMPC is the micropapillary component in which tumor cells are floating within alveolar spaces. B, SMPC. SMPC are tumor cells observed in the stroma and consisting of a papillary 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,

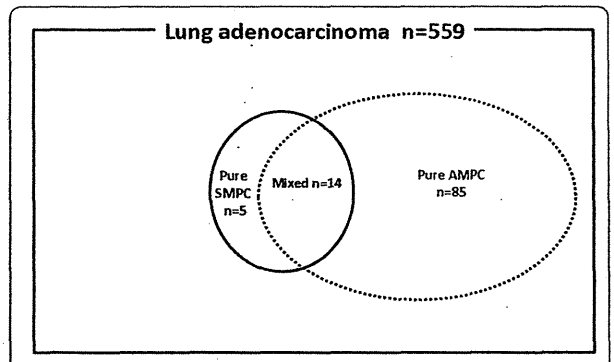

Figure 2 Venn diagram of patients included in the present study. Among the 559 cases of lung adenocarcinoma, 104 cases had MPC. Nineteen cases had SMPC (SMPC(+) tumors, the area enclosed by continuous line), and 99 had AMPC (AMPC(+) tumors, the area enclosed by dotted line). A mixture of SMPC and AMPC 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

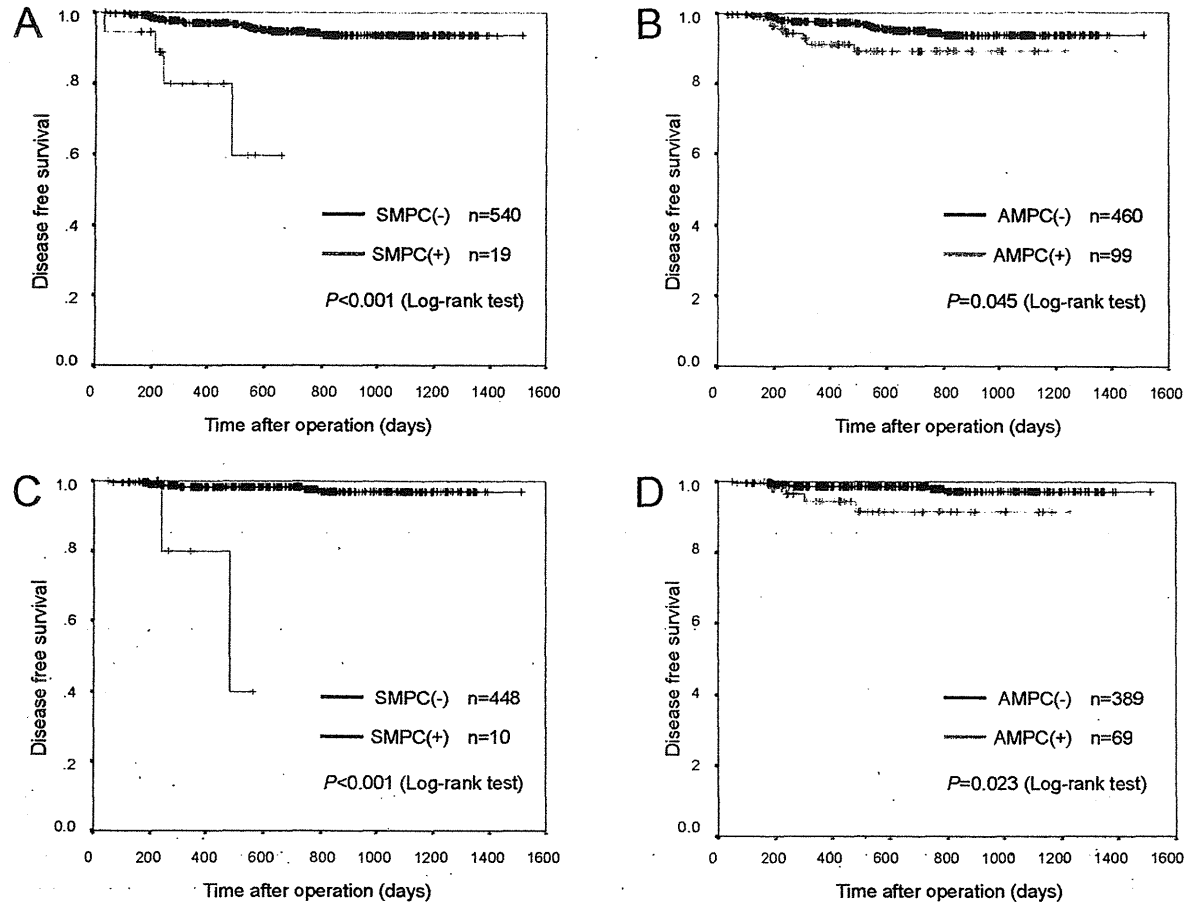

Figure 3 Cumulative disease-free survival rates of patients according to presence of SMPC and AMPC. A, B are cumulative disease-free survival rates in all stage, and C, D are that of in p-stage I. Cumulative disease-free survival rates stratified by presence of SMPC are shown in A and C; and those stratified 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(+)

Table 5 Staining Scores in SMPC, AMPC and nMPC lesions

Classification/Antibody	SMPC	AMPC	nMPC
Cellular adhesion molecules			
E-cadherin	215.3*	143.9	187.1
CD44	60.8‡	205.9¶	141.3
Laminin5γ2	69.4	36.9	60.3
Growth factor			
VEGF-C	294.4	296.4	282.1
Apoptosis-associated proteins			
bcl2	13.2	11.1	21.8
p53§	36.4	26.1	45.0
cleaved caspase-3§	0.3	0.2	0.4
Mucin-related proteins			
MUC1	169.7	182.5	202.1
MUC6	0.0	0.0	0.0
Hypoxia induced protein			
HIF-1α	1.8	2.4	2.9
Others			
TTF-1	267.9	289.3	248.6
SP-A	45.2‡	82.6	123.2
Vimentin	112.1	117.9	72.1
Ki-67§	22.6	16.9	16.2
LYVE1	98.9	107.2	101.9
c-Met	217.2	253.8	211.4
Phospho-c-Met	34.2‡	50.0	88.0

SMPC, stromal micropapillary component; AMPC, aerogeneous micropapillary component; nMPC, non-micropapillary component; 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.

§ Positivity rate of positive tumor cells in 300 tumor cells (percentage).

* The difference in staining scores between SMPC and AMPC is statistically significant ($P = 0.020$).

‡ The differences in staining scores between SMPC and nMPC are statistically significant for CD44 ($P = 0.011$), and SP-A ($P = 0.024$) and phospho-c-Met ($P = 0.011$) expression.

¶ The difference in staining scores between AMPC and nMPC is statistically significant ($P = 0.015$).

tumors as well as AMPC(+) tumors are associated with several biological factors including tumor size, lymph node metastasis, advanced stage disease, and pleural and lymphovascular invasion. Univariate analysis also revealed the presence of SMPC and AMPC as a significant predictor of unfavorable outcome. However, the most remarkable finding was observed in multivariate analysis: among the patients in p-stage I, patients with not AMPC but SMPC showed a significantly poorer DFS than those without MPC. We used immunohistochemistry with monoclonal antibody D2-40 against lymphatic endothelium in TMA specimens and found that lymphatic vessels are involved within SMPC areas in 4 (21%) of 19 SMPC(+) tumors (data not shown). When compared with AMPC(+) tumors, SMPC(+) tumors significantly more often showed pleural, lymphatic, and vascular invasion than AMPC(+) tumors (68% vs. 33%, $P = 0.004$; 74% vs. 30%, $P < 0.001$; 74% vs. 41%, $P = 0.010$, respectively). Therefore, these data suggest that a strong association between SMPC(+) tumors and pleural and lymphovascular invasion may in part explain their aggressive behavior.

Moreover, we investigated the immunohistochemical differences between SMPC and AMPC. In the study, we observed high E-cadherin expression and low CD44 expression in SMPC. Phospho-c-Met expression generally decreases in SMPC to a greater extent than in AMPC. Recently, it has been suggested that E-cadherin repression and CD44 expression are associated with the epithelial-mesenchymal transition (EMT), which was thought to lead to tumor invasion [19,20]. Additionally, Elliot et al. reported that hepatocyte growth factor (HGF) and c-Met signaling promotes EMT in breast cancer [21], and Orian-Rousseau et al. reported that CD44 is strictly required for c-Met activation by HGF in human carcinoma [22]. Consistent with these data, EMT may not occur in SMPC despite its existence in the stroma, or invasion of SMPC may occur through a different invasion mechanism from EMT. Our immunohistochemical findings of SMPC showed lower expression of SP-A than that of nMPC. Many studies have reported that SP-A deletion is correlated with patient survival, and reduced SP-A in MPC may be an excellent indicator for poor prognosis in small-size lung adenocarcinoma [23,24]. Reduced SP-A may contribute to an unfavorable outcome of SMPC(+) tumors.

Some studies have reported a significant association between the presence of MPC and *EGFR* mutations and effectiveness of EGFR tyrosine kinase inhibitor (EGFR-TKI) for MPC(+) tumors [25-28]. Since SMPC of lung adenocarcinoma may be associated with a high incidence of *EGFR* mutations, EGFR-TKI may be effective against SMPC(+) tumors. Patients with these pathological features of lung adenocarcinoma may benefit from EGFR-TKI as postoperative chemotherapy or first-line chemotherapy of relapsed lung adenocarcinoma.

In conclusion, we observed SMPC(+) adenocarcinoma. The incidence of SMPC(+) tumors is low, and SMPC(+) tumors have a different prognostic impact compared to AMPC(+) tumors. Particularly for the early stage tumors, SMPC(+) tumors have different pathobiological characteristics from AMPC(+) tumors, and SMPC(+) tumors frequently contain the *EGFR* mutation. Therefore, it is important to determine the presence of SMPC in lung adenocarcinoma, particularly p-stage I tumors, and the presence of SMPC should be noted in a pathology report to alert the clinician to the possibility of poor prognosis.

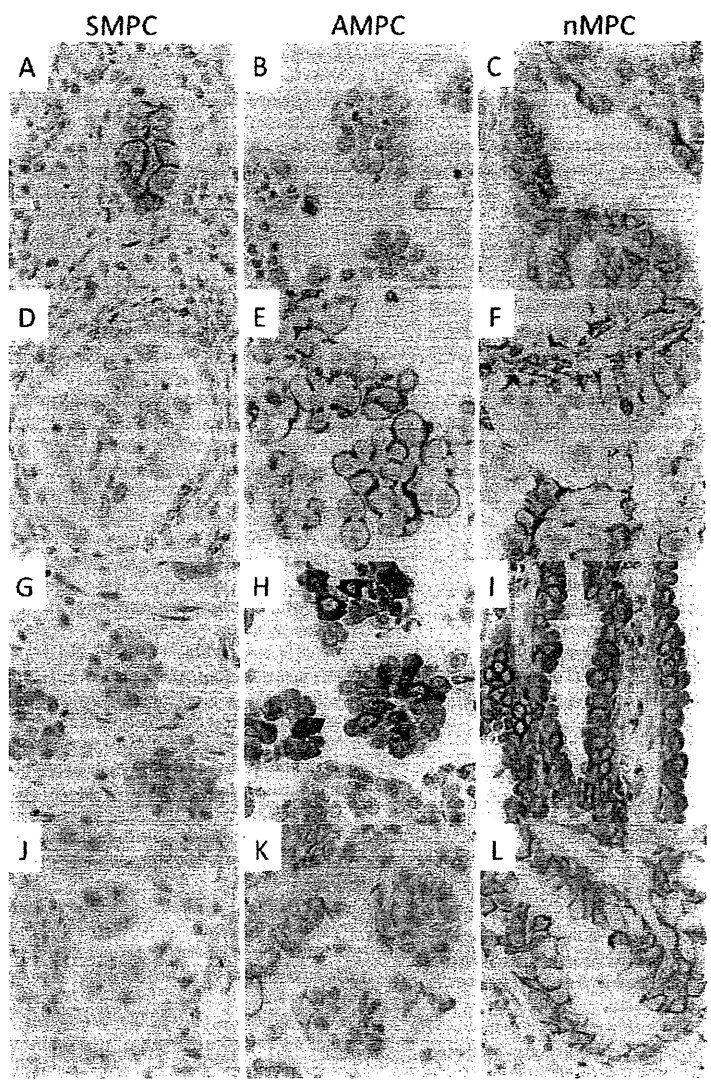

Figure 4 Photomicrographs of immunohistochemistry. E-cadherin (A-C); CD44 (D-F); SP-A (G-I); Phospho-c-Met (J-L). Compared with AMPC, increased E-cadherin and decreased CD44 membrane immunostaining were found in SMPC. Moreover, SP-A cytoplasm and Phospho-c-Met membrane immunostaining were decreased in SMPC (×400). SMPC, stromal micropapillary component, left panels; AMPC, aerogenous micropapillary component, middle panels; nMPC, non-micropapillary component, right panels.

Table 6 Mutation analysis

	total	%	SMPC(+) cases	%	SMPC(-) cases	%	*P* value
No.	33		19		14		
EGFR mutation							
Negative	13	39	5	26	8	57	0.076*
Positive	20	61	14	74	6	43	
ex19	7	35§	5	36§	2	33§	0.664*
ex21	13	65§	9	64§	4	67§	
KRAS mutation							
Negative	33	100	19	100	14	100	-
Positive	0	0	0		0		

No., number of patients; EGFR, epidermal growth factor receptor; SMPC, stromal micropapillary component

* Fisher's exact probability test

§ Rate of positive cases at ex19 and 21 in *EGFR* mutation positive cases, respectively.

List of abbreviations

AMPC: aerogenous micropapillary component; SMPC: stromal micropapillary component; MPC: micropapillary component; TMA: tumor tissue microarray; DFS: disease-free survival.

Acknowledgements

The authors thank Ms. Yoshihara for TMA synthesis.
This article was supported by Kanagawa Cancer Research Fund and Grant for Collaboration between Hospital and Research Institute, Kanagawa Cancer Center.

Author details

[1]Department of Pathology, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa, 241-0815, Japan. [2]Department of Thoracic Oncology, Kanagawa Cancer Center, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa, 241-0815, Japan. [3]Molecular Pathology and Genetics Division, Kanagawa Cancer Center Research Institute, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa, 241-0815, Japan. [4]Cancer Prevention and Cancer Control Division, Kanagawa Cancer Center Research Institute, 1-1-2 Nakao, Asahi-ku, Yokohama, Kanagawa, 241-0815, Japan. [5]Division of Clinical Oncology and Respiratory Medicine, Department of Internal Medicine, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane, 693-8501, Japan.

Authors' contributions

MO and TY designed the study, performed clinical and pathological investigation, and wrote the drafts. YS and YM participated in pathological and genetical investigation. NO participated in statistical investigation. SO performed the histological and immunohistochemical evaluation. CH assisted the clinical investigation. HN participated in managing and operating the patients. YK assisted the pathological investigation. KY participated in collecting clinical data and images. TI participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 3 November 2011 Accepted: 6 January 2012
Published: 6 January 2012

References

1. Travis WD, *et al*: **International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society international multidisciplinary classification of lung adenocarcinoma.** *J Thorac Oncol* 2011, 6:244-285.
2. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MK, Park BJ, Rusch VW, Travis WD: **Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases.** *Mod Pathol* 2011, 24:653-664.
3. Amin MB, Tamboli P, Merchant SH, Ordóñez NG, Ro J, Ayala AG, Ro JY: **Micropapillary component in lung adenocarcinoma: a distinctive histologic feature with possible prognostic significance.** *Am J Surg Pathol* 2002, 26:358-364.
4. Miyoshi T, Satoh Y, Okumura S, Nakagawa K, Shirakusa T, Tsuchiya E, Ishikawa Y: **Early-stage lung adenocarcinoma with micropapillary pattern, a distinct pathologic marker for a significantly poor prognosis.** *Am J Surg Pathol* 2003, 27:101-109.
5. Maeda R, Isowa N, Onuma H, Miura H, Harada T, Touge H, Tokuyasu H, Kawasaki Y: **Lung adenocarcinomas with micropapillary components.** *Gen Thorac Cardiovasc Surg* 2009, 57:534-539.
6. Nagano T, Ishii G, Nagai K, Ito T, Kawase A, Takahashi K, Nishimura Y, Noshiwaki Y, Ochiai A: **Structural and biological properties of a papillary component generating a micropapillary component in lung adenocarcinoma.** *Lung Cancer* 2010, 67:282-289.
7. Roh MS, Lee JI, Choi PJ, Hong YS: **Relationship between micropapillary component and micrometastasis in the regional lymph nodes of patients with stage I lung adenocarcinoma.** *Histopathology* 2004, 45:580-586.
8. Kamiya K, Hayashi Y, Douguchi J, Hashiguchi A, Yamada T, Izumi Y, Watanabe M, Kawamura M, Horinouchi H, Shimada N, Kobayashi K, Sakamoto M: **Histopathological features and prognostic significance of the micropapillary pattern in lung adenocarcinoma.** *Mod Pathol* 2008, 21:992-1001.
9. Ohe M, Yokose T, Sakuma Y, Osanai S, Hasegawa C, Washimi K, Nawa K, Woo T, Hamanaka R, Nakayama H, Kameda K, Yamada K, Isobe T: **Stromal micropapillary pattern predominant lung adenocarcinoma - A report of two cases.** *Diagn Pathol* 2011, 6:92.
10. Kuroda N, Hamaguchi N, Takeuchi E, Ohara M, Hirouchi T, Mizuno K: **Lung adenocarcinoma with a micropapillary pattern: a clinicopathological study of 25 cases.** *APMIS* 2006, 114:381-385.
11. In *TNM classification of malignant tumors*.. seventh edition. Edited by: Sobin LH, Gospodarowicz MK, Wittekind C. Wiley-Blackwell; 2009:.
12. Tsuta K, Ishii G, Nitadori J, Murata Y, Kodama T, Nagai K, Ochiai A: **Comparison of the immunophenotypes of signet-ring cell carcinoma, solid adenocarcinoma with mucin production, and mucinous bronchioloalveolar carcinoma of the lung characterized by the presence of cytoplasmic mucin.** *J Pathol* 2006, 209:78-87.
13. Matsukuma S, Yoshihara M, Kasai F, Kato A, Yoshida A, Akaike M, Kobayashi O, Nakayama H, Sakuma Y, Yoshida T, Kameda Y, Tsuchiya E, Miyagi Y: **Rapid and simple detection of hot spot point mutations of epidermal growth factor receptor, BRAF, and NRAS in cancers using the loop-hybrid mobility shift assay.** *J Mol Diagn* 2006, 8:504-512.
14. Walsh MM, Bleiweiss IJ: **Invasive micropapillary carcinoma of the breast: eighty cases of an under-recognized entity.** *Hum Pathol* 2001, 32:583-589.
15. Johansson SL, Borghede G, Holmang S: **Micropapillary bladder carcinoma: a clinicopathological study of 20 cases.** *J Urol* 1999, 161:1798-1802.
16. Kim M, Hong S, Jang S, Yu E, Kim J, Kim K, Gong G, Ro J: **Invasive colorectal micropapillary carcinoma: an aggressive variant of adenocarcinoma.** *Hum Pathol* 2006, 37:809-815.
17. Nora S, Presmanes MC, Monroy V, Alandro MH, Alvarez-Fernandez E: **Micropapillary lung adenocarcinoma: a distinctive histologic subtype with prognostic significance. Cases series.** *Hum Pathol* 2008, 39:324-330.
18. Kamiya K, Hayashi Y, Douguchi J, Hashiguchi A, Yamada T, Izumi Y, Watanabe M, Kawamura M, Horinouchi H, Shimada N, Kobayashi K, Sakamoto M: **Histopathological features and prognostic significance of the micropapillary pattern in lung adenocarcinoma.** *Mod Pathol* 2008, 21:992-1001.
19. Nieto MA: **Epithelial-mesenchymal transitions in development and disease: old views and new perspectives.** *Int J Dev Biol* 2009, 53:1541-1547.
20. Ponta H, Sherman L, Herrlich PA: **CD44: from adhesion molecules to signaling regulators.** *Nat Rev Mol Cell Biol* 2003, 4:33-45.
21. Elliot BE, Hung WL, Boag AH, Tuck AB: **The role of hepatocyte growth factor (scatter factor) in epithelial-mesenchymal transition and breast cancer.** *Can J Physiol Pharmacol* 2002, 80:91-102.
22. Orian Rousseau V, Chen L, Sleeman JP, Herrlich P, Ponta H: **CD44 is required for two consecutive steps in HGF/c-Met signaling.** *Genes Dev* 2002, 16:3074-3086.
23. Jiang F, Caraway NP, Bekele BN, Zhang HZ, Khanna A, Wang H, Li R, Fernancez RL, Zaidi TM, Johnston DA, Katz RL: **Surfactant protein A gene deletion and prognostics for patients with stage I non-small cell lung cancer.** *Clin Cancer Res* 2005, 11:5417-5424.
24. Tsutsumida H, Nomoto M, Goto M, Kitajima S, Kubota I, Hirotsu Y, Wakimoto J, Hollingsworth MA, Yonezawa S: **A micropapillary pattern is predictive of a poor prognosis in lung adenocarcinoma, and reduced surfactant apoprotein A expression in the micropapillary pattern is an excellent indicator of a poor prognosis.** *Modern Pathol* 2007, 20:638-647.
25. Ninomiya H, Hiramatsu M, Inamura K, Nomura K, Okui M, Miyoshi T, Okumura S, Satoh Y, Nakagawa K, Nishio M, Horai T, Miyata S, Tsuchiya E, Fukuyama E, Ishikawa Y: **Correlation between morphology and EGFR mutations in lung adenocarcinomas Significance of the micropapillary pattern and the hobnail cell type.** *Lung Cancer* 2009, 63:235-240.
26. Inamura K, Ninomiya H, Ishikawa Y, Matsubara O: **Is the epidermal growth factor receptor status in lung cancers reflected in clinicopathologic features?** *Arch Pathol Lab Med* 2010, 134:66-72.
27. Motoi N, Szoke J, Riely GJ, Seshan VE, Kris MG, Rusch VW, Gerald WL, Travis WD: **Lung adenocarcinoma: modification of the 2004 WHO mixed subtype to include the major histologic subtype suggests correlations between papillary and micropapillary adenocarcinoma subtypes, EGFR mutations and gene expression analysis.** *Am J Surg Pathol* 2008, 32:810-827.

28. Kim YH, Ishii G, Goto K, Nagai K, Tsuta K, Shiono S, Nitadori J, Kodama T, Nishiwaki Y, Ochiai A: **Dominant papillary subtype is a significant predictor of the response to gefitinib in adenocarcinoma of the lung.** *Clin Cancer Res* 2004, 10:7311-7317.

doi:10.1186/1746-1596-7-3
Cite this article as: Ohe *et al.*: Stromal micropapillary component as a novel unfavorable prognostic factor of lung adenocarcinoma. *Diagnostic Pathology* 2012 7:3.

Leukemia Research 35 (2011) 1205–1211

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

Predictability of the response to tyrosine kinase inhibitors via *in vitro* analysis of Bcr-Abl phosphorylation

Masaru Shibata[a], Sachiko Ezoe[a,*], Kenji Oritani[a], Keiko Matsui[a], Masahiro Tokunaga[a], Natsuko Fujita[a], Yuri Saito[a], Takayuki Takahashi[b], Masayuki Hino[c], Itaru Matsumura[d], Yuzuru Kanakura[a]

[a] *Hematology and Oncology, Osaka University, Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[b] *Kobe City Medical Center General Hospital, Kobe, Japan*
[c] *Department of Hematology, Graduate School of Medicine, Osaka City University, Osaka, Japan*
[d] *Department of Internal Medicine, Kinki University School of Medicine, Sayama, Japan*

ARTICLE INFO

Article history:
Received 16 December 2010
Received in revised form 12 January 2011
Accepted 14 January 2011
Available online 26 March 2011

Keywords:
CML
Tyrosine kinase inhibitor
Bcr-Abl
Crkl
Phosphorylation
Immunoblot

ABSTRACT

It would be of great value to predict the efficacy of tyrosine kinase inhibitors (TKIs) in the treatment of individual CML patients. We propose an immunoblot system for detecting the phosphorylation of Crkl, a major target of Bcr-Abl, in blood samples after *in vitro* incubation with TKIs. When the remaining phosphorylated Crkl after treatment with imatinib was evaluated as the "residual index (RI)", high values were found in accordance with imatinib resistance. Moreover, RI reflected the outcome of imatinib- as well as second generation TKIs with a high sensitivity and specificity. Therefore, this system should be useful in the selection of TKIs.

1. Introduction

The introduction of tyrosine kinase inhibitors (TKIs) targeting Bcr-Abl have dramatically improved the treatment of CML. Imatinib mesylate (Gleevec; Novartis Pharmaceuticals, East Hanover, NJ) was shown to induce high rates of cytogenetic and molecular responses, resulting in greatly prolonged survival in CML patients [1,2]. However, despite the remarkable improvement in survival and responsiveness with imatinib-treatment, a considerable proportion of the patients treated with imatinib have been reported to exhibit either primary or secondary resistance or intolerance [3–5]. Clinical resistance to imatinib can result from mutations in the Abl kinase domain at residues that directly contact imatinib or that influence imatinib binding [6]. As resistance can also arise in the absence of Bcr-Abl mutations, other mechanisms of resistance and disease progression may exist, including Bcr-Abl-independent signaling in CML cells [7]. To overcome the resistance and intolerance to imatinib, efforts have been made to develop second- and third-generation TKIs. Examples of such inhibitors include nilotinib (Tasigna, Novartis) [8], dasatinib (Sprycel, Bristol-Myers Squibb) [9] and other TKIs under clinical investigation such as bosutinib [10] and INNO-406 [11]. These TKIs are significantly more potent than imatinib and have exhibited efficacy against many types of imatinib-resistant Bcr-Abl mutants. Furthermore, they are also candidates for first-line therapy, as there is a need to improve the results achieved with imatinib [12–14]. In parallel with the entrance of new therapeutic compounds, an important question is which TKI is the most appropriate to each CML patient.

To establish a system with which we can predict the response of each patient to TKIs, we investigated in this study the phosphorylation of Crkl, a major target of Bcr-Abl, after *in vitro* incubation with or without TKIs in peripheral blood (PB) samples from patients either newly diagnosed or resistant to imatinib. It is demonstrated that this *in vitro* analysis system is highly reflective of the clinical response to TKIs of CML patients, and these data should prove useful in selecting TKIs in individual cases.

2. Patients, materials and methods

2.1. Patient blood samples

Thirty-one patients with CML in the chronic phase (CP) were included in this study (Table 1). The optimal response, response and resistance were defined in accordance with the European Leukemia Net (ELN) recommendations [15,16]. Briefly, an "optimal response" to imatinib means achieving a complete hematological response (CHR) at 3 months or complete cytogenetic response (CCyR) at

* Corresponding author. Tel.: +81 6 6879 3871; fax: +81 6 6879 3879.
E-mail address: sezoe@bldon.med.osaka-u.ac.jp (S. Ezoe).

0145-2126/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.leukres.2011.01.012

6 months after the induction of imatinib, and resistance means failure to achieve such a response. On the other hand, in nilotinib- or dasatinib-treated patients, a "response" means a minor cytogenetic response (mCyR) at 3 months or partial cytogenetic response (PCyR) at 6 months after the induction of the second generation TKI, and resistance means failure to achieve this response.

Ten microliters of the PB samples were obtained from patients with informed consent at the beginning or before the initiation of imatinib, nilotinib or dasatinib. Half of each sample was used for examination of the Bcr-Abl sequence, which was performed by the SRL Co. (Tokyo, Japan), and the other half was used for immunoblot analysis.

Approvals for the study were obtained from the institutional review boards of all the participating facilities.

2.2. Reagents

Imatinib, methanesulfonate salt was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland), and nilotinib and dasatinib were purchased from LC laboratories (Boston, MA). The antibodies used in this study were as follows: anti-Lyn, anti-phospho-Crkl, anti-phospho-c-Abl from Cell Signaling Technology (Beverly, MA), anti-phospho-Lyn(Y396) from Epitomics (Burlingame, CA), anti-Crkl, anti-β-actin from Santa Cruz Biotechnology (Santa Cruz, CA), and the secondary antibodies, anti-Rabbit IgG HRP and anti-Goat IgG HRP were from Promega (Madison, WI). Pervanadate was purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Cell line

A Bcr-Abl positive human cell line, K562, was used in the preliminary experiments in this study. K562 cells were maintained in RPMI1640 (nacalai tesque, Kyoto, Japan) supplemented with 10% fetus bovine serum (FBS) (EQUITECH-BIO, Kerrville, TX).

2.4. Immunoblot assays of patients' samples

Whole blood cell samples from patients were used within 3 h after blood had been drawn. Red cells were lysed with Whole Blood Lysing Reagents (Beckman Coulter, Brea, CA), and white blood cells were cultured with or without imatinib, nilotinib or dasatinib. After 5-h incubation, the cell lysates were collected and subjected to immunoblot assays. Gel electrophoresis and immunoblot assays were performed according to methods described previously [17,18]. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (PerkinElmer Life Sciences, Boston, MA).

2.5. Evaluation of phosphorylation intensity and determination of the "residual index (RI)"

The intensity of each blot of immunoreactive protein was quantified using ChemiDoc XRS+ with Image Lab Software (Bio Rad, Tokyo Japan). The RI values of each patient to TKIs were determined in accordance with the numerical expression, as indicated in Fig. 2A.

2.6. Statistical analysis

Analysis of variance was used to assess data reproducibility. The Mann–Whitney rank sum was used to define differences between groups.

3. Results

3.1. Immunoblot analysis of phosphorylated Crkl in CML patients

To assess the drug response of the CML patients, we performed immunoblot assays detecting phosphorylated Crkl, a direct target of Bcr-Abl kinase. To establish the experimental procedures, preliminary experiments were performed with K562, a CML blast crisis cell line, or blood sample from a newly diagnosed CML patient (Patient A), 98% of whose PB cells were Bcr-Abl-positive on fluorescence *in situ* hybridization (FISH). First, to determine the optimum incubation period for the TKIs, PB cells were incubated with or without TKIs for varying time periods. A two-hour incubation was not sufficient because imatinib did not completely suppress the phosphorylation of Crkl, while 24-h incubation was too long because the PB neutrophils appeared to die (Fig. 1A, left panel). A five-hour incubation completely eliminated the phosphorylation of Crkl without cell death. On the other hand, simultaneous treatment with a phosphatase inhibitor sustained the phosphorylation of Crkl even after treatment for 24 h (Fig. 1A, right panel). Thus, we decided to incubate cells for 5 h without phosphatase inhibitors. Next, to build an *in vitro* simulation model for the estimation of the activities of TKIs in the body, we fixed the concentrations of TKIs at the peak value of plasma concentrations in patients (Cmax) after administration of the recommended dose of TKIs. The Cmax of imatinib in CML patients after taking orally 400 mg of the drug is 3.0–4.8 μM, and that of nilotinib after taking 400 mg is 2.9–4.0 μM. In the case of

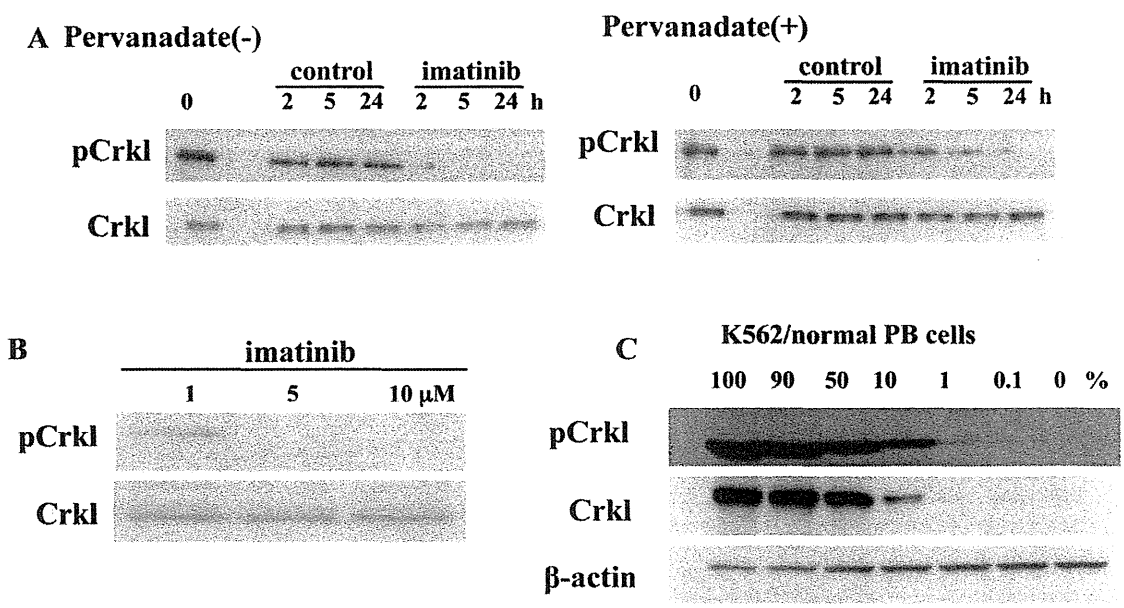

Fig. 1. Optimization of western blot after TKI-incubation. (A and B) Blood sample from Patient A was incubated with or without 5 μM imatinib supplemented with (right panel) or without (left panel) 10 μM of pervanadate for the indicated periods (A) or incubated with imatinib at the indicated concentrations for 5 h (B). The treated cells were lysed and subjective to immunoblot analysis using the indicated antibodies. (C) K562 cells were mixed into normal human PB cells at the indicated ratios. Then the samples were subjective to immunoblot analysis.