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Development and external validation of a nomogram for overall survival after curative resection in serosa-negative, locally advanced gastric cancer

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Background: Few nomograms can predict overall survival (OS) after curative resection of advanced gastric cancer (AGC), and these nomograms were developed using data from only a few large centers over a long time period. The aim of this study was to develop and externally validate an elaborative nomogram that predicts 5-year OS after curative resection for serosa-negative, locally AGC using a large amount of data from multiple centers in Japan over a short time period (2001–2003).

Patients and methods: Of 39 859 patients who underwent surgery for gastric cancer between 2001 and 2003 at multiple centers in Japan, we retrospectively analyzed 5196 patients with serosa-negative AGC who underwent Resection A according to the 13th Japanese Classification of Gastric Carcinoma. The data of 3085 patients who underwent surgery from 2001 to 2002 were used as a training set for the construction of a nomogram and Web software. The data of 2111 patients who underwent surgery in 2003 were used as an external validation set.

Results: Age at operation, gender, tumor size and location, macroscopic type, histological type, depth of invasion, number of positive and examined lymph nodes, and lymphovascular invasion, but not the extent of lymphadenectomy, were associated with OS. Discrimination of the developed nomogram was superior to that of the TNM classification (concordance indices of 0.68 versus 0.61; $P < 0.001$). Moreover, calibration was accurate.

Conclusions: We have developed and externally validated an elaborative nomogram that predicts the 5-year OS of post-operative serosa-negative AGC. This nomogram would be helpful in the assessment of individual risks and in the consideration of additional therapy in clinical practice, and we have created freely available Web software to more easily and quickly predict OS and to draw a survival curve for these purposes.

Key words: advanced gastric cancer, external validation, multivariable analysis, nomogram, prognosis, survival analysis

introduction

Gastric cancer was the second leading cause of cancer death in both sexes worldwide in 2008 (736 000 deaths, 9.7% of the total) [1]. In Japan, 49 830 people died of gastric cancer in 2011, ranking gastric cancer second among all cancer deaths [2]. The prognosis of gastric cancer greatly differs according to the spread or characteristics of the tumor. The identification of prognostic factors of gastric cancer is useful for clinical decision-making and improved survival rates [3].

The American Joint Committee on Cancer (AJCC) classified gastric cancer into nine groups in the seventh edition of their classification system [4]. The classification is based on the depth of invasion, number of metastatic lymph nodes, and metastasis. This system represents an excellent common language in the field of gastric cancer, but it does not include other prognostic factors such as age, tumor size, or other tumor characteristics [3, 5–9].

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A nomogram is a useful tool that predicts the probability of a clinical event via a simple, clear figure and is widely used in the field of cancer [10]. A few good nomograms that predict the survival rate or recurrence of gastric cancer have been developed [11–13]. However, most nomograms of gastric cancer were developed using data from only a few large centers over a long time period (from the 1980s to 2000s); such a long time period introduces external variations in long-term outcomes [14].

The aim of this study was to develop and externally validate a more elaborative nomogram that predicts 5-year overall survival (OS) in patients who have undergone curative resection with a high probability of cure (namely, Resection A) for serosa-negative, locally advanced gastric cancer (AGC). We obtained data from multiple centers in Japan during a short time period (2001–2003). This short time period enables us to reduce bias that arises from variation in survival outcomes between centers and during different time periods. We selected patients with serosa-negative, locally AGC who underwent Resection A, because such patients were considered to have a better prognosis and might benefit more from adjuvant therapy than would other patient populations. We expected that this nomogram could contribute to the selection of good candidates for adjuvant therapy. For the analysis, we adopted a wide variety of factors regarded as prognostic factors to enhance the accuracy of prediction [3, 5–9].

patients and methods

patients

The Japanese Gastric Cancer Association (JGCA) collects data annually from multiple centers in Japan. Of 39 859 patients who underwent gastric cancer surgery from 2001 to 2003 at multiple centers in Japan, we collected the data of 5196 patients with AGC who underwent Resection A without missing values on predicted prognostic factors. The data of 3085 patients who underwent surgery from 2001 to 2002 were used as a training set for the construction of a nomogram and Web software. The data of 2111 patients who underwent surgery in 2003 were used as an external validation set.

factors

The definition and documentation of factors were based on the Japanese Classification of Gastric Carcinoma, 13th edition [15]. To predict the probability of 5-year OS, we used the following variables: age at operation, gender, tumor size, main tumor location (upper third, middle third, or lower third), macroscopic type, histological type, depth of invasion, number of positive lymph nodes, number of examined lymph nodes, lymphovascular invasion, and extent of lymphadenectomy.

The macroscopic types were classified into six categories. Types 1–4 were equivalent to the Borrmann classification. Type 0 indicated superficial, flat tumors with or without minimal elevation or depression, and type 5 indicated unclassifiable carcinomas that could not be classified into any of the above types.

The histological types were classified into six categories: mucinous adenocarcinoma; tubular adenocarcinoma; poorly differentiated adenocarcinoma, solid type; poorly differentiated adenocarcinoma, nonsolid type; signet-ring cell carcinoma; and papillary adenocarcinoma. Considering the fact that poorly differentiated adenocarcinoma, nonsolid type and signet-ring cell carcinoma often coexisted in the resected specimens, we grouped these two histological types into one category for the multivariable analysis.

All curative resections in this study were Resection A. Resection A for AGC is defined by no residual disease, a high probability of cure, and satisfaction of all of the following conditions: (i) depth of invasion is the proper muscle or subserosa; (ii) N0 treated by D1 or more lymph node dissection or N1 treated by D2 or more lymph node dissection; (iii) no distant organ metastasis including on peritoneal wash cytology; and (iv) proximal and distal resection margins of >10 mm.

statistical analysis

OS was estimated using the Kaplan–Meier method. Cox proportional hazards regression was used for multivariable analysis. We adopted a piecewise linear hazards model to detect the cut points of continuous variables, because this model is more flexible than the ordinary linear regression model [16]. Cubic splines and fractional polynomials might be useful, but require the estimation of many parameters to construct models. Furthermore, it is known that the inclusion of unnecessary terms and parameters in hazard models leads to decreasing statistical powers of interesting risk factors. Therefore, we did not adopt them. Piecewise linear variables were generated from continuous variables. For example, seven piecewise linear variables $\langle Age - i \rangle$ ($i = 30, 40, 50, 60, 70, 80, 90$) were generated by age. If age was $< i$ years, the piecewise linear variable $\langle Age - i \rangle$ represents 0. Otherwise, the variable $\langle Age - i \rangle$ represents age $- i$. In the same way, we generated $\langle Size - i \rangle$ ($i = 2, 4, 6 \ldots 24$), $\langle Positive\ lymph\ nodes - i \rangle$ ($i = 1, 2, 3 \ldots 34$), and $\langle Examined\ lymph\ nodes - i \rangle$ ($i = 10, 20, 30 \ldots 130$). We conducted variable selection by means of the forward stepwise method using not only the original variables, but also the generated piecewise linear variables. We then developed a nomogram and Web software predicting OS after curative resection of AGC based on the results.

External validation of the nomogram was carried out by discrimination and calibration using the data of patients who had undergone surgery in 2003. Discrimination was evaluated by the concordance index (c-index), which estimates the probability of concordance between predicted and observed responses [17]. A value of 0.5 indicates no predictive discrimination, and a value of 1.0 indicates perfect separation of patients with different outcomes. The bootstrapping method was used to reduce estimate bias. Calibration was carried out by grouping all patients with respect to their nomogram-predicted OS by quartile and then comparing the mean of the group with the observed Kaplan–Meier estimate of actual OS.

All analyses were carried out using SPSS ver. 20 (IBM Corp., Armonk, NY) and R 2.14.1 software with Package Hmisc ver. 3.4–2. In all statistical analyses, a P-value of <0.05 was considered significant.

results

Descriptive statistics for the AGC cohort are listed in Table 1. Variables to fit the Cox model were selected from all variables in Table 1 and the piecewise variables using the forward stepwise method ($P < 0.05$).

Table 2 shows the results of the variable selection with hazard ratios and P-values. Age at operation, gender, tumor size, main tumor location, macroscopic type, histological type, depth of invasion, number of positive lymph nodes, number of examined lymph nodes, and lymphovascular invasion were statistically significant, whereas the extent of lymphadenectomy was not statistically significant. Some selected predictors included piecewise linear variables. We grouped macroscopic types 2 and 3 into one category to make the model fit on the basis of the Akaike Information Criterion value [18].

Figure 1 shows the nomogram based on the result of the Cox proportional hazards regression. Each point can be determined

Table 1. Descriptive statistics for advanced gastric cancer cohort

Variables	Training set ($N = 3085$)		External validation set ($N = 2111$)	
	n	%	*n*	%
Age (years)				
Median (range)	67 (21–94)		68 (15–93)	
Gender				
Male	2232	72.4	1520	72.0
Female	853	27.6	591	28.0
Tumor size (cm)				
Mean ± standard deviation	4.6 ± 2.5		4.7 ± 2.6	
Main tumor location				
Upper	834	27.0	557	26.4
Middle	1225	39.7	863	40.9
Lower	1026	33.3	691	32.7
Macroscopic type				
0	759	24.6	523	24.8
1	207	6.7	162	7.7
2 + 3	1857	60.2	1275	60.4
4	91	2.9	47	2.2
5	171	5.5	104	4.9
Histological type				
pap	132	4.3	97	4.6
tub	1514	49.1	1022	48.4
por1	511	16.6	360	17.1
por2 + sig	847	27.5	576	27.3
muc	81	2.6	56	2.7
Depth of invasion				
Proper muscle	1480	48.0	1059	50.2
Subserosa	1605	52.0	1052	49.8
Number of positive lymph nodes				
Mean ± standard deviation	1.2 ± 2.5		1.1 ± 2.5	
Number of examined lymph nodes				
Mean ± standard deviation	34.7 ± 19.6		34.4 ± 19.6	
Lymphovascular invasion				
Negative	553	17.9	437	20.7
Positive	2532	82.1	1674	79.3
Extent of lymphadenectomy				
D1	299	9.7	200	9.5
D1 + α	241	7.8	207	9.8
D1 + β	163	5.3	162	7.7
D2	2289	74.2	1504	71.2
D3	93	3.0	38	1.8

D1 + α included lymph nodes along the left gastric artery irrespective of the location of lesions as well as lymph nodes along the common hepatic artery in patients with lesions located in the lower third of the stomach. D1 + β included lymph nodes along the left gastric artery, the common hepatic artery, and around the celiac artery.

muc, mucinous adenocarcinoma; tub, tubular adenocarcinoma; por1, poorly differentiated adenocarcinoma, solid type; por2, poorly differentiated adenocarcinoma, nonsolid type; sig, signet-ring cell carcinoma; pap, papillary adenocarcinoma.

Table 2. Variable selection carried out by Cox proportional hazards regression

Selected variables	Hazard ratio	*P*
Age (years)		
<Age − 60>[a]	1.07	<0.0001
Gender		
Male	Ref	
Female	0.66	0.0002
Tumor size (cm)		
<Size − 12>[a]	2.15	0.0006
<Size − 14>[a]	0.49	0.0241
Main tumor location		
Upper	Ref	
Middle	0.67	0.0003
Lower	0.70	0.0017
Macroscopic type		
0	Ref	
1	2.07	0.0001
2 + 3	1.33	0.0360
4	1.73	0.0323
5	0.94	0.8213
Histological type		
muc	Ref	
tub	1.75	0.1032
por1	1.68	0.1469
por2 + sig	2.23	0.0217
pap	2.17	0.0421
Depth of invasion		
Proper muscle	Ref	
Subserosa	1.55	<0.0001
Number of positive LNs		
Positive LNs	1.23	<0.0001
<Positive LNs − 5>[a]	0.82	<0.0001
Number of examined LNs		
Examined LNs	0.87	<0.0001
<Examined LNs − 10>[a]	1.14	0.0002
Lymphovascular invasion		
Negative	Ref	
Positive	1.52	0.0085

Interpretation of piecewise variables: for example for age, a hazard ratio of <60 years of age is regarded as a reference, and the hazard ratio becomes 1.07 times higher for every 1-year increase in age when the age of the patient is >60 years. For tumor size, a hazard ratio of <12 cm is a reference and the hazard ratio becomes 2.15 times higher for every 1-cm increase in tumor size from 12 to 14 cm. When the tumor size is >14 cm, the hazard ratio becomes 1.05 times ($1.05 = 2.15 \times 0.49$) higher for every 1-cm increase in tumor size. The number of positive lymph nodes and the number of examined lymph nodes are interpreted in the same way.

[a]Piecewise variable.

LNs, lymph nodes; muc, mucinous adenocarcinoma; tub, tubular adenocarcinoma; por1, poorly differentiated solid adenocarcinoma; por2, poorly differentiated nonsolid adenocarcinoma; sig, signet-ring cell carcinoma; pap, papillary adenocarcinoma.

by drawing a line straight upward from each predictor to the point axis, and the total points are then calculated by summing each point. The probability of 2-year and 5-year OS can be found by drawing a line straight down from the total points axis.

The nomogram was externally validated using the independent dataset listed in Table 1. The c-index was 0.68 [95% confidence interval (CI) 0.66–0.71] in the external validation set. Furthermore, we compared the discrimination of our nomogram with that of the seventh AJCC TNM classification. The discrimination of our nomogram was superior to that of the seventh AJCC TNM classification (TNM classification c-index, 0.61; 95% CI 0.59–0.64).

Supplementary Figure S1, available at *Annals of Oncology* online shows calibration plots for the AGC nomogram. The dashed line indicates the ideal line at which the nomogram-predicted probability equals the actual survival probability. In the 2-year OS graph, the dotted line indicates a 5% margin from the ideal line. In the 5-year OS graph, the dotted line indicates a 7.5% margin from the ideal line. Both calibrations were accurate, because the 95% CIs of the actual outcomes were within their margins.

Supplementary Figure S2, available at *Annals of Oncology* online shows the Web software that predicts the probability of 1-year to 5-year OS and draws a survival curve (http://vps2.takatyan.info/surv/main.rb). These predictions can be calculated by entering clinicopathologic variables on the website.

discussion

This study makes a significant contribution through using a large cohort of patients with AGC who were treated in Japan from 2001 to 2003 was used to develop the nomogram. Compared with previous studies, the number of patients with

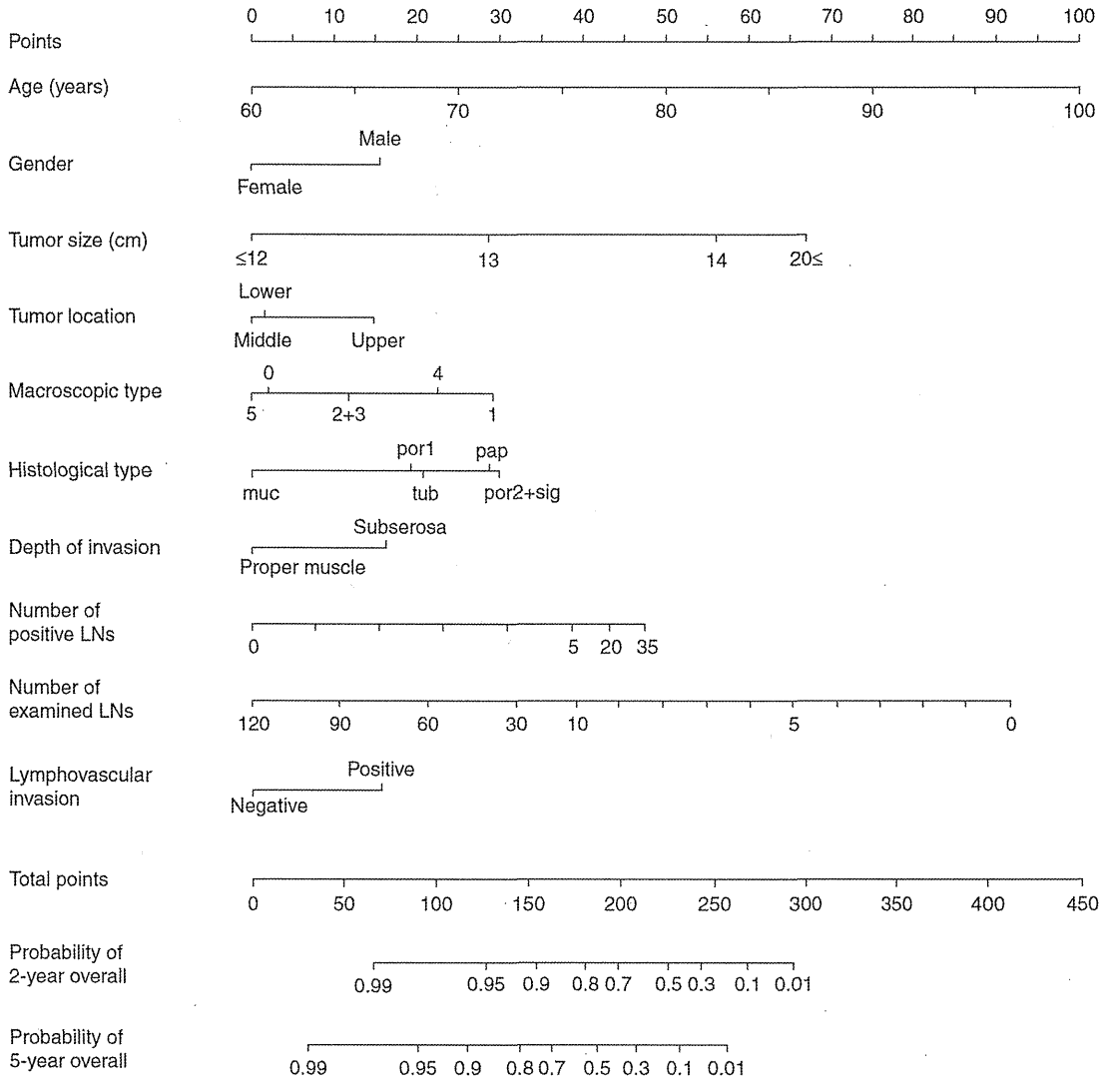

Figure 1. Nomogram predicting the probability of 2-year and 5-year overall survival of postoperative advanced gastric cancer. Each point can be determined by drawing a line straight upward from each predictor to the point axis. The total points are then calculated by summing each point. The probability of 2-year and 5-year overall survival can be found by drawing a line straight down from the total points axis.

AGC (5196) was the largest, and the time span of the dataset (3 years) was the shortest. This nomogram was more significantly predictive than the seventh AJCC stage grouping, with a c-index of 0.68 and good calibration. To avoid the complexity of drawing lines and adding points on the nomogram, we also constructed Web software that could be freely used.

In this study, patients with serosa-negative, locally AGC were selected and mainly assigned to stage II according to the Japanese Classification of Gastric Carcinoma [15]. Compared with the 5-year OS rate of 73% after surgery alone for this patient population, adjuvant chemotherapy with uracil-tegafur significantly improved 5-year OS up to 86% in a randomized, controlled trial [19]. The Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer, another randomized phase III trial with similar eligibility criteria, confirmed the effectiveness of 1-year postoperative treatment of S-1 compared with surgery alone in patients with stage II gastric cancer who underwent D2 gastrectomy, with the 5-year OS rate after surgery being 71.3% in the surgery-only group and 84.2% in the S-1 group [20]. However, whether all patients at this stage require adjuvant chemotherapy is uncertain. Our nomogram may be a useful tool to select patients who are unlikely to benefit from adjuvant chemotherapy. For example, adjuvant chemotherapy may be unnecessary if the predicted OS according to this nomogram is >85%.

Some variables that were not used in previous nomograms were selected as independent prognostic factors and entered into our nomogram. In serosa-negative, locally AGC, macroscopically advanced types (types 1, 2, 3, and 4) were more unfavorable than the macroscopically superficial type (type 0), which seemed to be an intuitively plausible and useful variable for the nomogram. The number of patients with type 4 tumors was extremely small, and the hazard ratio was unexpectedly low (1.73); this was probably because only patients whose depth of invasion was limited to the muscle proper or subserosa were included in this study. Unlike previous studies that use the Lauren classification [12] or simple grading by tumor differentiation [11], histological type was selected as an independent prognostic factor in this study. When subclassified into five subtypes, poorly differentiated adenocarcinoma, nonsolid type including signet-ring cell histology, and papillary adenocarcinoma were significantly associated with poorer survival. It seemed strange that both a differentiated and an undifferentiated histology were simultaneously unfavorable prognostic factors; however, this might explain the reason why histological type was not selected as a significant prognostic factor in previous studies. Lymphovascular invasion was also selected as an independent prognostic factor in this study. Although it was not included in previous nomograms [11–13], its influence on postoperative survival is known [6, 9]. Based on these results, the presence or absence of lymphovascular invasion was used in our nomogram. The results of other prognostic factors such as sex, number of positive and examined lymph nodes, and depth of invasion were consistent with those of previous nomograms. The significance of number of examined or negative lymph nodes was reaffirmed for prognosis.

The c-index of our nomogram was 0.68, slightly lower than those reported in previous studies (0.70–0.80 in different patient populations) [11–13]. It is unclear why the c-index of our nomogram was lower than others. One possible explanation is the difference in patient populations. Previous studies included many patients with early gastric cancer whose prognosis was excellent (22–65.1% of each patient population) [11, 12]. This study, on the other hand, included only patients with serosa-negative, locally AGC whose prognoses varied widely. When patients with early gastric cancer were included in this study, the c-index of our nomogram was as high as 0.84 (data not shown). Our nomogram discrimination was superior to that of the AJCC stage grouping (0.68 versus 0.61, $P < 0.001$).

Our study accurately predicts survival. The calibration plot of the external validation set indicated that actual survival corresponded closely with predicted survival and was always within a 7.5% margin from the ideal reference line; this suggests that the nomogram was more predictive than that of Han et al. [11]. In addition, by using the JGCA nationwide registry, the data of 5196 patients over a 3-year period could be collected from various institutions. The average number of patients recruited in this study was 1732 per year, which is much higher than patient numbers in previous studies (45–361 patients per year) [11, 12]. The nomogram can be widely used because this data collection method may minimize the effect of patients' historical backgrounds and institutional differences.

There are some limitations to our study. First, our nomogram was developed and validated using data from almost exclusively Japanese patients with relatively less advanced disease. Therefore, it would be necessary for our nomogram to be externally validated by datasets in other countries for more widespread use, although disease stages of the datasets should be unified as in this study. Second, recent studies confirmed that adjuvant chemotherapy improved survival in patients with locally AGC [20, 21]. However, we could not evaluate the effect of adjuvant chemotherapy, because it was not common in this study period. Adjuvant chemotherapy should be added as a prognostic factor for nomograms in future studies.

conclusion

We have developed a nomogram and accompanying Web software that predict 5-year OS after curative resection for serosa-negative, locally AGC. External validation demonstrated that calibration of the nomogram was accurate. This nomogram and Web software should be helpful for the clinical assessment of individual risks and the consideration of adjuvant therapy. Our Web software enables users to more easily and quickly predict OS and to draw a survival curve, without cumbersome procedures. Furthermore, we revealed that not only traditional variables used for previously reported nomograms, but also histological type, macroscopic type, and lymphovascular invasion were independent prognostic factors. We hope that this study will help other researchers to investigate the prognosis of gastric cancer and that the nomogram will be widely applied to predict survival of this disease in clinical practice.

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disclosure

The authors have declared no conflicts of interest.

references

1. International Agency for Research on Cancer, World Health Organization. Estimated cancer Incidence, Mortality, Prevalence and Disability-adjusted life years (DALYs) Worldwide in 2008. http://globocan.iarc.fr/ (17 August 2013, date last accessed).
2. Vital Statistics Japan (Ministry of Health, Labour and Welfare). http://ganjoho.jp/professional/statistics/statistics.html (17 August 2013, date last accessed).
3. Son T, Hyung WJ, Lee JH et al. Clinical implication of an insufficient number of examined lymph nodes after curative resection for gastric cancer. Cancer 2012; 118: 4687–4693.
4. Edge SB BD, Compton CC, Fritz AG et al. AJCC Cancer Staging Manual, 7th ed. New York: Springer 2010.
5. Zu H, Wang F, Ma Y et al. Stage-stratified analysis of prognostic significance of tumor size in patients with gastric cancer. PLoS One 2013; 8(1): e54502
6. Kunisaki C, Makino H, Kimura J et al. Impact of lymphovascular invasion in patients with stage I gastric cancer. Surgery 2010; 147: 204–211.
7. Li C, Oh SJ, Kim S et al. Macroscopic Borrmann type as a simple prognostic indicator in patients with advanced gastric cancer. Oncology 2009; 77: 197–204.
8. Kunisaki C, Akiyama H, Nomura M et al. Clinicopathologic characteristics and surgical outcomes of mucinous gastric carcinoma. Ann Surg Oncol 2006; 13: 836–842.
9. Talamonti MS, Kim SP, Yao KA et al. Surgical outcomes of patients with gastric carcinoma: the importance of primary tumor location and microvessel invasion. Surgery 2003; 134: 720–727; discussion 727–9.
10. Iasonos A, Schrag D, Raj GV et al. How to build and interpret a nomogram for cancer prognosis. J Clin Oncol 2008; 26: 1364–1370.
11. Han DS, Suh YS, Kong SH et al. Nomogram predicting long-term survival after d2 gastrectomy for gastric cancer. J Clin Oncol 2012; 30: 3834–3840.
12. Katlan MW, Karpeh MS, Mazumdar M et al. Postoperative nomogram for disease-specific survival after an R0 resection for gastric carcinoma. J Clin Oncol 2003; 21: 3647–3650.
13. Kim JH, Kim HS, Seo WY et al. External validation of nomogram for the prediction of recurrence after curative resection in early gastric cancer. Ann Oncol 2012; 23: 361–367.
14. Kim DH, Kim SM, Hyun JK et al. Changes in postoperative recurrence and prognostic risk factors for patients with gastric cancer who underwent curative gastric resection during different time periods. Ann Surg Oncol 2013; 20: 2317–2327.
15. Japanese Gastric Cancer Association. Japanese Classification of Gastric Carcinoma, 13 ed. Tokyo: Kandera 1999.
16. Akazawa K, Nakamura T, Palesch Y. Power of logrank test and Cox regression model in clinical trials with heterogeneous samples. Stat Med 1997; 16: 583–597.
17. Harrell FE, Jr, Califf RM, Pryor DB et al. Evaluating the yield of medical tests. JAMA 1982; 247: 2543–2546.
18. Akaike H. Information Theory and an Extension of the Maximum Likelihood Principle. Budapest: Hungary Akademiai Kiado 1973.
19. Nakajima T, Kinoshita T, Nashimoto A et al. Randomized controlled trial of adjuvant uracil-tegafur versus surgery alone for serosa-negative, locally advanced gastric cancer. Br J Surg 2007; 94: 1468–1476.
20. Sakuramoto S, Sasako M, Yamaguchi T et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. N Engl J Med 2007; 357: 1810–1820.
21. Bang YJ, Kim YW, Yang HK et al. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. Lancet 2012; 379: 315–321.

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Expression profiling stratifies mesothelioma tumors and signifies deregulation of spindle checkpoint pathway and microtubule network with therapeutic implications

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Background: Malignant pleural mesothelioma (MPM) is a lethal neoplasm exhibiting resistance to most treatment regimens and requires effective therapeutic options. Though an effective strategy in many cancer, targeted therapy is relatively unexplored in MPM because the therapeutically important oncogenic pathways and networks in MPM are largely unknown.

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ORIGINAL ARTICLE

Determination of the optimal cutoff percentage of residual tumors to define the pathological response rate for gastric cancer treated with preoperative therapy (JCOG1004-A)

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Abstract

Background Pathological response rate (pathRR) is a common endpoint used to assess the efficacy of preoperative therapy for gastric cancer. PathRR is estimated based on the percentage of the residual tumor area in the primary tumorous bed. Various cutoff definitions used in previous trials (e.g., 10, 33, 40, 50, 67 %) often impair the comparability of pathRRs between trials.

Methods Individual patient data were used from four JCOG trials evaluating preoperative chemotherapy (JCOG0001, JCOG0002, JCOG0210, JCOG0405). Pathological specimens were evaluated from 173 out of 188 patients (92 %) who underwent surgery. Residual tumor area and primary tumorous beds were traced on a virtual microscopic slide by one pathologist and another confirmed these areas. The hazard ratio (HR) in overall survival was calculated for each cutoff percentage by stratified Cox regression analysis, including the study as a stratification factor, and concordance probability estimates (CPE) were calculated.

Results The numbers of patients with 0%, 1–10 %, 11–33 %, 34–50 %, 51–66 %, and 67–100 % residual tumors were 8, 35, 33, 27, 23, and 47, respectively. HRs in 10, 33, 50, and 67 % cutoffs were 1.91, 1.70, 1.55, and 1.71 for the overall population, and CPEs were 0.56, 0.56, 0.55, and 0.55, respectively. In patients with R0 resection, HRs in 10, 33, 50, and 67 % cutoffs were 1.87, 1.54, 1.24, and 1.38, and CPEs were 0.56, 0.55, 0.52, and 0.52. In subgroup analyses, the 10 % cutoff did not predict survival well for type 4 (linitis plastica) tumors.

Conclusions The 10 % cutoff should be the global standard cutoff of %residual tumor to determine pathRR. PathRR might not be recommended for clinical trials where the main subjects are type 4 tumors.

Keywords Stomach neoplasms · Induction chemotherapy · Treatment outcome

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Introduction

Gastric cancer is the fourth most commonly occurring cancer and second most common cancer-related cause of death in the world. The incidence of gastric cancer was estimated at 989,600 cases in 2008, with 61 % of the new cases being derived from Eastern Asia including China, Japan, and Korea [1].

Standard treatments for locally advanced gastric cancer differ among various regions: surgery followed by adjuvant chemoradiotherapy in the USA [2], pre- and postoperative chemotherapy in European countries [3], and postoperative chemotherapy in Asian countries [4, 5]. Preoperative chemotherapy is a part of the standard treatment used in Europe and has been evaluated in many clinical trials in other countries. However, there are no established short-term endpoints to screen the efficacy of preoperative chemotherapy regimens. The response rate based on the Response Criteria in Solid Tumor (RECIST) [6] is one of the standard short-term endpoints, but is not always applicable to gastric cancer because locally advanced gastric cancer does not necessarily have a measurable lesion.

Pathological response rate (pathRR) is another commonly used endpoint in the preoperative settings of gastric cancer because it can be used even when there is no measurable lesion. PathRR is evaluated microscopically in a resected specimen of the stomach and is estimated based on the percentage of the residual tumor area in the primary tumorous bed. Although many phase II trials have adopted pathRR as the primary endpoint, there is no globally accepted consensus regarding the optimal cutoff percentage to determine the responder. Various definitions regarding the cutoff percentage of residual tumors such as 10 % [7–12], 40 % [13], 50 % [9, 14], or 67 % [15–23] have been used in previous clinical trials. According to the criteria proposed by Becker et al. [24, 25], 10 or 50 % is typically used as the cutoff percentage in Western countries, while 33 or 67 % is commonly used in Asian countries following the definition specified in the Japanese Classification of Gastric Carcinoma [26]. These differences in definitions between the East and West essentially impair the comparability of pathRRs between trials.

It is essential to establish a good short-term endpoint that predicts survival well in phase II trials in order to increase the success probability of phase III trials. Based on these backgrounds, we estimated the percentage of residual tumors on virtual microscopic slides as a continuous variable and determined which cutoff definition was the best to predict overall survival.

Methods

Included studies

The Stomach Cancer Study Group of JCOG has conducted a series of clinical trials evaluating preoperative chemotherapy. In this study, we used individual patient data from four phase II trials [17, 20, 22, 23] to evaluate the efficacy of preoperative chemotherapy. Details of the studies included are shown in the Table 1. The main subjects of JCOG0002-DI and JCOG0210 were patients with Borrmann type 4 (linitis plastica) cancer, while those of JCOG0001 and JCOG0405 were patients with non-type 4 cancer with extended lymph node metastases.

Pathological diagnosis

Hematoxylin-eosin (H&E)-stained pathological sections of resected tumors were collected from 24 participating institutions. Sections corresponding to the cut surface with the largest tumor diameter on the resected specimen were selected in each patient and were digitally captured on a virtual microscopic slide. The Japanese Criteria of Gastric Carcinoma [26] were used for pathological diagnosis of the residual tumor and primary tumorous bed. For example, the primary tumorous bed volume was defined by microscopic findings such as necrosis, macrophage accumulation, or interstitial fibrosis below the submucosal layer. Inflammatory changes caused by peptic ulcer disease were excluded from the primary tumorous bed volume. Degenerative cancer cells were evaluated as viable cancer cells. The definition of viable tumor cells was sometimes difficult, and any tumor cells identifiable under the microscope were regarded as viable unless the tumor cells were totally necrotic, cyto-/karyolitic, or apoptotic. An example of non-viable cells is shown in Fig. 1a. The validity of the detailed criteria was examined by four pathologists (TK, TS, RK, HT) with a small number of cases prior to the consecutive pathological diagnosis for this study. According to the consensus criteria, the residual tumor area and primary tumorous bed were traced on a virtual microscopic slide by one pathologist (TK) and another (TS) confirmed these areas. If the opinions of the two pathologists differed, a consensus-based decision was made. The square measures of these two areas were automatically calculated on the software for virtual microscopic diagnosis (NanoZoomer Virtual Microscopy System, Hamamatsu Photonics). The percentage of the residual tumor, the square measure of the residual tumor divided by that of the primary tumorous bed, was then calculated. Tumor cells, particularly those in type 4 tumors, often exist sparsely in the interstitial area and sometimes the density of tumor cells is very low, for example, less than 0.1. These areas were identified separately, and the square measure multiplied by 0.1 was added to the sum of the residual tumor area. An example of a pathological diagnosis is shown in Fig. 1b.

Statistical consideration

According to the four typical cutoff percentages (10, 33, 50, 67 %), patients were classified into a responder and non-responder group. The primary outcome was overall

Table 1 Details of included clinical trials

Trial	Phase	Subjects	Treatment	Number of patients
JCOG0001	II	Extended nodal metastasis	Irinotecan + Cisplatin	55
JCOG0002	II	Type 4	S-1	55
JCOG0210	II	Type 4 and large type 3	S-1 + Cisplatin	50
JCOG0405	II	Extended nodal metastasis	S-1 + Cisplatin	53

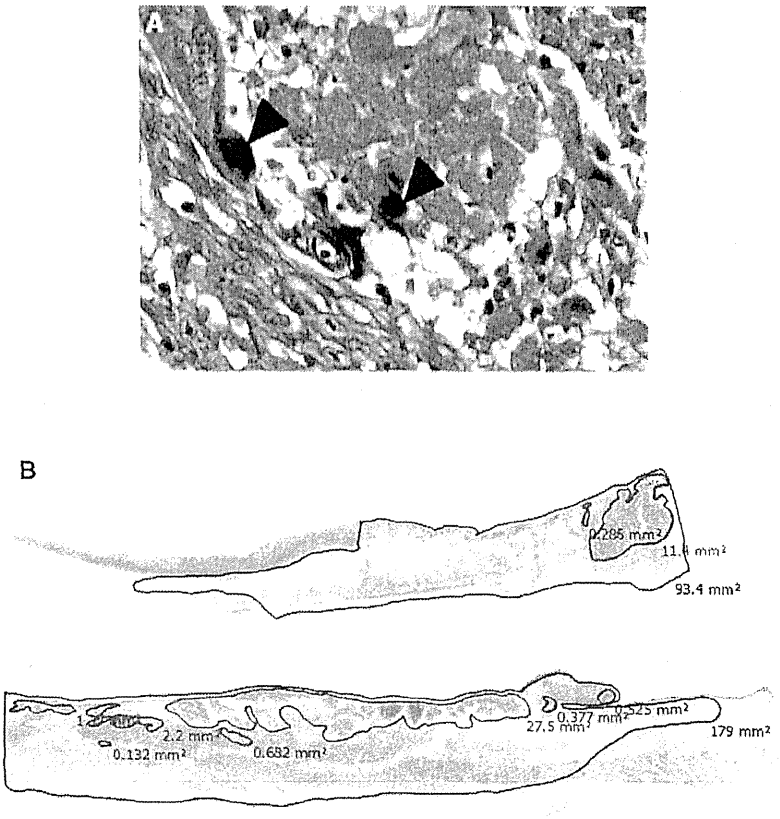

Fig. 1 **a** Example of tumor cells diagnosed as non-viable (*arrows*); **b** example of a pathological diagnosis of the residual tumor area and primary tumorous bed in a macroscopic type 3 tumor

survival, which was defined as the time from patient registration to death from any cause and was censored at the last day for surviving patients. All patients were followed up for at least 3 years. The hazard ratio (HR) of non-responders to responders in overall survival was calculated for each cutoff percentage by stratified Cox regression analysis including the study as a stratification factor. Adjusted HRs by the multivariate stratified Cox model were also estimated including age, sex, performance status, pathological type, and macroscopic type as covariates. Concordance probability estimates (CPE) were also calculated from the stratified Cox model for each cutoff to investigate how well each cutoff discriminated overall survival [27]. If the patient in the responder group lived longer, then the pair was regarded as concordant. CPE was the fraction of all pairs that were concordant and ranged from 0.5 to 1.0, with 0.5 indicating no association and 1.0 indicating a perfect association. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC).

Results

A total of 188 patients from all the enrolled patients ($n = 213$) in the four trials underwent surgery, and pathological specimens were evaluated in 173 (92 %) out of 188 operated patients (Fig. 2).

The characteristics of all analyzed patients are shown in the Table 2. Approximately two-thirds of patients had the histological diffuse type and 39 % of patients had Borrmann type 4 tumors. A total of 39 (23 %) out of 173 analyzed patients underwent R1/R2 resection.

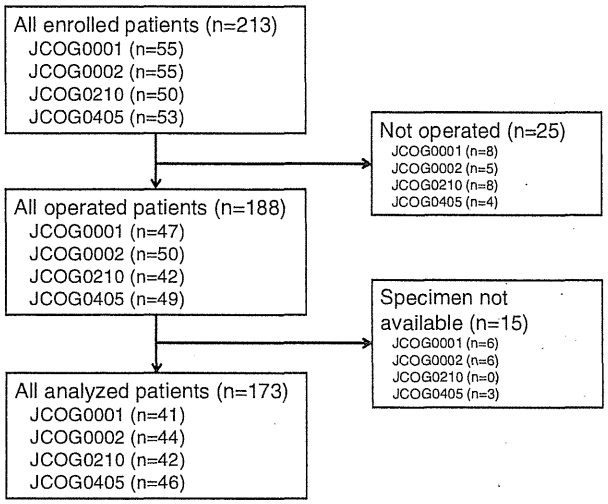

Fig. 2 Flow diagram of the study population

Table 2 Patient characteristics

Characteristic	No.	%
Age (years)		
<65	108	62
≥65	65	38
Sex		
Male	116	67
Female	57	33
Performance status		
0	160	92
1	13	8
Pathological type		
Intestinal type	58	34
Diffuse type	115	66
Macroscopic type		
Type 4	68	39
Non-type 4		
Type 0	3	2
Type 1	1	1
Type 2	27	16
Type 3	69	40
Type 5	5	3
Residual tumor		
R0	134	77
R1/R2	39	23

Pathological complete response rate was observed in eight patients only (4.6 %). There were 35 patients (20.2 %) with 1–10 % residual tumor, 33 patients (19.1 %) with 11–33 %, 27 patients (15.6 %) with 34–50 %, 23 patients (13.3 %) with 51–66 %, and 47 patients (27.2 %) with 67–100 %. Pathological response rates for the 10, 33, 50, and 67 % cutoffs were 25, 44, 60, and 73 %, respectively. Areas with a low density of tumor cells were identified in 36 patients (20.8 %) for whom the square measure of such areas was multiplied by 0.1 and was then added to the sum of the residual tumor area.

Prediction of overall survival

The HRs and CPEs for each cutoff percentage are shown in Fig. 3. HR for the overall population was the largest in the 10 % cutoff, which was the same even in the multivariate analysis, and CPEs were almost the same in each cutoff. When patients who underwent R1/R2 resection were excluded, both HR and CPE were the largest in the 10 % cutoff.

Subgroup analyses

HRs and CPEs in the subgroup analyses for the macroscopic type (type 4/non-type 4) and histological type (intestinal/diffuse) are shown in Fig. 4. The 10, 33, or 50 % cutoffs did not predict survival well in the subgroup analysis for type 4 tumors, while the 67 % cutoff predicted survival moderately well. All cutoff percentages worked well in the subgroup analysis for non-type 4 tumors. All cutoff percentages worked well in the diffuse type, while only 10 % predicted overall survival moderately well in the intestinal type.

As a sensitivity analysis, we simply added low cellularity area to the residual tumor area and calculated HRs and CPEs. The HRs with the cutoff of 10, 33, 50, and 67 % were 0.92, 1.01, 1.40, and 1.36 for type 4 tumors and 2.57, 2.25, 1.91, and 1.75 for non-type 4 tumors. CPEs with respective cutoffs were 0.50, 0.50, 0.54, and 0.53 for type 4 tumors and 0.59, 0.60, 0.58, and 0.55 for non-type 4 tumors. These results were quite similar to those when multiplying by 0.1 for low cellularity area.

Discussion

In the present study, the 10 % cutoff was the best in terms of the hazard ratio in both the overall population and patients who underwent R0 resection, while CPEs were almost the same between 10 and 33 %. Based on these results, the 10 % cutoff was recommended in terms of predicting survival. In addition, the 10 or 33 % cutoff did not predict survival well in the subgroup analysis for type 4 tumors, which implied that the diagnosis of %residual tumor may not have been as accurate as that of non-type 4 tumors.

Several short-term endpoints have been used in clinical trials to evaluate preoperative therapy. The response rate is

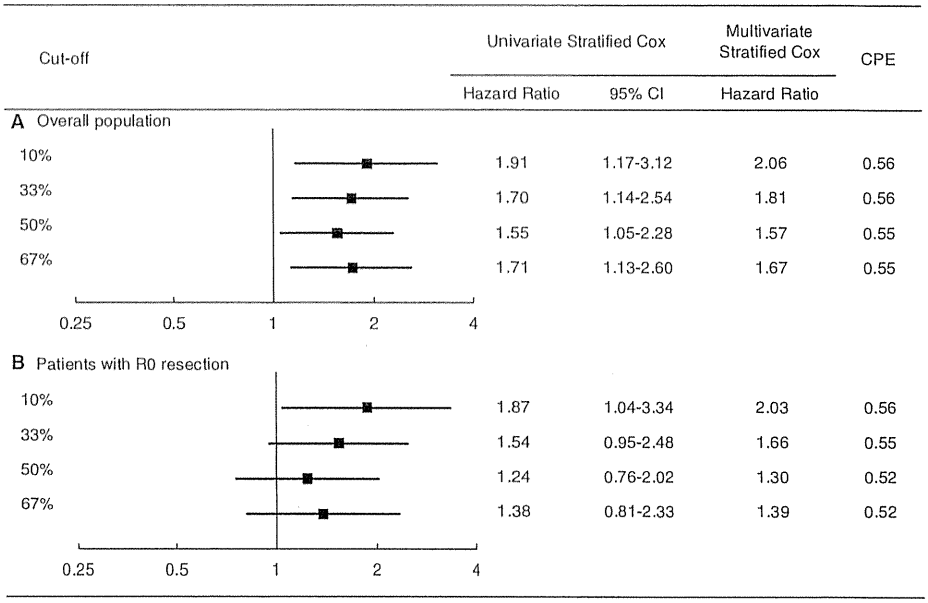

Fig. 3 **a** Hazard ratio of overall survival and concordance probability estimates (CPEs) for the overall population ($n = 173$); **b** hazard ratio of overall survival and CPE for patients with R0 resection ($n = 134$)

not applicable in many trials on gastric cancer because subjects include patients without measurable lesions. The R0 resection rate is another candidate, but the R0 resection rate is affected by selection bias. The complete response rate (CR rate) can also be used as a candidate; however, because it is commonly less than 10 % in gastric cancer, it is not a good endpoint to screen the efficacy of preoperative chemotherapy. The CR rate was only 4.6 % in the present study.

PathRR does not need any special modality and can be used without measurable lesions. Kurokawa et al. [28] demonstrated that response assessment validity was higher with pathRR with a cutoff of 67 % than with the response rate with RECIST. Becker et al. [25] showed in their multivariate analysis that pathRR with the 10 % cutoff remained a prognostic factor while R0 resection rate did not. Therefore, pathRR has currently become a common endpoint in preoperative settings in gastric cancer. However, different definitions of the cutoff percentage of residual tumors between the East and West have impaired the comparability of the results of different trials.

The 10 % cutoff was the best in terms of the hazard ratio in the overall population in the present study, while CPEs were almost the same between 10 and 33 %. Based on these results, the 10 % cutoff was recommended due to the larger hazard ratio observed in this study, the ease of the pathological diagnosis, and standardization of the definition between the East and West. By harmonizing the definitions used in the East and West, it may become possible to compare the results of phase II trials from both, which would enable the more efficient development of treatment screening. In the current version of the Japanese Classification of Gastric Carcinoma, the %residual tumors with both 1–10 % and 10–33 % were included in grade 2. Therefore, we propose a modification to the Japanese Classification of Gastric Carcinoma to include the 10 % cutoff in the histological grading system for preoperative chemotherapy.

A macroscopic type 4 tumor, linitis plastica type cancer, is a particular type of gastric cancer. It has been referred to as a scirrhous type, with tumor cells often existing sparsely in the interstitial area. Thus, identifying both the residual tumor area and primary tumorous bed was assumed to be difficult. In this study, the area with a low density of tumor cells was identified separately by multiplying the area by 0.1 and adding the sum of the residual tumor area. Nevertheless, the 10 or 33 % cutoff did not work well in the subgroup analysis for type 4 tumors, which implied that the diagnosis of %residual tumor may not have been as accurate as that of non-type 4 tumors. The area with a low density of tumor cells was multiplied by 1 for sensitivity analysis, and the results revealed the same trends for both type 4 and non-type 4 tumors. Thus, the pathological response rate is not recommended for clinical trials in which most subjects have macroscopic type 4 tumors.

The present study has some limitations. First, virtual microscopic slides were used to identify the areas determining %residual tumor considering the reproducibility of the results. There may be a difference between the area diagnosis on the virtual slides and that on microscopic diagnosis in clinical practice. The reproducibility of the pathological area diagnosis in a clinical practice should be

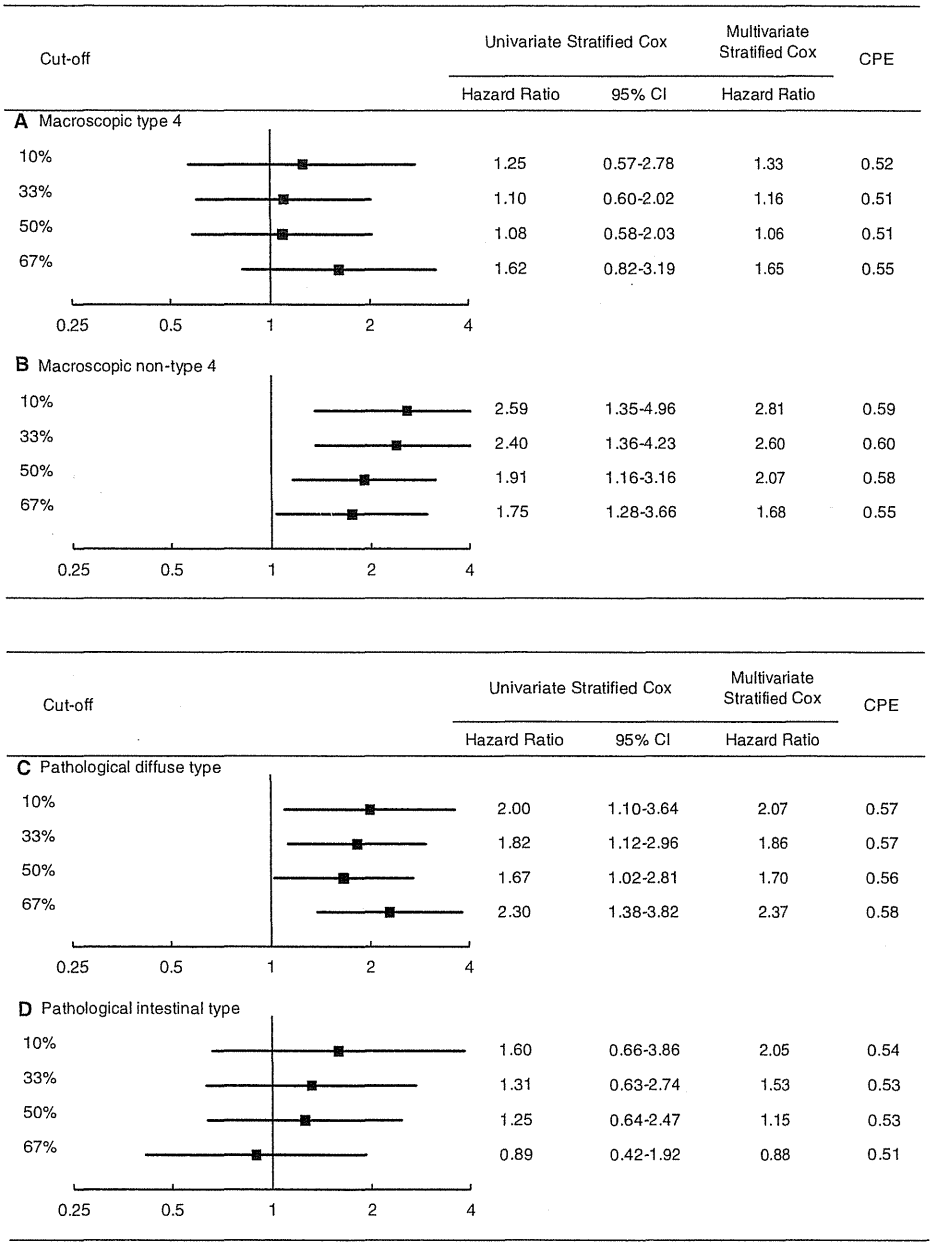

Fig. 4 Hazard ratio of overall survival and CPEs in subgroups: **a** macroscopic type 4 ($n = 68$), **b** macroscopic non-type 4 ($n = 105$), **c** pathological diffuse type ($n = 115$), and **d** pathological intestinal type ($n = 58$)

verified in a multiinstitutional setting. Second, determining the primary tumorous bed volume is generally harder than determining residual tumor volume. In addition to the criteria for evaluating primary tumorous bed volume employed in this study, we also believe that including some clinical findings, especially endoscopic findings, may be helpful to improve the understanding of primary tumorous bed volume. However, collecting the information for central review was not feasible in this multiinstitutional study, and pathological evaluation was performed based only on pathological specimens.

In conclusion, the 10 % cutoff should be the global standard cutoff of %residual tumor to determine the pathological response rate. The pathological response rate might not be recommended for clinical trials where the main subjects are type 4 tumors.

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References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer. 2010;127:2893–917.
2. Smalley SR, Benedetti JK, Haller DG, Hundahl SA, Estes NC, Ajani JA, et al. Updated analysis of SWOG-directed intergroup study 0116: a phase III trial of adjuvant radiochemotherapy versus observation after curative gastric cancer resection. J Clin Oncol. 2012;30:2327–33.
3. Cunningham D, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, et al. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. N Engl J Med. 2006;355:11–20.
4. Sasako M, Sakuramoto S, Katai H, Kinoshita T, Furukawa H, Yamaguchi T, et al. Five-year outcomes of a randomized phase III trial comparing adjuvant chemotherapy with S-1 versus surgery alone in stage II or III gastric cancer. J Clin Oncol. 2011;29:4387–93.
5. Bang YJ, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, et al. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. Lancet. 2012;379:315–21.
6. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer. 2009;45:228–47.
7. Reim D, Gertler R, Novotny A, Becker K, zum Buschenfelde CM, Ebert M, et al. Adenocarcinomas of the esophagogastric junction are more likely to respond to preoperative chemotherapy than distal gastric cancer. Ann Surg Oncol. 2012;19:2108–18.
8. Pera M, Gallego R, Montagut C, Martin-Richard M, Iglesias M, Conill C, et al. Phase II trial of preoperative chemoradiotherapy with oxaliplatin, cisplatin, and 5-FU in locally advanced esophageal and gastric cancer. Ann Oncol. 2012;23:664–70.
9. Valenti V, Hernandez-Lizoain JL, Beorlegui MC, Diaz-Gozalez JA, Regueira FM, Rodriguez JJ, et al. Morbidity, mortality, and pathological response in patients with gastric cancer preoperatively treated with chemotherapy or chemoradiotherapy. J Surg Oncol. 2011;104:124–9.
10. Ajani JA, Mansfield PF, Janjan N, Morris J, Pisters PW, Lynch PM, et al. Multi-institutional trial of preoperative chemoradiotherapy in patients with potentially resectable gastric carcinoma. J Clin Oncol. 2004;22:2774–80.
11. Ajani JA, Mansfield PF, Crane CH, Wu TT, Lunagomez S, Lynch PM, et al. Paclitaxel-based chemoradiotherapy in localized gastric carcinoma: degree of pathologic response and not clinical parameters dictated patient outcome. J Clin Oncol. 2005;23:1237–44.
12. Diaz-Gonzalez JA, Rodriguez J, Hernandez-Lizoain JL, Ciervide R, Gaztanaga M, San Miguel I, et al. Patterns of response after preoperative treatment in gastric cancer. Int J Radiat Oncol Biol Phys. 2011;80:698–704.
13. Brenner B, Shah MA, Karpeh MS, Gonen M, Brennan MF, Coit DG, et al. A phase II trial of neoadjuvant cisplatin-fluorouracil followed by postoperative intraperitoneal floxuridine-leucovorin in patients with locally advanced gastric cancer. Ann Oncol. 2006;17:1404–11.
14. Ferri LE, Ades S, Alcindor T, Chasen M, Marcus V, Hickeson M, et al. Perioperative docetaxel, cisplatin, and 5-fluorouracil (DCF) for locally advanced esophageal and gastric adenocarcinoma: a multicenter phase II trial. Ann Oncol. 2012;23:1512–7.
15. Tsuburaya A, Nagata N, Cho H, Hirabayashi N, Kobayashi M, Kojima H, et al. Phase II trial of paclitaxel and cisplatin as neoadjuvant chemotherapy for locally advanced gastric cancer. Cancer Chemother Pharmacol. 2013;71:1309–14.
16. Hirakawa M, Sato Y, Ohnuma H, Takayama T, Sagawa T, Nobuoka T, et al. A phase II study of neoadjuvant combination chemotherapy with docetaxel, cisplatin, and S-1 for locally advanced resectable gastric cancer: nucleotide excision repair (NER) as potential chemoresistance marker. Cancer Chemother Pharmacol. 2013;71:789–97.
17. Iwasaki Y, Sasako M, Yamamoto S, Nakamura K, Sano T, Katai H, et al. Phase II study of preoperative chemotherapy with S-1 and cisplatin followed by gastrectomy for clinically resectable type 4 and large type 3 gastric cancers (JCOG0210). J Surg Oncol. 2013;107:741–5.
18. Li GL, Liu K, Bao Y, Cao JM, Xu J, Wang XL, et al. Retrospective analysis of 56 patients with advanced gastric cancer treated with combination of intravenous and intra-arterial intensified neoadjuvant chemotherapy. Chin Med J. 2012;125:780–5.
19. Jia Y, Dong B, Tang L, Liu Y, Du H, Yuan P, et al. Apoptosis index correlates with chemotherapy efficacy and predicts the survival of patients with gastric cancer. Tumour Biol. 2012;33:1151–8.
20. Tsuburaya A, Mizusawa J, Tanaka Y, Fukushima N, Nashimoto A, Sasako M. Neoadjuvant chemotherapy with S-1 and cisplatin followed by D2 gastrectomy with para-aortic lymph node dissection for gastric cancer with extensive lymph node metastasis. Br J Surg. 2014;101:653–60.
21. Yoshikawa T, Omura K, Kobayashi O, Nashimoto A, Takabayashi A, Yamada T, et al. A phase II study of preoperative chemotherapy with S-1 plus cisplatin followed by D2/D3 gastrectomy for clinically serosa-positive gastric cancer (JACCRO GC-01 study). Eur J Surg Oncol. 2010;36:546–51.
22. Yoshikawa T, Sasako M, Yamamoto S, Sano T, Imamura H, Fujitani K, et al. Phase II study of neoadjuvant chemotherapy and extended surgery for locally advanced gastric cancer. Br J Surg. 2009;96:1015–22.
23. Kinoshita T, Sasako M, Sano T, Katai H, Furukawa H, Tsuburaya A, et al. Phase II trial of S-1 for neoadjuvant chemotherapy against scirrhous gastric cancer (JCOG 0002). Gastric Cancer. 2009;12:37–42.
24. Becker K, Mueller JD, Schulmacher C, Ott K, Fink U, Busch R, et al. Histomorphology and grading of regression in gastric carcinoma treated with neoadjuvant chemotherapy. Cancer. 2003;98:1521–30.
25. Becker K, Langer R, Reim D, Novotny A, Meyer zum Buschenfelde C, Engel J, et al. Significance of histopathological tumor regression after neoadjuvant chemotherapy in gastric adenocarcinomas: a summary of 480 cases. Ann Surg. 2011;253:934–9.

26. Sano T, Aiko T. New Japanese classifications and treatment guidelines for gastric cancer: revision concepts and major revised points. Gastric Cancer. 2011;14:97–100.
27. Gönen M. H G. Concordance probability and discriminatory power in proportional hazards regression. Biometrika. 2005;92: 965–70.
28. Kurokawa Y, Shibata T, Sasako M, Sano T, Tsuburaya A, Iwasaki Y et al. Validity of response assessment criteria in neoadjuvant chemotherapy for gastric cancer (JCOG0507-A). Gastric Cancer. 2013 [Epub ahead of print].

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ORIGINAL ARTICLE

Overexpression of Ephrin A2 receptors in cancer stromal cells is a prognostic factor for the relapse of gastric cancer

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Abstract
Background Microenvironments control cancer growth and progression. We explored the prognostic impact of stromal reaction and cancer stromal cells on relapse risk and survival after curative gastrectomy in gastric cancer patients.
Methods Tissue samples were obtained from 107 patients with gastric adenocarcinoma who underwent curative (R0) gastrectomy. Primary stromal cells isolated from gastric cancer tissue (GCSC) and normal gastric tissue (Gastric stromal cell: GSC) in each patient were cultured and subjected to comprehensive proteome (LC–MS/MS) and real-time RT-PCR analysis. Expression of Ephrin A2 receptors (EphA2) in cancers and GCSC was evaluated immunohistochemically. Intermingling of EphA2-positive cancer cells and GCSC (IC/A2+) and overexpression of EphA2 in cancer cells (Ca/A2+) in invasive parts of tumors were assessed, as were relationships of IC/A2+, Ca/A2+, and clinicopathological factors with relapse-free survival and overall survival.
Results Proteome analysis showed that EphA2 expression was significantly higher in GCSC than GSC. Real-time RT-PCR analysis showed that levels of EphA1/A2/A3/A5 and EphB2/B4 were ≥2.0-fold higher in GCSC than GSC. Ca/A2 and IC/A2 were positive in 65 (60.7 %) and 26 (24.3 %) patients, respectively. Relapse was significantly more frequent in IC/A2-positive than in IC/A2-negative (HR, 2.12; 95 % CI, 1.16–5.41; $p = 0.0207$) patients. Among the 54 patients who received S-1 adjuvant chemotherapy, relapse-free survival (RFS) was significantly shorter in those who were IC/A2-positive than in those who were IC/A2-negative and Ca/A2-negative (HR, 2.83; 95 % CI, 1.12–12.12; $p = 0.0339$). Multivariable analysis indicated that pathological stage ($p = 0.010$) and IC/A2+ ($p = 0.008$) were independent risk factors for recurrence.
Conclusion IC/A2+ was predictive of relapse after curative (R0) gastrectomy.

Keywords EphA2 · Gastric cancer · Stromal reaction · Predictive marker · Cancer-associated fibroblasts

S. Kikuchi and N. Kaibe contributed equally to this work.

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Introduction

Gastric cancer is the second leading cause of cancer deaths in both sexes worldwide (736,000 deaths, 9.7 % of the total). Sufficient surgical resection plus regional lymph node dissection in experienced centers have been shown to significantly improve overall survival (OS). Complete resection of the primary tumor and any local spread, including lymph node metastasis, is essential for cure. Other therapeutic approaches, such as chemotherapy, hormonal therapy, and radiotherapy, are insufficiently effective in preventing tumor recurrence. Combination chemotherapy regimens consisting of two or three cytotoxic agents results in an OS of 10–13 months in patients with unresectable or metastatic gastric cancer [1, 2]. Some patients who undergo R0 (curative) resection followed by postoperative therapy experience tumor relapse. At present, only tumor node metastasis (TNM) classification is predictive of relapse after adjuvant treatment.

Erythropoietin-producing hepatocellular (Eph) receptors are novel targets for anti-cancer agents, because EphA2 is frequently overexpressed in a variety of human epithelial cancer [3–5]. This overexpression is often associated with an aggressive tumor phenotype [6, 7]. Eph receptors are the largest known family of receptor tyrosine kinases (RTKs), and are activated by interacting with cell-surface ligands, termed ephrins. Eph receptors are classified into A-type (EphA1–8 and EphA10) and B-type (EphB1–4 and EphB6) based on their interactions with ephrin ligands, which are also classified as A-type and B-type. Eph receptors and ephrin ligands control cell morphology, adhesion, migration, and invasion by modifying the organization of the actin cytoskeleton and influencing the activities of integrins and intercellular adhesion molecules in bidirectional signaling pathways [8].

Cancer tissue is composed of cancer cells and stromal cells such as fibroblasts, monocyte/macrophages, endothelial cells, and immune cells (lymphocytes and neutrophils). Cancer progression is not solely determined by the cancer cells themselves, but also by the surrounding stromal cells [9–15.] We found that EphA2 was locally overexpressed in both cancer cells and gastric cancer tissue (GCSC) in invasive parts of tumors, but the function of EphA2 in these cells is still unclear. We therefore assessed the prognostic impact of stromal reaction and EphA2 expression in cancer cells and GCSC. To our knowledge, this study is the first to assess whether EphA2 overexpression in GCSC is predictive of the risk of relapse after curative gastrectomy in patients with gastric cancer.

Methods

Patients

We retrospectively analyzed findings in 107 patients who underwent curative (R0) resection of primary gastric adenocarcinoma at Hyogo College of Medicine, Japan, between 2008 and 2010. Patients were included if they had histologically proven T2–T4b gastric cancer; had undergone R0 resection with D2 or more extensive lymph-node dissection; had no distant metastases or tumor cells on peritoneal lavage cytology; and did not receive treatment prior to surgery. Following surgery, 79 patients (73.8 %) were eligible for adjuvant chemotherapy, and 54 patients (50.5 %) received adjuvant chemotherapy without severe adverse effects, consisting of 80 mg/m^2 S-1 (tegafur–gimeracil–oteracil potassium) administered orally on days 1–28 every 6 weeks, for eight cycles. The median follow-up period was 1,279 days (range 163–2,106 days). During the study, 13 patients died from gastric cancer relapse and five from other causes. Tumor recurrence was observed in 39 patients, including 15 with blood-borne, 14 with peritoneal, six with lymph node, and four with other types of recurrence. Patients were followed up monthly for the first year and every 3 months thereafter. Relapse was determined by imaging modalities, including ultrasonography, computed tomography (CT, performed every 3–6 months), gastrointestinal radiography, endoscopy, positive emission tomography/CT scan, blood tests (every 1–3 months) and ascites cytology.

Study design and treatment

The study protocol was approved by the Institutional Medical Ethics Committees of Hyogo College of Medicine. Written informed consent was obtained from all patients. The primary endpoint was relapse-free survival (RFS) and overall survival (OS). Clinicopathological data on all patients were collected prospectively. Tumors were classified according to the TNM system of the International Union Against Cancer (UICC), 7th edition, and the Japanese Classification of Gastric Carcinoma, 14th edition [16].

Pathological classification and IC

Formaldehyde (10 %)-fixed and paraffin-embedded specimens of surgically resected samples were used for hematoxylin–eosin (HE) staining and immunohistochemistry. All samples were histologically analyzed by a pathologist, to determine pathological diagnosis and intermingling of scattered cancer cells and GCSC (IC), with or without EphA2 expression. ICs were defined as (1) scattered cancer cells intermingled with GCSC in invasive parts of tumors; and (2) staining for EphA2 in cancer cells or GCSC that was equal to or stronger than in the soma of Auerbach's plexus, in which EphA2 is normally expressed. ICs positive and negative for EphA2 expression in invasive parts of tumors were designated IC/A2+ and IC/A2−, respectively. IC/A2 consists of double-positive cancer cells and GCSC

in invasive parts of tumor. EphA2 staining of >50 % of cancer cells throughout the tumor was designated as Ca/A2+. We analyzed the cancer–stromal mixture in 107 cases, but we couldn't find any case of EphrinA2-positive only in GCSC and not in cancer cells. Although gastric cancer has been defined as intestinal and diffuse types, most of these tumors consisted of heterogeneous tissue. If the pathological type of the major and invasive parts of the tumor differed, the tumor was defined as transitional type.

Primary cultured stromal cells

Following resection, tissue samples were prepared from cancerous lesions (GCSC) and non-cancerous areas at least 50 mm removed from the tumor (GSC). The specimens were trimmed of fat and necrotic tissues, minced, and transferred to 12-well microplates. Cells were cultured in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10 % fetal calf serum. Isolated fibroblasts were transferred to other dishes and used for experiments within eight passages.

Proteome analysis of stromal cells

Liquid chromatography–tandem mass spectrometry (LC–MS/MS analysis)

Sample preparation for LC–MS/MS analysis Stromal cells (2 × 10e6 cells) were lysed in 100 mM Tris–HCl, pH 8.8, 7 M urea, 2 % SDS. Cell lysates were sonicated using Bioraptor and subjected to protein assay (BCA method) and clarified by centrifugation for 20 min at 13,500 rpm. Proteins (200 mg) were then precipitated using methanol-chloroform, resuspended in a buffer containing 7 M guanidium hydroxide in 0.5 M triethylammonium hydrogen carbonate pH 8.5, and incubated for 15 min at 85 °C. The sample was diluted with fourfold volumes of water and digested with Lys-C at 37 °C for 4 h; this was followed by twofold dilution and trypsin digestion at 37 °C overnight (enzyme-to-protein ratio of 1:100 [w/w]). After reduction with 0.625 mM TCEP and alkylation with 3.125 mM iodoacetamide (IAA), digests were stored at −80 °C until analysis.

LC–MS/MS analysis All samples were analyzed by Q Exactive (Thermo Fisher Scientific), equipped with an AdvanceLC HPLC pump and HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). L-column C18 materials (3 μm, CERI Japan) were packed into self-pulled fused silica capillaries (100 μm inner diameters, 20 cm length) by using a high pressure chamber equipped with an HPLC pump at constant pressure of 230 bar. All samples were dissolved in 0.1 % TFA, 2 % acetnitrile, and injected to pre-column (L-column micro: 0.3 mm inner diameter, 5 mm length, CERI Japan), washed with the same buffer, and eluted with a linear gradient of 5–35 % B for 90 min, 35–95 % B for 1 min, and 95–95 % B for 10 min (A: 0.1 % formic acid, 2 % acetonitrile, B: 0.1 % formic acid, 90 % acetnitrile) at a flow rate of 200 nl/min. The Q Exactive was operated in the data-dependent mode with survey scans acquired at a resolution of 70,000 at *m/z* 200. The top ten most abundant ions were selected with an isolation window of 1.5 Thomsons and fragmented by higher energy collisional dissociation with normalized collision energies of 35. The ion target values were set to 1e6 for survey scan and 5e5 for MS/MS scan, respectively. The maximum ion injection times for both survey scan and MS/MS scan were 60 ms. Dynamic exclusion times was 60 s. Fibroblasts (2×10^6 cells) were lysed and sonicated. Following the determination of protein concentrations using the bicinchoninic acid method, 200 mg proteins were denatured and digested. All samples were analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS), using Q Exactive (Thermo Fisher Scientific), equipped with an AdvanceLC HPLC pump and HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). L-Column C18 materials (3 μm, CERI Japan) were packed into self-filling fused silica capillaries using a high pressure chamber equipped with an HPLC pump. The Q Exactive was operated in the data-dependent mode with survey scans. The ten most abundant ions were selected with an isolation window and fragmented by higher energy collisional dissociation with normalized collision energies. The ion target values were set to survey scan and MS/MS scan, respectively.

Real-time RT-PCR and RNA microarray of stromal cells

Total RNA was extracted from three sets of CAFs and NGFs using Trizol reagent (Gibco BRL, Rockville, MD). Real-time RT-PCR analysis were performed according manufacture's protocol (Power SYBR® Green PCR, applied biosystems, Warrington, UK) using Ephrin primers (Supplementary Table 1) by ABI SDS-7900HT (ABI). For RNA Microarray analysis, synthesis of cRNA, hybridization, scanning and data analysis were performed by Hokkaido System Science Co., Ltd. (Sapporo, Japan). Briefly, cyanine-3 (Cy3) labeled cRNA was prepared from total RNA (0.05 μg) using the Low Input Quick Amp Labeling Kit (Agilent) according to the manufacturer's instructions. These Cy3-labeled cRNAs (0.60 μg) were fragmented and hybridized to Agilent SurePrint G3 Human Gene Expression Microarrays (8 × 60 K ver.2.0). All fibroblast samples were assayed in triplicate. Those samples on the microarrays that showed significantly different expression when hybridized with labeled cRNA from GCSC and GSC

Table 1 Proteome analyses of GCSC and GCS, performed by LC–MS/MS procedure

Gene symbol	Description	Ratio (GSC)	SD	*P* value	Ratio (GCSC/GSC)	SD	*P* value
Experiment#1							
TRPV2	(ref_NP_057197 Gl:20127551) TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL_SUBFAMILY V	1	3.464	1.00E+00	140.118	248.059	1.43E−09
KRI8	(spr_K2C8_HUMAN P05787) KERATIN 8	1	1.511	1.00E+00	7.590	6.476	2.91E−06
N/A	(ens_P00000346026) ###_NO_DESCRIPTION_###	1	1.511	1.00E+00	7.590	6.476	2.91E−06
N/A	(tre_Q8NAB7) HYPOTHETICAL PROTEIN FLJ3563S	1	0.231	1.00E+00	6.586	1.241	4.95E−08
POSTN	(Spr_POSN_HUMAN Q15063_2) SPLICE ISOFORM 2 OF Q15063 PERIOSTIN PRECURSOR	1	0.074	1.00E+00	6.189	0.485	2.00E−15
MIPS	(tre_Q72525) D_MYO_INOSITOL_3_PHOSPHATE SYNTHASE	1	0.458	1.00E+00	2.773	0.887	3.64E−11
KRT15	(spt_K1CO_HUMAN P19012) KERATIN_TYPE 1 CYTOSKELETAL 15	1	0.237	1.00E+00	2.609	0.415	6.74E−05
LOX	(spt_LYOX_HUMAN P28300) PROTEIN_LYSINE 6_OXIDASE PRECURSOR	1	0.039	1.00E+00	2.411	0.126	9.90E−20
PPME1	(spr_PME1_HUMAN Q9Y570_2) SPLICE ISOFORM 2 OF Q9Y570 PROTEIN PHOSPHATASE METHYLESTER	1	0.215	1.00E+00	2.254	0.402	8.17E−06
EPHA2	(spr_EPA2_HUMAN P29317) EPHRIN TYPE_A RECEPTOR 2 PRECURSOR	1	0.312	1.00E+00	2.221	0.484	5.70E−04
ANXA3	(spr_ANX3_HUMAN P12429) ANNEXIN A3	1	0.080	1.00E+00	2.217	0.217	9.90E−20
PCDH12	(spr_PC12_HUMAN Q9NPG4) PROTOCADHERIN 12 PRECURSOR	1	0.307	1.00E+00	2.177	0.478	7.23E−08
N/A	(tre_Q9HBQ4) HYPOTHETICAL PROTEIN	1	0.031	1.00E+00	2.149	0.117	8.19E−08
N/A	(ref_NP_004872 GI22538444) QUINONE OXIDOREDUCTASE HOMOLOG	1	0.278	1.00E+00	2.111	1.250	1.43E−01
MFGE8	(spr_MFGM_HUMAN Q08431) LACTADHERIN PRECURSOR	1	0.252	1.00E+00	2.027	0.325	3.23E−09
EDIL3	(spr_E0I3_HUMAN 0438S4_2) SPLICE ISOFORM 2 OF 0438S4 EGF_LIKE REPEATS AND DISCOIDIN	1	0.463	1.00E+00	2.020	0.565	2.18E−03
Experiment#2							
TNFRSF11B	(spr_T11B_HUMAN 000300) TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 11B PRECUR	1	0.808	1.00E+00	32.112	15.504	9.90E−20
EPHA5	(spr_EPA5_HUMAN P54756_2) SPLICE ISOFORM 2 OF P54756 EPHRIN TYPE_A RECEPTOR 5 PRECUR	1	1.934	1.00E+00	10.610	13.107	7.70E−04
CHRM2	(spr_ACM2 HUMAN P08172) MUSCARINIC ACETYLCHOLINE RECEPTOR M2	1	0.970	1.00E+00	8.709	6.770	4.71E−05
EPHA2	(spr_EPA2 HUMAN P293I7) EPHRIN TYPE_A RECEPTOR 2 PRECURSOR	1	0.111	1.00E+00	4.967	0.546	3.14E−14
PVR	(spr_PVR.HUMAN P15151_4) SPLICE ISOFORM DELTA OF P15I5I POLIOVIRUS RECEPTOR PRECURSO	1	0.284	1.00E+00	3.370	1.298	4.55E−15

EphA2 was upregulated 2.221-fold (experiment #1) and 4.967-fold (experiment #2). EphA5 was upregulated 10.610-fold in experiment #2, and keratins 8 and 15 and lysine-6-oxidase were upregulated in experiment #1. Taken together, these findings suggest that re-organization of the cytoskeleton and extracellular matrix, including collagens, was activated in GCSC

were quantified using the peak area of precursor ion extracted at 5 ppm tolerance.

Antibodies and immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples were cut 3 μm thick using the Ventana BenchMark XT system (Ventana Medical Systems, Tucson, AZ, USA). After antigen retrieval and quenching by immersion in 3 % hydrogen peroxide, the tissue sections were incubated with primary antibodies (Abs) to EphA2 (Santa Cruz Biotechnology; Santa Cruz, CA, USA), pan-keratin (AE1/AE3; clone PCK26), α-smooth muscle actin (α-SMA; clone 1A4) and vimentin (clone V9) (all from Roche; Basel,

Switzerland), and D2-40 (podoplanin, 760-4395, CELL MARQUE; Rocklin, CA, USA). Binding was detected using the iVIEW DAB Universal Kit, according to the manufacturer's instructions, and all sections were counterstained with hematoxylin.

Statistical analysis

The χ^2 test was used to analyze possible associations of Eph receptor expression with clinicopathological variables. RFS was calculated using the Kaplan–Meier method and compared using the log-rank test. Multivariate proportional Cox models were used to assess the prognostic significance of factors on RFS. P values less than 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics 19 software (IBM Inc., Armonk, NY, USA).

Results

Proteome analysis and expression analysis of stromal cells

Proteome analyses were performed using LC–MS/MS methods. Upregulated peptides (GCSC/GSC ratios > 2.0, $p < 0.05$) are shown in Table 1. Interestingly, only 16 and six peptides were upregulated in GCSC in experiments 1 and 2, respectively. EphA2 was upregulated in these two experiments 2.221-fold and 4.967-fold, respectively. EphA5 was upregulated 10.610-fold in experiment 2, whereas keratins 8 and 15 and lysine-6-oxidase were upregulated in experiment 1.

Real-time RT-PCR analysis showed that EphA1, EphA2, EphA3, EphA5, EphB2, and EphB4 were upregulated 3.50-, 4.76-, 2.36-, 3.57-, 2.93-, and 2.86-fold, respectively, in GCSC relative to GSC in each patient (Fig. 1). Quantitative RNA microarray analysis of the levels of expression of all isoforms of the ephrin family showed that EphA2 and EphB2 were upregulated 2.28-fold and 2.22-fold, respectively, in GCSC relative to GSC in each patient. The GCSC/GSC expression ratios of other ephrin receptor isoforms ranged from 0.5 to 2.0 ($n = 3$). All sets of fibroblasts were obtained from diffuse-type gastric cancers (Supplementary Figure 1).

Immunohistochemistry

Scattered cancer cells and stromal cells, mostly consisting of fibroblasts, formed intermingled complexes in the invasive part of transitional-type tumors (Fig. 2a) and in all parts of diffuse-type tumors (Fig. 2c). Expression of EphA2 was observed in both cancer cells and GCSC

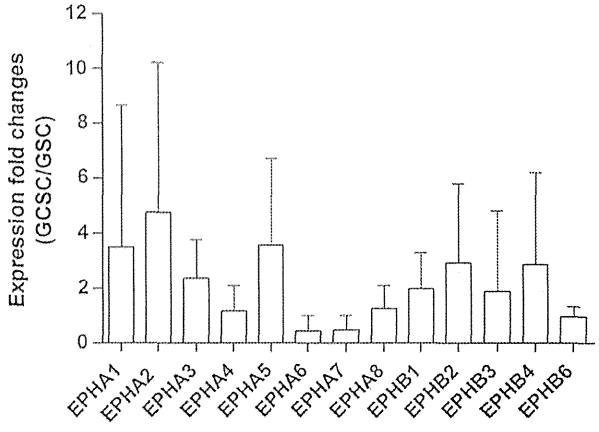

Fig. 1 Real-time RT-PCR analysis of Ephrin receptors of GCSC and GSC. Real-time RT-PCR analysis showed that EphA1, EphA2, EphA3, EphA5, EphB2, and EphB4 were upregulated 3.50-, 4.76-, 2.36-, 3.57-, 2.93-, and 2.86-fold, respectively, in GCSC relative to GSC in each patient. But there was no significance. All sets of stromal cells were obtained from diffuse-type gastric cancers ($n = 6$)

(brown, Fig. 2b, d). IC/A2+ in intestinal- type, transitional type tumors were mostly located at the extremities of the invasive parts. Cancer cells and GCSC expressed EphA2 (Fig. 3a, b). GCSC in ICs appeared as large or small in shape. EphA2 was normally expressed in the soma of Auerbach's plexus (Fig. 3h, black arrows). Keratin expression was observed in cancer cells, whereas vimentin expression was observed in GCSC, endothelial cells, and hematopoietic cells (Fig. 3c, d). Although cultured activated fibroblasts were believed to overexpress α-SMA, we found that large GCSC expressed α-SMA (Fig. 3e, asterisks), whereas most small-shaped GCSC in ICs did not (Fig. 3e). Large populations of EphrinA2-positive GCSC in IC were vimentin-positive (Fig. 3d), and keratin-, α-SMA-, CD31-, D2-40-negative (Fig. 3c, e, f, g). We examine double immunofluorescent staining with α-SMA (green) and EphA2 (red) in Fig. 4. In the left panel, all GCSC looks EphrinA2-positive and one cell strongly expressed α-SMA (white arrow head). In the right panel, all GCSC were EphA2 negative and some population of GCSC was α-SMA-positive (green, white arrow head). Primary cultured GCSC expressed different levels of α-SMA, and some populations strongly expressed α-SMA. These data suggest that the status of GCSC in IC/A2+ might be different from that in cultured and activated myofibroblasts.

Prognostic significance of Ca/A2 and IC/A2

IC/A2± and Ca/A2+ were found in 26 (24.3 %) and 65 (60.7 %) of the 107 patients, respectively (Supplementary

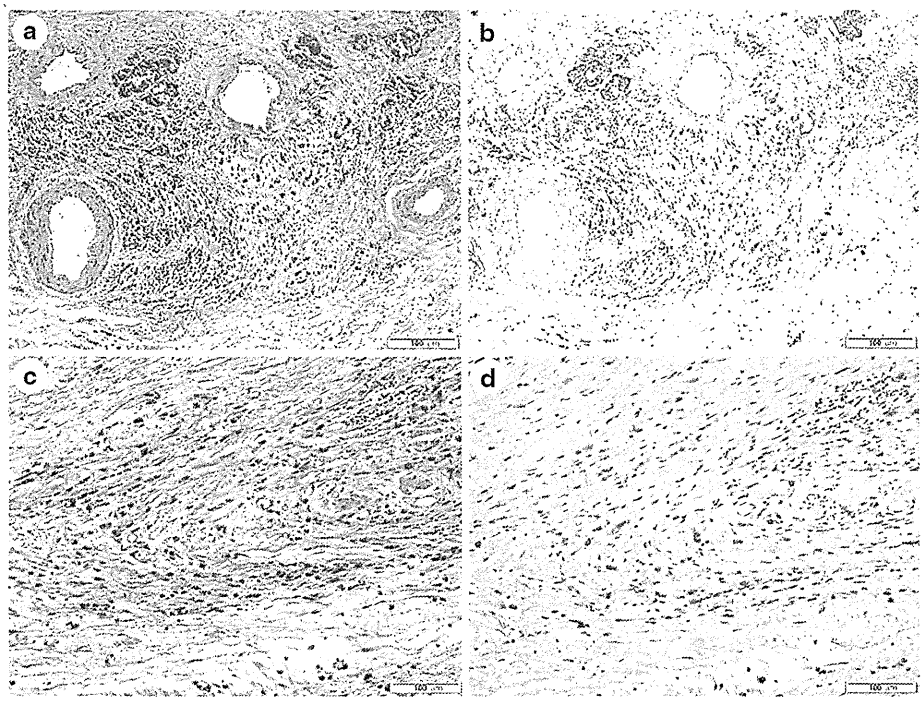

Fig. 2 Intermingling of scattered cancer cells and GCSC (IC). ICs were defined as 1) scattered cancer cells intermingled with GCSC in invasive parts of tumors; and 2) staining for EphA2 in cancer cells or GCSC being equal to or stronger than in the soma of Auerbach's plexus, in which EphA2 is normally expressed. ICs in transitional type (**a**, **b**) and diffuse type (**c**, **d**) gastric cancers. (**a**, **c**, HE staining; **b**, **d**, EphA2 staining). Original magnification ×20

Table 2). The relapse rate was significantly higher in IC/A2+ than in IC/A2− patients, with 14 of 26 IC/A2+ (53.8 %) patients relapsing after a median 347 days [hazard ratio (HR), 2.12; 95 % CI, 1.16–5.41; $p = 0.0207$, Fig. 5a]. Similarly, the relapse rate was higher in patients classified as Ca/A2+ than as Ca/A2− [HR, 1.96; 95 % CI, 0.99–3.51; $p = 0.0542$], with 28 of 65 (43.1 %) Ca/A2+ patients developing recurrence at a median 302 days (Fig. 5b). Overall survival was similar in IC/A2− and Ca/A2 positive or negative patients (Fig. 6a, b).

Prognostic significance of Ca/A2 and IC/A2 in patients who received adjuvant chemotherapy

Although Ca/A2+ was not significantly prognostic in patients who received adjuvant chemotherapy (Figs. 4d, 5d), IC/A2+ was significantly associated with poorer RFS during adjuvant chemotherapy (HR, 3.00; 95 % CI, 1.47–17.03; $p = 0.0108$, Fig. 5c). Stromal reaction may have been prognostic, because patients classified as IC/A2+ had significantly reduced median RFS than those classified as IC/A2−Ca/A2+ (median OS, 378 vs. 1,120 days; HR, 2.99; 95 % CI, 1.22–13.63; $p = 0.0269$, Fig. 5c). Almost half of the patients classified as IC/A2+ experienced recurrence within 1 year after R0 resection, even during the course of adjuvant chemotherapy. Interestingly, overall survival showed minor significant difference in three groups and median survival was 633 days in IC/A2+ and 1,398 days in IC/A2− with adjuvant chemotherapy (Fig. 6c). We suppose that the number of patients was too small to show a statistical difference in this study. Even for a high-risk patient, second or third line chemotherapy was effective in IC/A2+ patients.

Prognostic factors including IC/A2

Univariate analysis showed that IC/A2+ ($p = 0.012$), T3–T4 ($p = 0.048$), and pathological stage ($p = 0.003$) were significant prognostic factors. In multivariate analysis, IC/A2+ (HR, 2.550; 95 % CI, 1.278–5.090; $p = 0.008$) and pathological stage (HR, 1.390; 95 % CI, 1.080–1.788; $p = 0.010$) remained independently prognostic (Supplementary Table 3). Correlations between the expression of EphA2 and clinicopathological variables are shown in Supplementary Table 2. Overexpression of EphA2 in IC was independent of other factors.

Discussion

EphA2 is highly expressed in a variety of cancers, including breast, lung, prostate, urinary bladder, ovarian, esophageal, pancreatic, and colorectal cancers [17–24]. Overexpression of EphA2 is associated with tumor progression or poor patient survival. Recently, overexpression of EphA2 or A4 was also reported to be associated with poor prognosis in patients with gastric cancer [25]. We observed expression of EphA2 in cancer cells from 65 of 107 (60.7 %) patients with gastric cancer, suggesting that EphA2 may be a good molecular target in gastric cancer,