Table 2. Comparison of the clinicopathological features between p-STAT3-positive and -negative gastric cancer patients

	p-STAT3 expression		
	Negative	Positive	*P*-value
Tumour location			0.5642
Lower	4	4	
Mid	4	8	
Upper	6	5	
Lauren's classification			0.9809
Intestinal type	5	6	
Diffuse type	9	11	
Stage			0.1102
I	5	2	
II	1	4	
III	6	4	
IV	2	7	
Lymphatic invasion			0.1071
None	2	0	
Present	12	17	
Venous invasion			0.0874
None	4	1	
Present	10	16	
Lymph node metastasis			**0.0490**
None	6	2	
Present	8	15	
IL-22 expression			**0.0364**
Negative	5	1	
Positive	9	16	
IL-22R1 expression			0.4310
Negative	2	1	
Positive	12	16	

Abbreviations: IL-22 = interleukin-22; IL-22R1 = IL-22 receptor 1. *P*-values <0.05 are indicated by bold entries.

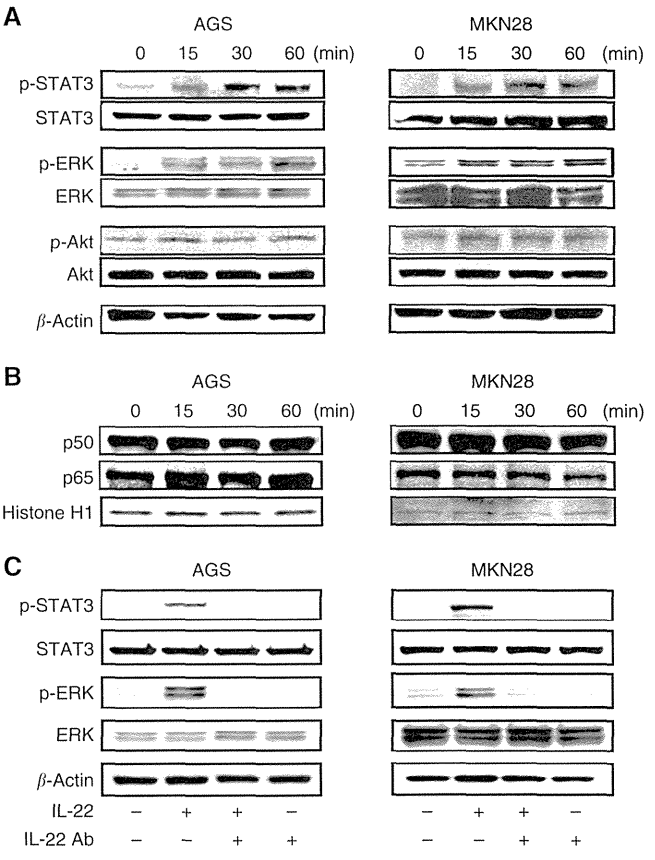

Figure 2. Effect of IL-22 treatment on intracellular signalling in gastric cancer cells. (**A**) Phosphorylation of STAT3, ERK and Akt in AGS and MKN28 cells treated with IL-22. (**B**) Expression of p50 and p60 in AGS and MKN28 cells treated with IL-22. AGS cells (1×10^6) and MKN28 cells (1×10^6) were cultured in 6-cm dishes, treated with IL-22 (10 ng ml^{-1}) for the indicated time, and extracted protein was analysed using western blotting. (**C**) Effect of anti-IL-22 antibody on IL-22-induced STAT3 and ERK phosphorylation in AGS and MKN28 cells. AGS and MKN28 cells were pretreated with IL-22 antibody (20 μg ml^{-1}) for 45 min and then stimulated with IL-22 (10 ng ml^{-1}) for 30 min.

signalling on possible major pathways including STAT3, ERK, Akt and NF-κB in gastric cancer cell lines (Lejeune *et al*, 2002; Andoh *et al*, 2005; Zhang *et al*, 2008). The expression of p-STAT3 was strongly enhanced from 15 min after IL-22 stimulation in both AGS and MKN28 cells (Figure 2A). The expression of p-ERK was also enhanced in AGS and MKN28 cells. On the other hand, the effect of IL-22 stimulation on p-Akt expression was negligible in both cell types. With regard to NF-κB signalling, IL-22 stimulation affected neither nuclear p50 nor p60 expression in the two gastric cancer cell lines (Figure 2B). We moreover examined the effect of anti-IL-22 antibody on gastric cancer cells and confirmed that the increased phosphorylation of STAT3 and ERK by IL-22 stimulation was abolished by concomitant administration of anti-IL-22 antibody (Figure 2C).

Interleukin-22 stimulation promotes the invasive ability of gastric cancer cells. We pursued a function of IL-22 invasive ability of gastric cancer cells as IL-22-positive stromal cells were increased at the invasive tumour front. We examined the invasive ability of gastric cancer cells stimulated with IL-22 using a Matrigel invasion assay. Gastric cancer cells that had invaded across the membrane were observed below the membrane (Figure 3A). When AGS cells were stimulated with IL-22, the number of invasive cells was significantly increased in a dose-dependent manner (Figure 3A). Similarly, the invasive ability of MKN28 cells was significantly enhanced dose-dependently by IL-22 stimulation (Figure 3A). Then, to inhibit the effect of IL-22, AGS cells were treated concomitantly with anti-IL-22 antibody (10–40 μg ml^{-1}). This abolished the increase in the number of invasive AGS cells stimulated with IL-22 (Figure 3B), and similar findings were also obtained for MKN28 cells (Figure 3C). Moreover, we examined whether IL-22 upregulates the expression of *MMP7* and *MMP13*, which are likely to promote cell invasion in the downstream of IL-22 signalling (Howlett *et al*, 2005; Fukuda *et al*, 2011). As shown in Figure 3D, IL-22 stimulation enhanced the expression of *MMP7* and *MMP13* in MKN28 and AGS cells, respectively. In contrast, we showed that the increase of *MMP7* and *MMP13* expression was abolished by the addition of anti-IL-22 antibody. Regarding the effect of IL-22 on cell proliferation and survival, IL-22 treatment did not show any promoting effects for those cell lines under this experimental condition (Supplementary Figure 2).

Interleukin-22 stimulation promotes gastric cancer cell invasion via STAT3 and ERK signalling. Inhibition of STAT3 signalling by STAT3 siRNA significantly decreased the number of invasive AGS cells accelerated by IL-22 stimulation, showing that IL-22 promotes AGS cell invasion via STAT3 signalling. In addition, STAT3 siRNA significantly inhibited the invasive ability of AGS cells under unstimulated conditions, suggesting that STAT3 signalling is crucial for AGS cell invasion (Figure 4A).

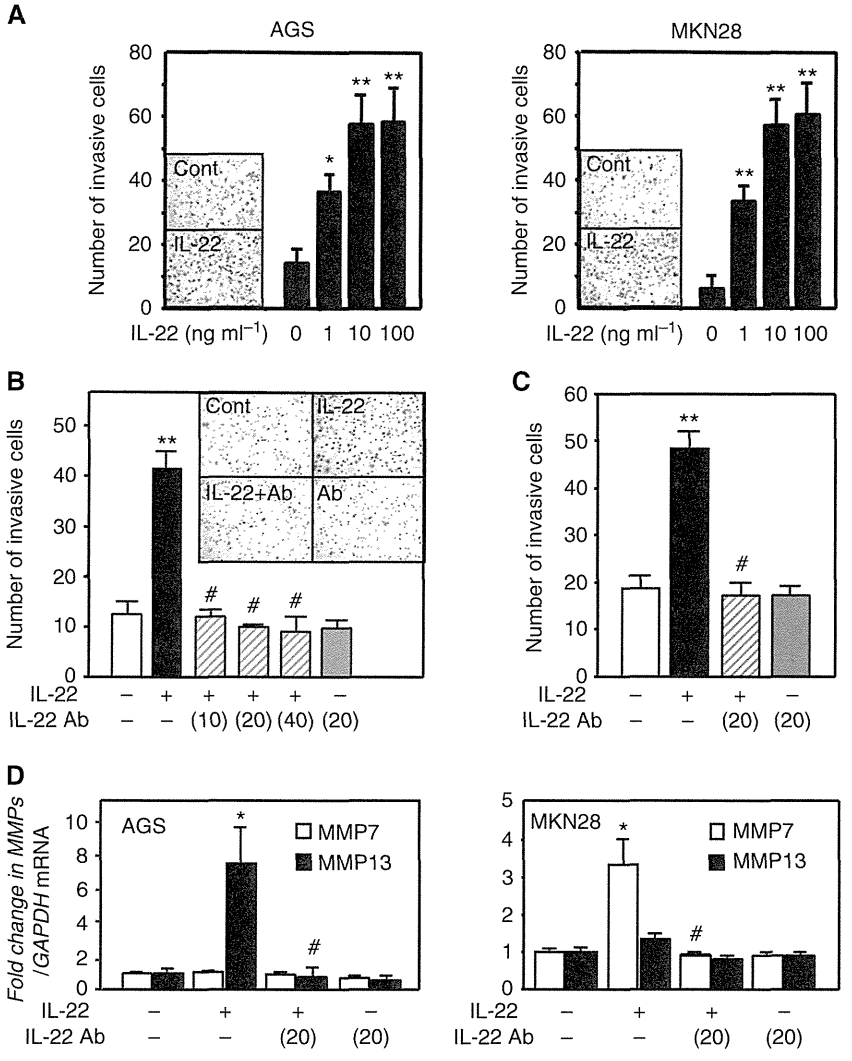

Figure 3. Effect of IL-22 on the invasive potential of gastric cancer cells. (**A**) Changes in the number of invasive AGS and MKN28 cells under IL-22 stimulation. A representative image showing that IL-22 stimulation promotes AGS and MKN28 cell invasion. Effect of anti-IL-22 antibody on IL-22 (10 ng ml^{-1})-induced invasion of AGS (**B**) and MKN28 cells (**C**). A representative image showing control AGS cells, IL-22-treated (10 ng ml^{-1}) cells, IL-22-treated (10 ng ml^{-1}) cells in the presence of anti-IL-22 antibody (10 μg ml^{-1}) and cells cultured with anti-IL-22 antibody alone. (**D**) Effect of IL-22 on *MMP7* and *MMP13* expression in gastric cancer cells. All the results are presented as the mean ± s.e.m. of four independent experiments. Significantly greater than control: $^{*}P<0.05$, $^{**}P<0.01$. Significantly lower than the IL-22-treated group: $^{\#}P<0.01$.

With regard to MKN28 cells, STAT3 siRNA partly but not completely decreased the number of invasive cells accelerated by IL-22 stimulation, suggesting that not only STAT3 but also some other forms of signalling may mediate the promotion of MKN28 cell invasion by IL-22 (Figure 4B). We furthermore examined whether MAPK signalling is involved in the promotion of gasric cancer cell invasion by IL-22. The increase in the number of invasive AGS cells after the treatment with IL-22 was partly attenuated by concomintant administration of the MEK inhibitor PD98059 (Figure 4C). On the other hand, the increase in the number of MKN28 cells after treatment with IL-22 was reduced to a level equivalent to that in the absebce of stimulation upon treatment with PD98059 (Figure 4D). Throughout the invasion assay, cell viability was >86% by trypan blue (data not shown). These findings suggest that IL-22 may promote gastric cancer cell invasion via STAT3 and MAPK signalling, although the predominant intracellular signalling mechanism may differ according to the cell line.

CAF cells promote gastric cancer cell invasion. We examined whether CAF cells produce IL-22 protein by ELISA. The concentration of IL-22 in culture supernatant was lower than the serum concentration from UC, whereas it was higher than the serum concentration from healthy control (Figure 5A). This finding suggests that CAF cells are at least possible to produce IL-22 protein. Moreover, we demonstrated that the level of *IL-22* expression was significantly greater in CAFs than in NGF cells (Figure 5B).

To establish an *in vitro* model for the invasive front of gastric cancer tissues, we prepared co-culture system using CAF1 and AGS cells. The number of invasive AGS cells was significantly increased when AGS cells were co-cultured with CAF1 cells (Figure 5C). Furthermore, addition of IL-22 antibody abolished the increase in number of invasive AGS cells under co-culture with CAF1 cells (Figure 5C).

DISCUSSION

Although IL-22 has recently been highlighted in the pathophysiology of inflammatory diseases, its biological role in carcinogenesis is poorly understood. In the present study, we first confirmed that

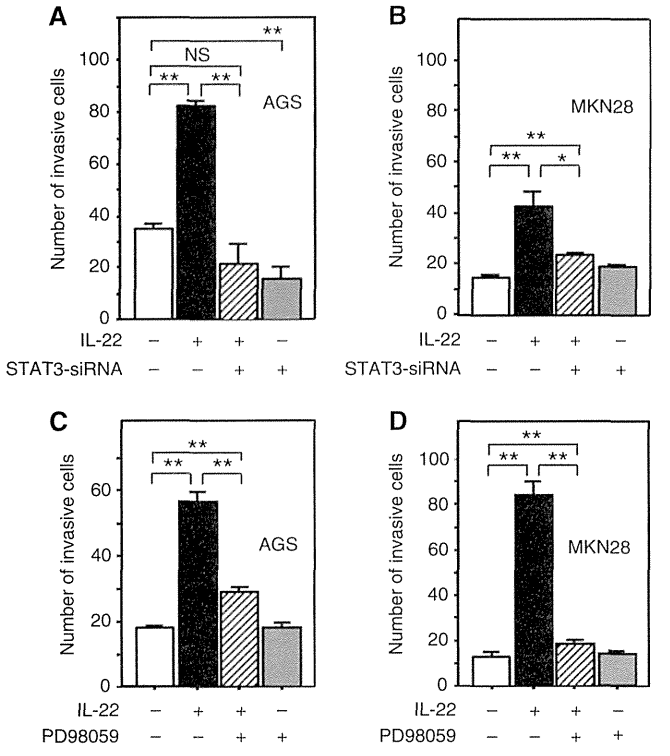

Figure 4. Effect of STAT3 siRNA treatment (**A** and **B**) and MEK inhibitor (**B** and **D**) on the IL-22-induced invasive potential of gastric cancer cells. AGS and MKN28 cells were transfected with STAT3 siRNA (or non-silencing siRNA as a control) for 48 h and used for invasion assay (**A** and **B**). The transfected AGS (5×10^4) and MKN28 (5×10^5) were seeded in the upper invasion chamber, stimulated by IL-22 (10 ng ml^{-1}) for 36 h, and evaluated as described in Materials and Methods. Similarly, AGS and MKN28 cells were cultured in the medium with or without PD98059 (20 μM) in the upper invasion chamber and stimulated by IL-22 (10 ng ml^{-1}; **C** and **D**). All the results are presented as the mean ± s.e.m. of four independent experiments. Significantly different between two groups: $^*P<0.05$, $^{**}P<0.01$.

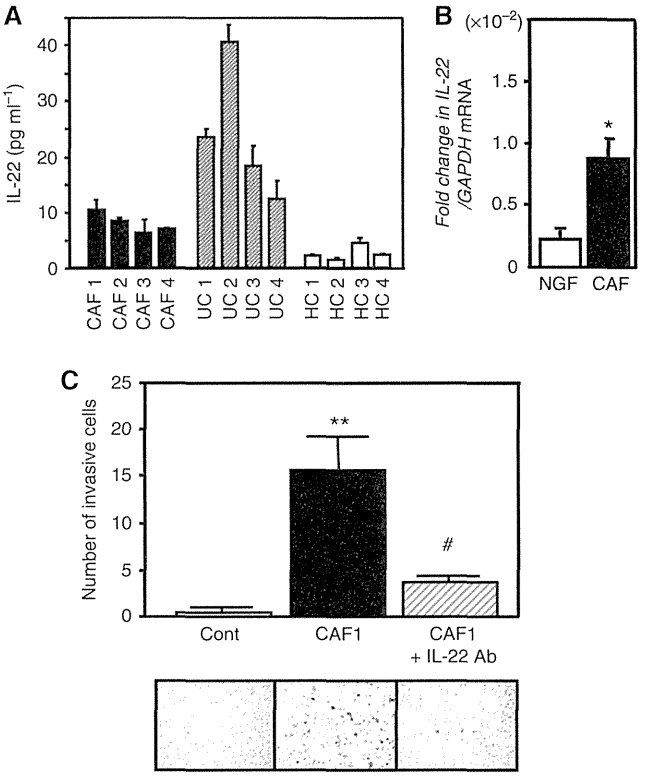

Figure 5. CAF cells promote AGS cell invasion via IL-22. (**A**) IL-22 production from CAF cells. CAF culture supernatant and serum from healthy control (HC) and UC patients were analysed by ELISA. (**B**) Expression of *IL-22* in NGF and CAF cells ($n=4$). (**C**) Effect of IL-22 on the link between CAF and AGS cells ($n=4$). CAF1 (1×10^5) cells were cultured in the lower chamber for 24 h. After washing with serum-free medium, upper invasion chambers were placed above the lower chambers and AGS cells (5×10^4) were then placed there in serum-free medium with or without anti-IL-22 antibody (10 μg ml^{-1}). After an additional 30 h of co-culture, the number of invaded AGS cells was evaluated as described in Materials and Methods. Photographs showing invasive gastric cancer cells in each group. All the results are presented as the mean ± s.e.m. Significantly greater than the control group: $^*P<0.05$, $^{**}P<0.01$. Significantly lower than the CAF1 co-cultured group: $^{\#}P<0.01$.

gastric cancer cells bear receptors for IL-22 *in vitro* and furthermore, using immunohistochemistry, we showed that human gastric cancer lesions indeed express IL-22 receptors, suggesting that gastric cancer cells may be reactive to IL-22 stimulation. We next investigated the expression of IL-22 in human gastric cancer tissues because little information is available about the clinicopathological significance of IL-22 expression in any human cancerous tissues. Interestingly, we observed strong expression of IL-22 in stromal cells at the invasive front of the tumour, suggesting that IL-22 signalling may be involved in gastric cancer cell invasion. In support of this hypothesis, gastric cancers that have IL-22-positive stromal cells at the invasive front showed not only a significantly high risk of lymphatic invasion but also a high tumour stage although this must be reconfirmed in a larger study including more number of gastric cancers without lymphatic invasion.

To clarify whether IL-22 promotes the invasion ability of gastric cancer cells, we stimulated two gastric cancer cell lines with IL-22 in an *in vitro* invasion assay. As shown in Figure 3, IL-22 stimulation significantly promoted the invasive ability of gastric cancer cells, and its effect was abolished by addition of IL-22 antibody, confirming that IL-22 is an invasion-promoting factor for gastric cancer cells. Although we did not test the effect of IL-22 in *in vivo* models, it is noteworthy that hepatocellular carcinoma co-transplanted with IL-22-expressing lymphocytes showed a high potential for invasion and metastasis (Jiang *et al*, 2011). Gastric cancer cells are known to have higher invasion and metastasis potential than other solid tumours. Therefore, IL-22 warrants further study as a potentially important mediator of invasion/metastasis in gastric carcinogenesis.

Previous studies have indicated that IL-22 may activate various pathways such as STAT3, MAPK, Akt and/or NF-κB signalling in different types of cells (Lejeune *et al*, 2002; Andoh *et al*, 2005; Zhang *et al*, 2008). In this regard, we examined the signalling pathways activated by IL-22 in human gastric cancer cells and subsequently showed that STAT3 and ERK phosphorylation was enhanced in two of the gastric cancer cell lines examined. Accumulating evidence suggests that IL-22 is possible to promote cell proliferation and anti-apoptosis via STAT3 and/or ERK signalling (Brand *et al*, 2006; Ziesché *et al*, 2007; Zhang *et al*, 2008; Sekikawa *et al*, 2010; Jiang *et al*, 2011). In addition, we have clarified in the present study that IL-22 significantly promoted the invasive ability of two gastric cancer cell lines via STAT3 and ERK signalling. These effects of IL-22 seem to be advantageous to tumour progression, and indeed, recent studies has reported that IL-22 is associated with tumour progression and prognosis in human malignancies (Kobold *et al*, 2013; Wen *et al*, 2014).

Interleukin-22 is normally expressed in immune cells such as IL-17-producing T (Th17), NK, dendritic, and lymphoid

tissue-inducer cells (Zenewicz and Flavell, 2011). However, it was noteworthy that IL-22 was strongly expressed in αSMA-positive fibroblasts neighbouring gastric cancer cells at the invasive front. The fibroblasts in such a tumour microenvironment are referred to as CAF cells and can be detected by their αSMA expression (Xing *et al*, 2010; Cirri and Chiarugi, 2012). In this context, it is tempting to speculate that IL-22 is produced in CAF cells in gastric cancer tissues. In support of this, we confirmed that IL-22 is expressed in isolated CAF cells from gastric cancer tissues. Although we are unable to explain why IL-22 is expressed in CAF cells, recent evidence has suggested that CAF cells originate from not only resident fibroblasts but also bone marrow-derived progenitors or transformed cells from endothelial or cancer cells (Xing *et al*, 2010; Cirri and Chiarugi, 2012). Thus, the heterogeneity of CAF cells may explain the unexpected expression of IL-22 in CAF cells.

CAF cells have recently received attention because of their pivotal roles in tumour growth, angiogenesis, invasion, and metastasis by interacting with tumour cells (Bhowmick *et al*, 2004; Xing *et al*, 2010; Cirri and Chiarugi, 2012). Although the mechanism of the interaction between CAF and tumour cells is not fully understood, growth factors, chemokines or extracellular matrix are thought to be important mediators by which such cells communicate with their microenvironment (Bhowmick *et al*, 2004; Xing *et al*, 2010; Cirri and Chiarugi, 2012). Regarding the role of IL-22 in cancerous cells, a few studies have indicated that IL-22 can function as a cell growth and/or anti-apoptotic factor *in vitro* (Brand *et al*, 2006; Zhang *et al*, 2008; Jiang *et al*, 2011), although the source of IL-22 in human cancerous tissues has remained unclear. In this context, we have shown for the first time that IL-22 promotes the invasive ability of gastric cancer cells via STAT3 and ERK activation, and that CAF is a possible source of IL-22 at the invasive tumour front. Although these two important findings were confirmed in different series of experiments, our co-culture experiment lent further support to the possibility that IL-22-expressing CAFs actually have a role in the promotion of gastric cancer cell invasion.

In summary, we have clarified that IL-22 promotes the invasive ability of gastric cancer cells via STAT3 and ERK activation. Moreover, we have shown that IL-22 is expressed in CAFs at the invasive front of gastric cancer lesions and that IL-22-expressing CAFs isolated from human gastric cancer promote invasion of the cancer cells. Together, these results suggest that IL-22 produced by CAFs promotes gastric cancer cell invasion via STAT3 and ERK signalling.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Gastric Cancer
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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 GI: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).

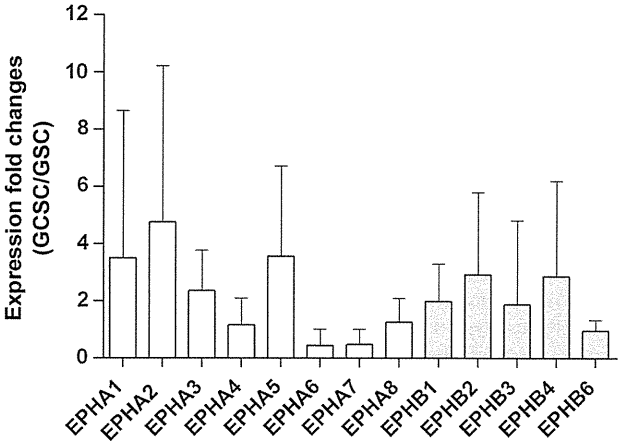

Fig. 1 Real-time RT-PCR analysis of Ephrin receptors of GCSC and GSC. Real-time RT-PCR analysis showed that EphA1, EphA2, EphA3, EphA5, EphB2, and EphB4 were upregulated 3.50-, 4.76-, 2.36-, 3.57-, 2.93-, and 2.86-fold, respectively, in GCSC relative to GSC in each patient. But there was no significance.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

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 (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

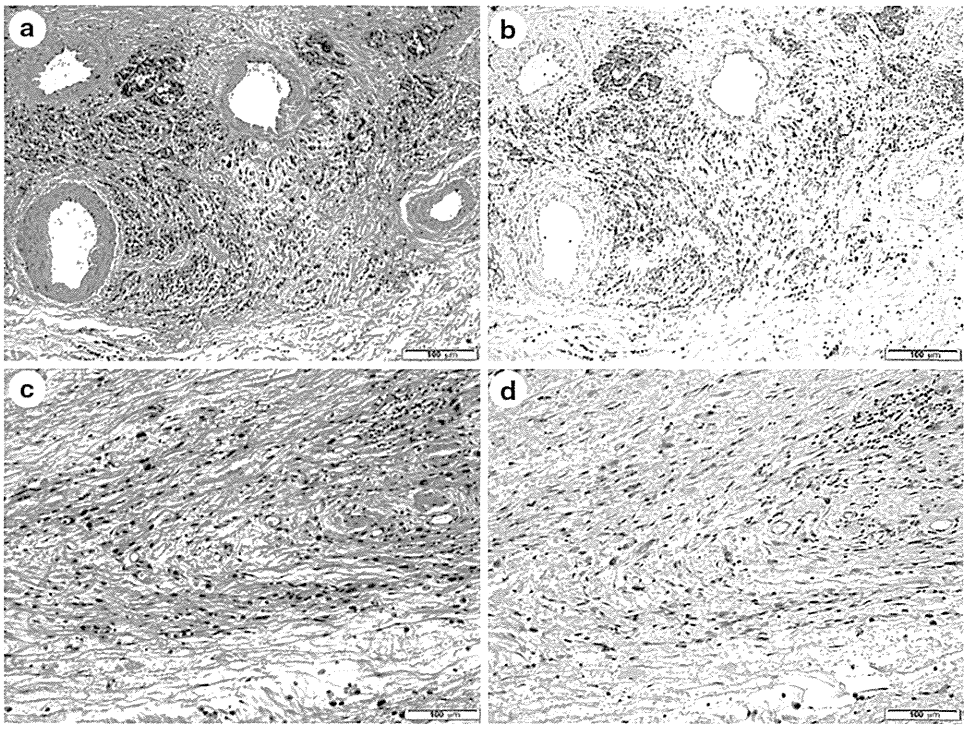

Fig. 2 Intermingling of scattered cancer cells and GCSC (IC). ICs were defined as 1) scattered cancer cells intermingled with GCSC in invasive parts of tumors; and 2) staining for EphA2 in cancer cells or GCSC being equal to or stronger than in the soma of Auerbach's plexus, in which EphA2 is normally 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,

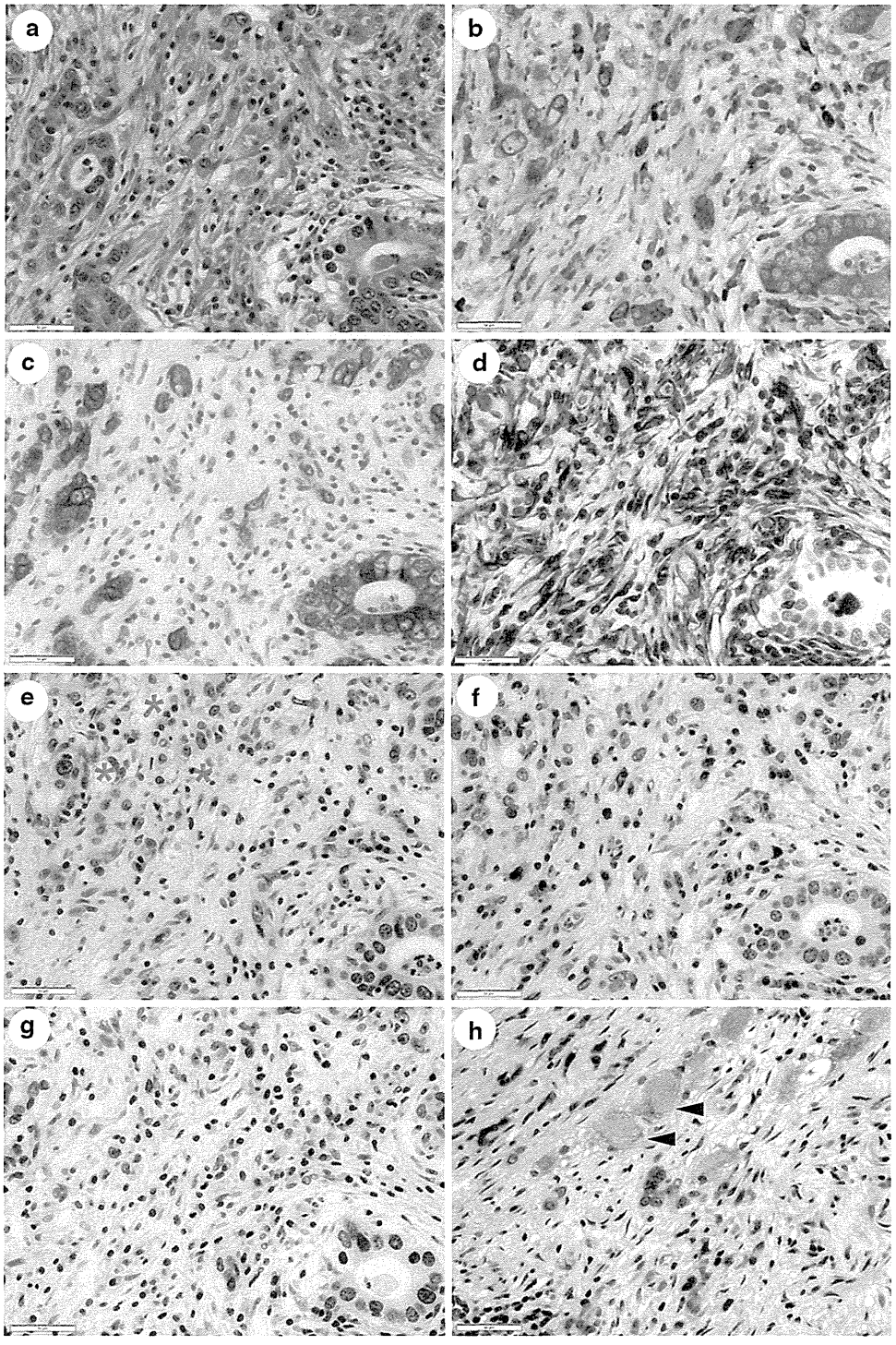

Fig. 3 Intermingling of scattered cancer cells and GCSC expressing Ephrin A2 receptors (IC/A2+). Immunohistochemistry analyzed phenotype of IC/A2-positive lesion. **a** Hematoxylin-eosin staining of ICs, showing intermingling of scattered cancer cells and stromal cells in invasive lesions. **b** EphA2 expression in both of cancer cells and stromal cells. Expression of keratin (**c**) and vimentin (**d**) in cancer cells and mesenchymal cells. Fibroblasts, endothelial cells, and hematopoietic cells were positive for vimentin, but negative for keratin. **e** alpha-SMA expression in vascular vessels and myofibroblasts. Most stromal cells were alpha-SMA negative in ICs. Large spread stromal cells were positive for alpha-SMA (*asterisks*), whereas other most small stromal cells in ICs were alpha-SMA negative. **f** CD31 was expressed in endothelial cells and hematopoietic cells, not in most stromal cells (**g**) D2-40 expression in lymphatic endothelial cells. **h** Expression of EphA2 in the soma of Auerbach's plexus (*black arrow heads*). Original magnification ×40

but that EphA2 expression alone is insufficient to distinguish patients at high risk of tumor recurrence. In contrast, IC/A2+ was significantly predictive of relapse. Of 26 patients classified as IC/A2+, 14 (53.8 %) developed recurrence, including eight with peritoneal metastasis, three with LN metastasis, and three with blood-borne metastasis, with a median RFS of 378 days, significantly shorter than in patients classified as IC/A2−Ca/A2− (1,298 days) or IC/A2−Ca/A2+ (1,120 days). Invisible/micro-metastases may have been present in IC/A2+ patients at the time of gastrectomy. These patients require another treatment strategy, since many relapsed while receiving adjuvant chemotherapy. Even for a high-risk patient, second or third line chemotherapy was effective in

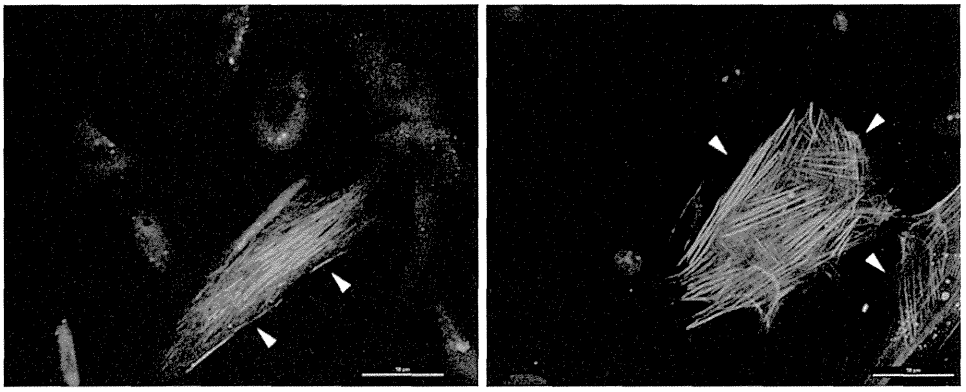

Fig. 4 Immunofluorescent microscopy of primary cultured GCSC. Expression of EphA2 (*red*) and α-SMA (*green*) was analyzed in primary cultured GCSC by immunofluorescent microscopy. In left panel, GCSC looks EphrinA2-positive (*red*) and one cell strongly expressed α-SMA (*white arrow head*). In *right panel*, all cells were EphA2 negative and widespread cells were α-SMA-positive (*white arrow head*). Primary cultured GCSC expressed different levels of α-SMA. These data suggested that the status of GCSC in IC/A2+ might be different from that in cultured and activated myofibroblasts. Original magnification ×40 (color figure online)

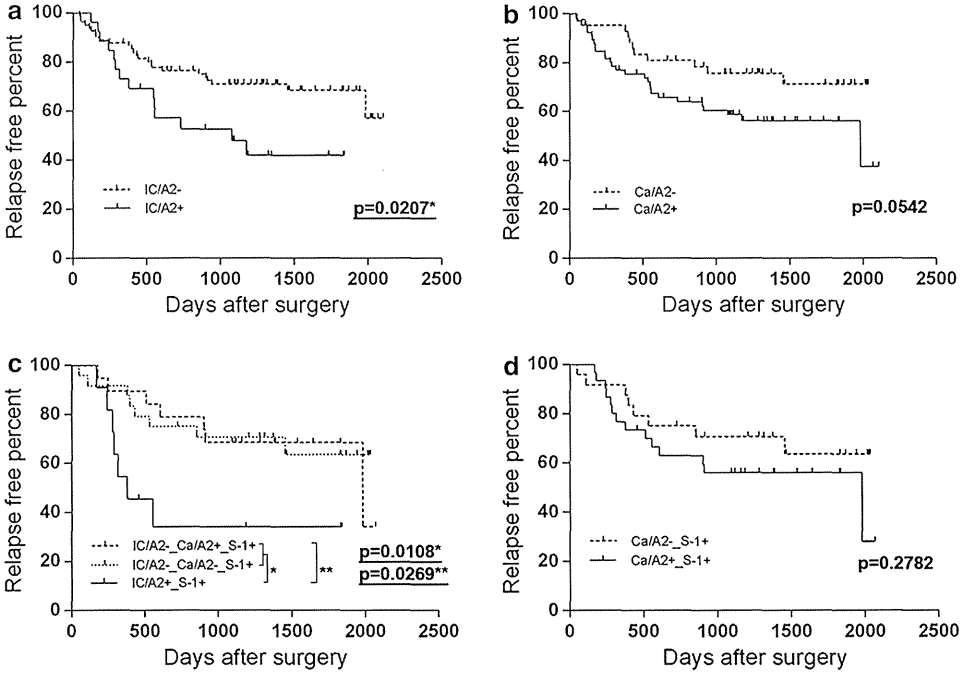

Fig. 5 Relapse free survival of all patients ($n = 107$) and of patients who received adjuvant chemotherapy (S-1, $n = 54$). Kaplan–Meier curves showing the relationship between RFS and expression of EphA2 in IC parts (**a**), in cancer cells (**b**) in all patients, and in patients who received adjuvant chemotherapy (**c**, IC/A2+ vs. IC/A2− Ca/A2± vs. IC/A2−Ca/A2−; **d**, Ca/A2). **a** The relapse rate was significantly higher in IC/A2+ than in IC/A2− patients (HR, 2.12; 95 % CI, 1.16–5.41; $p = 0.0207$). **b** The recurrence rate was higher in patients classified as Ca/A2+ than in those classified as Ca/A2− [HR, 1.96; 95 % confidence interval (CI), 0.99–3.51; $p = 0.0542$], not significant. **c** IC/A2+ was significantly associated with poorer RFS during adjuvant chemotherapy (HR, 3.00; 95 % CI, 1.47–17.03; $p = 0.0108$). Stromal reaction may have been prognostic, because patients classified as IC/A2+ had significantly reduced median RFS compared to those classified as IC/A2− (378 vs. 1,120 days; HR, 2.99; 95 % CI, 1.22–13.63; $p = 0.0269$). **c**, and **d** Fifty-four patients received six to eight cycles of adjuvant chemotherapy with S-1 (tegafur–gimeracil–oteracil potassium) without severe adverse effects. **b** Fourteen of 30 patients (46.7 %) classified as Ca/A2+ experienced recurrence during treatment, compared with seven of 11 (63.6 %) IC/A2+ patients. Almost half of the patients classified as IC/A2+ relapsed within 1 year after R0 operation, even while receiving adjuvant chemotherapy

IC/A2+ patients. Overall survival wasn't significantly different between IC/A2 and Ca/A2 without regard for adjuvant chemotherapy. When cancer was relapsed, most patients received additional chemotherapy, and our data proved effectiveness of additional chemotherapy in relapse cases. IC/A2+ in gastric cancer exactly indicated a risk of

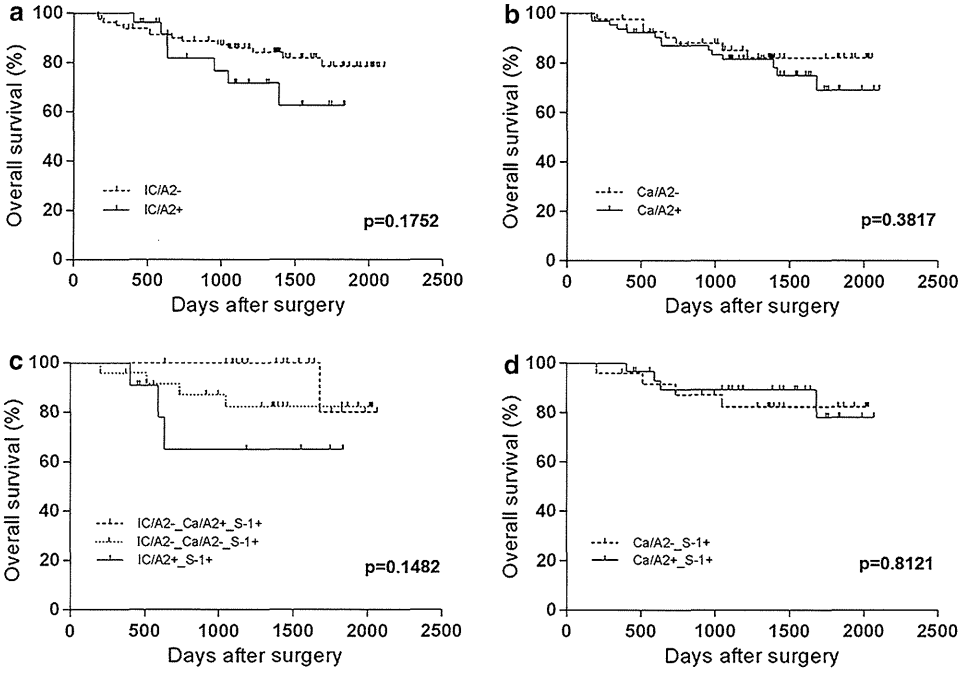

Fig. 6 Overall survival of all patients ($n = 107$) and of patients who received adjuvant chemotherapy (S-1, $n = 54$). Kaplan–Meier curves showing the relationship between overall survival (OS) and expression of EphA2 in IC parts (**a**), in cancer cells (**b**) in all patients, and in patients who received adjuvant chemotherapy (**c**, IC/A2+ vs. IC/A2− Ca/A2± vs. IC/A2−Ca/A2−; **d**, Ca/A2). **a**, and **b** Overall survival wasn't significant in IC/A2+ and IC/A2− patients ($p = 0.1752$), and in Ca/A2+ and Ca/A2− patients ($p = 0.3817$). **c** Median overall survival in IC/A2+ (633 days) was shorter than in IC/A2− (1,398 days), and not significant. **d** Ca/A2 wasn't a prognostic factor in patients who received adjuvant chemotherapy ($p = 0.8121$). When cancer was relapsed, most patients received additional chemotherapy, and these data had also proved effectiveness of second or third line chemotherapy in relapse cases. IC/A2+ in gastric cancer exactly indicated a risk of relapse, not the biological malignancy of cancer

relapse, not the biological aggressiveness of cancer. In patients who received adjuvant chemotherapy, median RFS and median OS in IC/A2+ was 378 and 633 days. However, median RFS and median OS in IC/A2− was 1,280 and 1,398 days, respectively. Our data indicated that careful follow-up and earlier diagnosis of relapse might improve survival of high-risk patients.

The EphA2–ephrin signaling axis regulates multiple events critical for the malignant transformation of normal cells. The key downstream molecules in this signaling pathway include the phosphatidylinositol 3′ kinases, Src family kinases, Rho and Rac1 GTPases, mitogen activated protein kinases and integrins. Moreover, there is cross talk between these molecules and other oncogene receptors (e.g., EGFR), which regulate cell adhesion, proliferation, and migration; modulate the cytoskeletal architecture, and control the development of vascular networks. The ephrin RTKs and their ephrin ligands have intriguing expression patterns in cancer and stromal cells, suggesting the importance of their bidirectional signals in many aspects of tumor development and progression. Targeting EphA2 overexpression may be beneficial in cancer therapeutics. Among the molecules targeting Eph receptors and ephrin currently in development are RTKs in the forward signaling pathway [26, 27], siRNA/oligonucleotides as inhibitors of Eph expression [28, 29], peptides/mAb that inhibit Eph–ephrin interactions [30, 31], cytotoxic mAbs and mAb conjugates [32, 33], and nanoparticles/mAb as imaging agents [34–36]. Most of these molecules were found to target cancer cells but not stromal cells, and their effects on tumor progression involving cancer stromal cell interactions were unclear. These interactions through the Eph–ephrin axis may be flexible and adaptable for survival in various microenvironments. EphA2 targeting should regulate deleterious cancer stromal cell interactions and be cytotoxic to cancer cells.

In conclusion, IC/EphA2 expression in invasive parts of tumors was useful in determining a high risk of relapse after curative (R0) surgery for gastric cancer. Further examination of the EphA2–ephrin signaling pathway in cancer stromal cells is essential in the development of agents that target Eph and ephrin.

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ORIGINAL REPORT

Lapatinib Plus Paclitaxel Versus Paclitaxel Alone in the Second-Line Treatment of *HER2*-Amplified Advanced Gastric Cancer in Asian Populations: TyTAN—A Randomized, Phase III Study

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See accompanying article on page 2074

A B S T R A C T

Purpose

In Asian countries, paclitaxel once per week is used as second-line treatment in advanced gastric cancer, including human epidermal growth factor receptor 2 (HER2) –positive tumors. The role of anti-HER2 agents, including lapatinib, in this setting and population is unclear.

Patients and Methods

TyTAN was a two-part, parallel-group, phase III study in Asian patients. An open-label, dose-optimization phase (n = 12) was followed by a randomized phase (n = 261), in which patients who were HER2 positive by fluorescence in situ hybridization (FISH) received lapatinib 1,500 mg once per day plus once-per-week paclitaxel 80 mg/m^2 or paclitaxel alone. The primary end point was overall survival (OS). Secondary end points included progression-free survival (PFS), time to progression (TTP), overall response rate (ORR), time to response, response duration, and safety. Analyses were based on immunohistochemistry (IHC) and gastrectomy status, prior trastuzumab therapy, and regional subpopulations.

Results

Median OS was 11.0 months with lapatinib plus paclitaxel versus 8.9 months with paclitaxel alone (P = .1044), with no significant difference in median PFS (5.4 *v* 4.4 months) or TTP (5.5 *v* 4.4 months). ORR was higher with lapatinib plus paclitaxel versus paclitaxel alone (odds ratio, 3.85; P < .001). Better efficacy with lapatinib plus paclitaxel was demonstrated in IHC3+ compared with IHC0/1+ and 2+ patients and in Chinese compared with Japanese patients. A similar proportion of patients experienced adverse events with each treatment (lapatinib plus paclitaxel, 100% *v* paclitaxel alone, 98%).

Conclusion

Lapatinib plus paclitaxel demonstrated activity in the second-line treatment of patients with HER2 FISH-positive IHC3+ advanced gastric cancer but did not significantly improve OS in the intent-to-treat population.

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INTRODUCTION

Gastric cancer remains the second most common cause of cancer mortality worldwide.[1,2] The incidence of gastric cancer is highest in Eastern Asia (19% and 12% of cancers [excluding nonmelanoma skin cancer] in men and women, respectively).[1,2] Relative 5-year survival is 18% in the United Kingdom and approximately 27% in the United States,[3,4] but it is somewhat higher in Japanese (40% to 60%) and Korean patients (approximately 60%), potentially related to earlier diagnosis.[5,6]

Cisplatin- or fluorouracil-based regimens are often used to treat metastatic gastric cancer, with median overall survival (OS) of 10 to 13 months and progression-free survival (PFS) of 6 to 7 months.[7,8] Many patients initially respond to chemotherapy, but treatment is not curative, and patients experience progression. Second-line once-per-week paclitaxel is widely used in this setting in Asian countries, with median OS of 151 days to 9.5 months and PFS of 2.6 to 3.6 months.[9-12]

Among patients with gastric cancer, positive expression of the human epidermal growth factor

receptor 2 (HER2) protein has been variably reported in 8.2% to 29.5% of patients by immunohistochemistry (IHC2+/IHC3+)[13-18] and 3.8% to 27.1% of patients by fluorescence in situ hybridization (FISH).[14,15,19] Trastuzumab plus chemotherapy is standard first-line treatment for advanced HER2-positive disease (median OS, 13.8 *v* 11.1 months with chemotherapy alone).[20] The role of anti-HER2 agents in the second-line treatment of HER2-positive advanced gastric cancer is unclear, and a new targeted therapy is needed.

Lapatinib (Tykerb/Tyverb; GlaxoSmithKline, Ware, United Kingdom) binds to the intracellular tyrosine kinase domains of epidermal growth factor receptor (ErbB1) and HER2 (ErbB2), blocking autophosphorylation and downstream signaling.[21] Here we report on TyTAN (Tykerb With Taxol in Asian HER2-Positive Gastric Cancer), the first randomized study to our knowledge comparing the efficacy and safety of lapatinib plus paclitaxel with paclitaxel alone in the second-line treatment of HER2-positive advanced gastric cancer.

PATIENTS AND METHODS

Study Design

TyTAN was a two-part, parallel-group, phase III study. In the dose-optimization, nonrandomized, open-label pilot study, patients were stratified according to gastrectomy status (no history of gastrectomy; gastrectomy, pylorus removed; or gastrectomy, pylorus preserved). Patients received oral lapatinib 1,500 mg once per day and 1-hour intravenous (IV) infusion of paclitaxel 80 mg/m^2 on days 1, 8, and 15 of a 28-day cycle.

Blood samples for lapatinib pharmacokinetic analyses were taken predose and 0.5 to 24 hours postdose on days 8 (treatments coadministered) and 14 (lapatinib administered alone); samples for paclitaxel analyses were taken on days 1 (paclitaxel administered alone) and 8 (treatments coadministered) at 0.5 to 24 hours postdose. In cases of toxicity, doses could be reduced in a stepwise manner (Appendix and Appendix Fig A1, online only).

In the randomized study, patients were enrolled using RAMOS (Registration and Medication Ordering System) and stratified according to prior trastuzumab therapy and gastrectomy status. Patients were randomly assigned using a telephone-based system and Randall-generated codes at a 1:1 ratio to oral lapatinib 1,500 mg once per day plus paclitaxel 80 mg/m^2 IV on days 1, 8, and 15 of a 4-week cycle or paclitaxel 80 mg/m^2 IV.

The study complied with the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use good clinical practice and the 2008 Declaration of Helsinki. The protocol was approved by a national or local ethics committee or institutional review board. Written informed consent was obtained from patients before study-specific procedures.

Study Population

The pilot study involved nine Japanese centers; patients with gastric cancer were enrolled irrespective of HER2 status. In the randomized study, 48 centers in mainland China, Japan, South Korea, and Taiwan enrolled patients with *HER2*-amplified gastric cancer. Patients in the intent-to-treat (ITT) population were FISH positive (HER2:CEP17 ratio $\geq$ 2) based on previous local or central laboratory tests. The modified ITT (mITT) population comprised all randomly assigned patients confirmed FISH positive by central laboratories. Other inclusion and exclusion criteria were identical.

Eligible patients were age $\geq$ 20 years, had experienced progression after prior gastric cancer treatment, had measurable lesions according to RECIST (version 1.0), had European Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 1, had predicted life expectancy $\geq$ 12 weeks from first study-drug dose, had normal left ventricular ejection fraction and organ function, and could swallow and retain medication. A washout period from previous treatments was required (2 to 6 weeks).

Patients were excluded if they were of childbearing potential (unless practicing acceptable birth control methods); had used epidermal growth factor receptor agents; had been previously exposed to taxanes; had unresolved or unstable toxicity resulting from prior cancer treatment; had a history of uncontrolled or symptomatic angina, arrhythmias, or congestive heart failure; or exhibited hypersensitivity or idiosyncrasy to drugs chemically related to paclitaxel.

Study End Points

Pilot study. The primary objective was to determine the optimal lapatinib plus paclitaxel dose, based on dose-limiting toxicities, and assess safety and tolerability. Evaluations included demographic and baseline characteristics, adverse events (AEs), ECOG PS, vital signs, clinical laboratory tests, and 12-lead electrocardiograms (ECGs). Pharmacokinetic profiling was performed at the time of blood sampling. A safety and efficacy review committee (SERC) proposed additional actions or patient inclusion as appropriate.

Randomized study. The primary efficacy end point was OS (time from random assignment to death). Secondary end points included PFS, time to progression (TTP), overall response rate (ORR; complete and partial responses), and response duration. Safety assessments involved AEs (including AEs of special interest; Appendix Table A1, online only), clinical laboratory tests, vital signs, 12-lead ECGs, and ECOG PS. When data on approximately 100 patients were available, the SERC assessed ORRs, 95% CIs, and safety data in futility analyses. A second interim analysis by an independent data monitoring committee (IDMC; at approximately 144 deaths) reviewed efficacy and safety data to advise on early study termination if lapatinib plus paclitaxel demonstrated superior efficacy.

Assessments

Disease assessments and tumor progression were recorded at screening, at baseline, every 8 weeks, and at end of therapy. Safety and laboratory assessments (including 12-lead ECGs and echocardiography or multigated acquisition scans) were performed at screening, at baseline and/or on day 1, once per week, once every 4 weeks, or once every 8 weeks (dependent on specific parameter), and at therapy end.

Pilot Study Pharmacokinetics

Noncompartmental model analysis from lapatinib plasma concentration-time data was used to calculate area under the concentration-time curve from time zero to 24 hours (AUC_{0-24}), maximum plasma concentration (C_{max}), and time to C_{max}. AUC from time zero to infinity ($AUC_{0-\infty}$), total body clearance, steady-state volume of distribution, and apparent elimination half-life were established from paclitaxel data.

Statistics

No statistical analyses were performed on pilot study data. The randomized study had a 24-month accrual period with a 12-month follow-up; 191 events would show superiority of lapatinib plus paclitaxel (hazard ratio [HR] $\leq$ 0.667 or 3-month increase in median OS). For primary analyses, 220 patients were required (110 patients per arm); 260 patients were enrolled (predicting 15% dropout) to achieve 80% power (one-sided α = 2.5%). IDMC interim analysis used a significance level of .001 (one sided); final analyses at 191 deaths used a significance level of .024967 (one sided) for stratified log-rank testing. AEs of special interest were assessed using descriptive statistics; 95% CIs were calculated for incidence rates per treatment group and treatment group differences in incidence rates.

Efficacy analyses examined the ITT (ie, patients randomly assigned to treatment) and mITT populations (ie, randomly assigned patients confirmed FISH positive by central laboratories); safety analyses assessed the randomly assigned population who received $\geq$ one medication dose. OS and PFS were summarized for subgroups (ie, gastrectomy status, HER2 IHC, prior trastuzumab therapy, and regional subpopulation [mainland China, Japan, South Korea, and Taiwan]).

Kaplan-Meier curves summarized OS, PFS, TTP, and response duration. OS was compared between treatments with stratified log-rank testing. ORR was based on complete or partial response. Secondary end point tests were two sided (α = 5.0%). The 95% CIs for tumor response rates were calculated, and the odds ratios (ORs) were compared using stratified Fisher's exact tests. Zelen's test for homogeneity of ORs across strata was performed to validate data.

Table 1. Baseline Patient Demographic and Clinical Characteristics

Characteristic	Pilot Study				Randomized Study					
	Cohort One (n = 6)		Cohort Three (n = 6)		Lapatinib Plus Paclitaxel (n = 132)		Paclitaxel Alone (n = 129)		Total (N = 261)	
	No.	%	No.	%	No.	%	No.	%	No.	%
Age, years										
Mean	57.5		57.2		60.8		60.4		60.6	
SD	11.31		10.98		9.45		10.96		10.21	
Median	60.0		59.5		61.0		62.0		61.0	
Range	35-66		36-66		32-79		22-80		22-80	
Sex										
Female	1	17	0	0	31	23	23	18	54	21
Male	5	83	6	100	101	77	106	82	207	79
Country of enrollment										
Japan	6	100	6	100	52	39	48	37	100	38
Mainland China	0	0	0	0	45	34	50	39	95	36
South Korea	0	0	0	0	22	17	24	19	46	18
Taiwan	0	0	0	0	13	10	7	5	20	8
Weight, kg										
Mean	60.10		58.27		55.32		55.88		55.60	
SD	6.27		6.86		10.38		9.60		9.99	
Median	60.25		62.00		55.00		55.80		55.00	
Range	53.0-68.8		46.3-63.2		30.5-90.8		34.1-84.0		30.5-90.8	
ECOG PS at screening										
0	4	67	5	83	60	45	48	37	108	41
1	2	33	1	17	72	55	81	63	153	59
HER2 IHC					(n = 100)		(n = 92)		(n = 192)	
0/1+	NR		NR		36	36	32	35	68	35
2+	NR		NR		12	12	11	12	23	12
3+	NR		NR		52	52	49	53	101	53
FISH positive*	NR		NR		132	100	129	100	261	100
Gastrectomy status										
No history	6	100	0	0	55	42	55	43	110	42
Yes, pylorus removed	0	0	6	100	66	50	63	49	129	49
Yes, pylorus preserved	0	0	0	0	11	8	11	9	22	8
Time since diagnosis, weeks					(n = 126)		(n = 124)		(n = 250)	
Median	64.43		55.57		9.6		9.9		9.8	
Range	20.0-84.6		24.0-109.7		1-69		1-146		1-146	
Primary tumor type at diagnosis										
Gastric	6	100	6	100	132	100	127	98	259	> 99
Other†	0	0	0	0	0	0	2	2	2	< 1
Disease stage at initial diagnosis					(n = 131)		(n = 126)		(n = 257)	
IV	6	100	3	50	87	66	80	62	167	64
Stage IV disease at screening	6	100	6	100	127	96	121	94	248	95
Type of gastric cancer										
Diffuse	NR		NR		44	34	44	34	88	34
Intestinal	NR		NR		56	43	54	42	110	43
Other‡	NR		NR		30	23	30	23	60	23
Prior therapy										
Chemotherapy	6	100	6	100	132	100	129	100	261	100
Surgery	0	0	6	100	77	58	74	57	151	58
Biologic therapy	2	33	0	0	6	5	8	6	14	5
Immunotherapy	0	0	0	0	4	3	2	2	6	2
Radiotherapy	0	0	0	0	6	5	7	5	13	5
Prior trastuzumab therapy										
Treated	NR		NR		8	6	7	5	15	6
Untreated	NR		NR		124	94	122	95	246	94

(continued on following page)

Table 1. Baseline Patient Demographic and Clinical Characteristics (continued)

Characteristic	Pilot Study: Cohort One (n = 6) No.	%	Pilot Study: Cohort Three (n = 6) No.	%	Randomized Study: Lapatinib Plus Paclitaxel (n = 132) No.	%	Randomized Study: Paclitaxel Alone (n = 129) No.	%	Total (N = 261) No.	%
Follow-up anticancer therapy										
Any therapy	NR		NR		76	58	82	64	158	61
Chemotherapy	NR		NR		73	55	71	55	144	55
Radiotherapy	NR		NR		11	8	21	16	32	12
Biologic therapy (monoclonal antibodies, vaccines)	NR		NR		7	5	15	12	22	8
Surgery	NR		NR		5	4	10	8	15	6
Immunotherapy	NR		NR		2	2	2	2	4	2
Unknown	NR		NR		2	2	1	< 1	3	1
Hormonal therapy	NR		NR		0	0	0	0	0	0

Abbreviations: ECOG PS, Eastern Cooperative Oncology Group performance status; FISH, fluorescent in situ hybridization; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; NR, not reported; SD, standard deviation.
*HER2:CEP17 ratio ≥ 2; local or central laboratory test.
†Includes cardia of stomach and carcinoma of gastric cardia.
‡Most patients categorized as other had adenocarcinoma.

RESULTS

Population Characteristics

In the pilot study (June 2007 to January 2009), six patients had no history of gastrectomy (cohort one), and six had undergone gastrectomy (pylorus removed; cohort three). Cohort two enrollment (gastrectomy, pylorus preserved) was halted based on preliminary safety and pharmacokinetic analyses of cohorts one and three. Baseline characteristics were similar between cohorts (Table 1).

In the separate, randomized study (March 2008 to January 2012), 261 patients were randomly assigned to lapatinib plus paclitaxel (n = 132) or paclitaxel alone (n = 129; Fig 1). The SERC presumed the blood concentration and tolerability of active treatments in patients who had undergone gastrectomy (pylorus preserved) would lie in the range observed in patients who had not undergone gastrectomy and in those who had undergone gastrectomy (pylorus removed), allowing inclusion of patients who had undergone gastrectomy with the pylorus removed (49%) or preserved (8%) as well as those with no history of gastrectomy (42%).

Demographic and baseline characteristics were balanced between treatment arms (Table 1). In each arm, 55% of patients received follow-up chemotherapy. More patients randomly assigned to paclitaxel alone than lapatinib plus paclitaxel received other follow-up therapies. Few patients in each treatment arm had previously received trastuzumab (lapatinib plus paclitaxel, 6%; paclitaxel alone, 5%). All randomly assigned patients had evidence of FISH positivity at baseline; central laboratory tests confirmed FISH positivity in 124 of 132 patients receiving lapatinib plus paclitaxel and 121 of 129 patients receiving paclitaxel alone. Among those with a FISH ratio < 2 (n = 8) or from 2.0 to 4.0 (n = 79), five (63%) and 47 (59%) patients, respectively, were also IHC0/1+ (Appendix Table A2, online only).

Pilot Study

Optimal dose. Because no dose-limiting toxicities requiring a dose reduction were observed, the SERC endorsed an optimally tolerated regimen of lapatinib 1,500 mg once per day plus paclitaxel 80 mg/m^2 once per week for 3 weeks of a 4-week cycle, regardless of gastrectomy status.

Pharmacokinetics. Lapatinib plus paclitaxel increased $AUC_{0\text{-}24}$ for lapatinib by 27% and 22% in cohorts one (n = 6) and three (n = 6), respectively, versus lapatinib alone and C_{max} by 38% and 52% in cohorts one and three, respectively. Lapatinib + paclitaxel showed a 30% increase of $AUC_{0\text{-}24}$ versus paclitaxel alone in cohort one, and 26% in cohort three, but no increase for C_{max} (Appendix Table A3, online only).

$AUC_{0\text{-}24}$ and C_{max} for lapatinib were lower in patients who had undergone gastrectomy with pylorus removed than in patients with

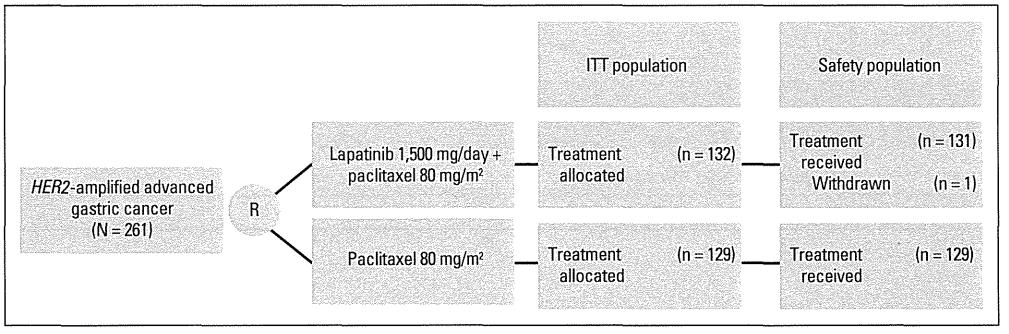

Fig 1. CONSORT diagram. *HER2*, human epidermal growth factor receptor 2; ITT, intent to treat; R, random assignment.

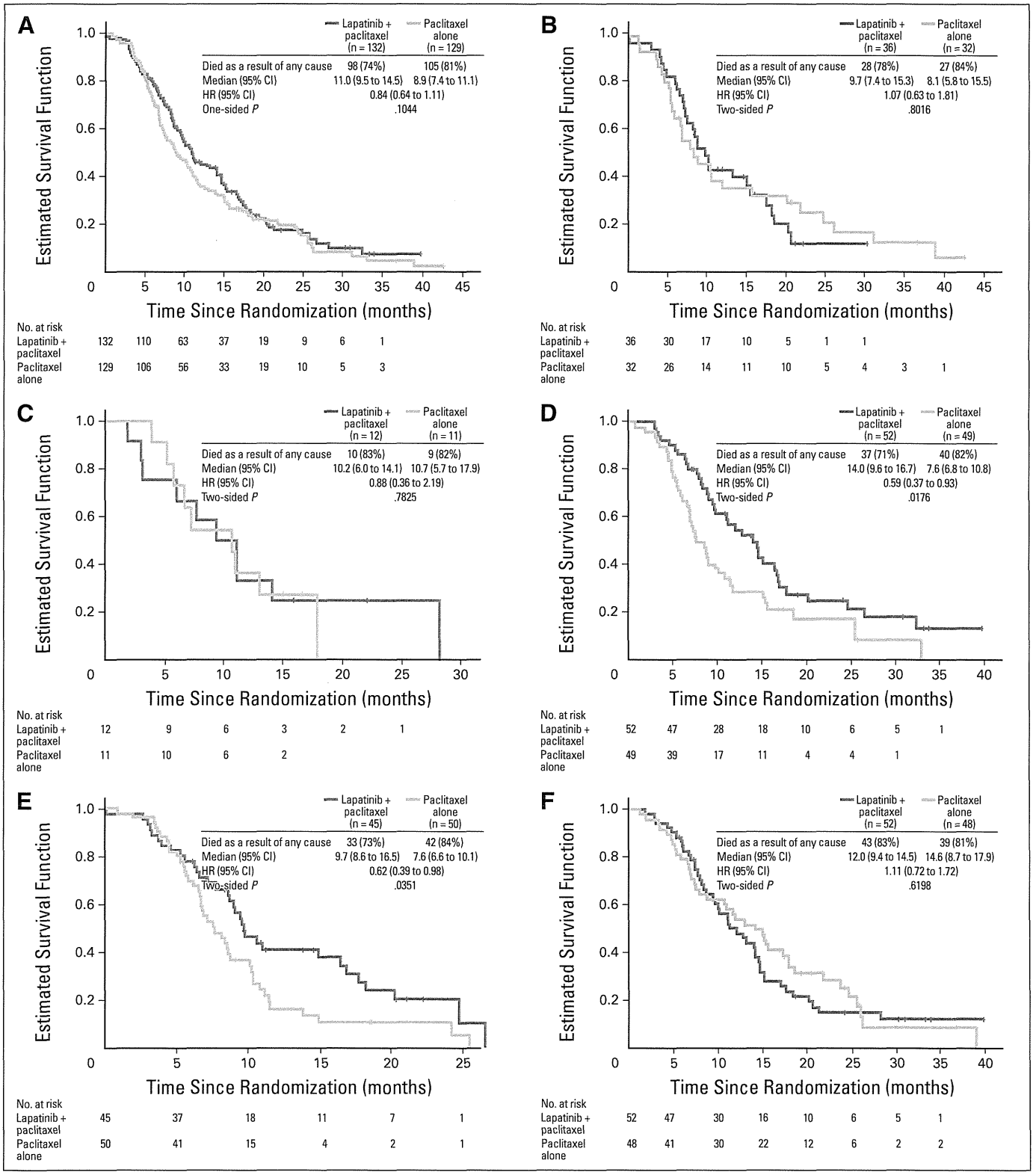

Fig 2. Kaplan-Meier overall survival curves for (A) intent-to-treat population and (B) human epidermal growth factor receptor 2 (HER2) immunohistochemistry (IHC) 0/1+, (C) HER2 ICH2+, (D) HER2 IHC3+ patients, (E) patients in mainland China, and (F) patients in Japan. HR, hazard ratio.