Table 1: Incidence of Hand-foot Syndrome on Comparative Studies of S-1

Study	Treatment Arms	Incidence of HFS (%) All Grades	Incidence of HFS (%) Grades 3–4
FLAGS (Ajani et al., 2010)[3]	S-1 + cisplatin (n=527)	5.4	0.19
	5-FU + cisplatin (n=526)	2.6	0.39
REAL-2 (Cunningham et al., 2008)[4]	epirubicin + cisplatin + capecitabine (n=250)	45.9	10.3
	epirubicin + oxaliplatin + capecitabine (n=244)	39.3	3.1
	epirubicin + cisplatin + 5-FU (n=263)	29.8	4.3
	epirubicin + oxaliplatin + 5-FU (n=245)	28.9	2.7
ML17032 (Ryu and Kang, 2009)[5]	capecitabine + cisplatin (n=160)	22	3.9
	5-FU + cisplatin (n=156)	4	0
SOX vs CAPOX (Kim et al., 2012)[6]	S-1 + oxaliplatin (n=65)	3.1	0
	capecitabine + oxaliplatin (n=64)	25	1.6

5-FU = 5-fluorouracil; FLAGS = First-Line advanced gastric cancer study; REAL2 = Randomized ECF for Advanced and Locally Advanced Esophagogastric Cancer study 2; S-1 = tegafur/gimeracil/oteracil combination (Teysuno™).

Four avenues have been exploited in order to improve the therapeutic index of 5-FU. The first avenue consisted of biochemical modulation with methotrexate, leucovorin and interferon.[1,2] The second avenue involved optimising the dosing schedule. For many years, it has been believed that continuous infusion of 5-FU channels its mechanism of action toward the DNA pathway – the basis of its anti-tumour activity – whereas a bolus dose diverts its activity toward the RNA pathway, which is believed to be the main cause of toxicity. Fifty-six years later, this is not an established fact, merely a supposition. The third avenue involved the development of oral agents for the convenience of clinicians and patients through the development of the 5-FU prodrugs. Finally, the fourth avenue focused on the metabolism of the agent. An increased understanding of each step of the 5-FU metabolism has allowed the selection of modulators targeting key enzymes involved in 5-FU degradation and activation.

Advances in understanding of the mechanism of action of 5-FU have led to the development of strategies that increase its anticancer activity. 5-FU alone is inactive and needs to be activated to the deoxy-nucleotide level, with the formation of 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), by the action of thymidine phosphorylase (TP). FdUMP blocks thymidylate synthase (TS), the enzyme responsible for the last and crucial step of catalysis in DNA synthesis. 5-FU can also be activated via its conversion to 5-fluorouridine-5′-monophosphate (FUMP), which is incorporated into RNA as a nucleotide analogue that subsequently induces malfunction. Normally, 85 % of 5-FU is catabolised without being activated through its degradation to fluoro-beta-alanine by the enzyme dihydropyrimidine dehydrogenase (DPD). Without this degradation the level of active metabolites will increase substantially, causing intolerable toxicity.

S-1 is the international non-proprietary name (INN) of a fixed combination of three compounds that was selected to enhance 5-FU therapeutic effect while reducing toxicity. The first component is a 5-FU prodrug tegafur that has a sugar moiety attached to the fluorinated base and allows a reproducible high level absorption of 5-FU-based compound in the gastrointestinal (GI) tract. The second component is 5-chloro-2,4-dihydroxypyridine (CDHP or gimeracil), which inhibits dihydropyrimidine dehydrogenase (DPD). The third component is a potassium oxonate (OXO or oteracil) compound that inhibits orotate phosphoribosyl transferase (OPRT) at the start of the pathway of activation to FUMP. S-1 is marketed under different brand names around the world: TS-1® in Japan and Teysuno® in Europe.

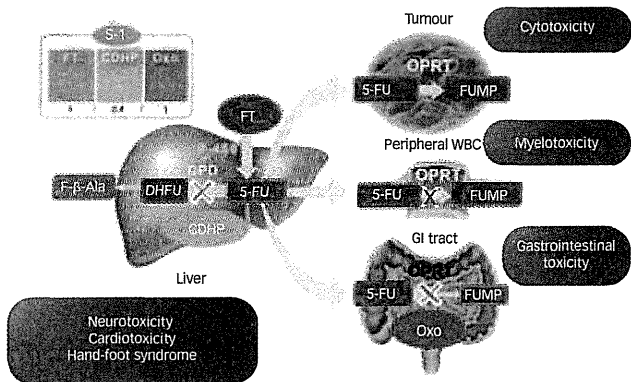
The formulation of S-1 has three possible beneficial consequences. Firstly, in blocking DPD, gimeracil allows a dramatic decrease in the quantity of 5-FU prodrug needed to achieve the active AUC of 5-FU compared with the dose of 1,000 mg/m² bid for capecitabine. In fact, the 25 mg/m² bid dose of tegafur provided by S-1 is 40 fold lower than that of capecitabine, resulting in a comparative dose of metabolised 5-FU of 2.5 %. Additionally, the direct action on DPD decreases the accumulation of fluoro-beta-alanine (Yamada et al., 2003) and the consequent occurrence of hand-foot syndrome (HFS) and other toxicities associated with this 5-FU catabolite. Secondly, oteracil blocks OPRT, leading to a decrease in activation of 5-FU into FUMP, especially in normal tissues, and, therefore, a decrease in gastrointestinal and haematological toxicities. Thirdly, 5-FU can be transformed in the tumour cells in its active form so that it is no longer broken down and is channelled towards FdUMP, triggering a more selective antineoplastic activity. *Figure 1* summarises the mechanism of action of this agent and demonstrates the way in which S-1 interferes and affects the metabolism of 5-FU in a targeted way.

In terms of reducing fluoro-beta-alanine levels by blocking the catabolism of 5-FU, all clinically available fluoropyrimidines can be distinguished into two classes: those that inhibit degradation, known as DPD inhibitory fluoropyrimidines (DIF), including tegafur-uracil, eniluracil and S-1, and those that do not contain a catabolism inhibitor such as IV 5-FU and capecitabine. Essentially, the DIF type of blocking agents substantially reduces fluoro-beta-alanine production and would be expected to reduce toxicity such as the incidence of HFS.

Patients receiving drugs of the DIF category have very low levels of HFS all grades, whereas patients receiving capecitabine show a high incidence of HFS, as do recipients of continuous infusion FU with bolus. When DIF compounds, including S-1, are employed, the incidence of grade 3-4 HFS is reduced to almost zero, as opposed to a 10 % level of severe toxicity with non-DIF compounds. In cancer treatments, theory and preclinical data rarely correspond exactly; but these findings are an example of a correspondence between a postulated mechanism and actual clinical data.

Animal studies involving treatment with a fluoropyrimidine without interference with the RNA-directed pathway, including the activation modulator, resulted in a serious degradation of the intestinal villi and crypts. When oteracil was added however, the anti-RNA effect was blocked and the intestinal villi were protected, as well as the crypts. In a study of 3,800 patients with gastric cancer who were receiving at least fluoropyrimidine plus oteracil treatment and listed in Japanese registry, the incidence of Grade 3–4 diarrhoea was only 2 %.[8] This

Figure 1: The Mechanism of Action of 5-fluorouracil showing the Inhibitory Action of S-1 (Teysuno®)



5-FU = 5-fluorouracil; CDPH = 5-chlorodihydro-pyrimidine; DHFU = 5', 6'- dihydro-5-fluorouracil; DPD = dihydropyrimidine dehydrogenase; FT = 1-(2-tetrahydrofuryl)-5-fluorouracil; FUMP = 5-fluorouridine-5'-monophosphate; OPRT = orotate phosphoribosyl transferase; Oxo = potassium oxonate; WBC = white blood cells. Source: data presented by Y Yamada.

value is consistent with clinical experience but has not been observed in randomised trials. More accurate reporting of the occurrence of diarrhoea in clinical trials conducted in Caucasians is needed to demonstrate the shorter duration and better response to treatment of these symptoms with S-1 than with other 5-FU preparations.

Pharmacokinetic analysis of 5-FU showed that to achieve similar drug exposure, expressed as area under the curve (AUC), in non-Asian patients as in Asian patients, the dose should be reduced. This finding indicates that the metabolism of S-1 is substantially different between Western and Asian populations. As a result of this observation, investigators repeated the entire series of clinical trials that had been carried out in Asian patient populations to adapt the drug to Western populations. Pharmacokinetic studies showed that compared with continuous infusion, the levels of fluoro-beta-alanine obtained with S-1 were approximately five times lower than those obtained with 5-FU. The levels for capecitabine, however, were approximately ten-fold higher.

Several studies have compared S-1 with capecitabine both indirectly and directly. In terms of efficacy, the indirect comparisons in randomised Phase III trials have provided strong evidence to support the use of S-1. The ML17032 study evaluating capecitabine plus cisplatin versus 5-FU plus cisplatin found non-inferiority of the capecitabine + cisplatin regimen.[5] The large (n=1,053) First-line advanced gastric cancer study (FLAGS) evaluating S-1 plus cisplatin versus 5-FU plus cisplatin showed non-inferiority of the S-1 plus cisplatin regimen.[3] From these results it may be concluded that in efficacy terms, capecitabine is roughly equal to IV 5-FU and that S-1 is equal to IV 5-FU. However, it cannot be concluded that S-1 is completely equal to capecitabine. In terms of safety, the FLAGS study found that S-1 had a significantly improved safety profile compared to IV 5-FU and cisplatin.[3]

To obtain meaningful safety comparisons between different cancer treatments, a comparable criterion must be defined. HFS is one of the most frequent adverse events necessitating a dose reduction or treatment interruption for capecitabine. The frequency of this parameter and the ability to monitor it in patients has made it a criterion of choice. Moreover, capecitabine-based regimens have shown an overall incidence of HFS of approximately 50 % and a severe incidence of approximately 10 %, whereas, in the FLAGS study, S-1 showed an incidence of less than 6 % with severe grade incidence of less than 1 % (see *Table 1*).

In addition to the randomised Phase III studies, a Phase II study conducted in South Korea (n=129) compared S-1 + oxaliplatin (SOX) with capecitabine + oxaliplatin (CAPOX) in advanced gastric cancer patients.[6] Both the SOX and CAPOX regimens were equally active and well tolerated. A 25 % incidence of all grades of HFS was observed for CAPOX versus only 3.1 % for SOX. In addition, a 1.6 % incidence of grade 3/4 HFS was seen in the CAPOX group versus 0 % in the SOX group. Grade 3/4 neuropathy, nausea, vomiting and asthenia were also less frequent with SOX.

The occurrence of a relevant adverse event, HFS, in indirect Phase III and direct Phase II comparisons of the different oral 5-FUs raises the following question for oncologists: "What will be your choice in daily practice for the treatment of your patients with advanced gastric cancer?"

S-1 (Teysuno®) – 10 Years of Savoir-faire in Asia

The mortality rate due to gastric cancer in Japan has decreased continuously since the 1960s. Gastric cancer, however, remains the second highest cause of cancer-related death, ranking second for males and third for females in Japan.[9] The five-year survival rates for gastric cancer between 1990 and 1994 at the National Cancer Center Hospital in Japan were not satisfactory at any disease stage, particularly for stage IV for which the rate is less than 10 %.[10]

In the 1960s, the fully active agent 5-FU became available. At the time, drugs were approved by the Japanese Pharmaceutical and Medical Devices Agency (PMDA) according to their response rates, and there was no requirement for Phase III survival benefits data. In the 1990s, irinotecan and taxanes were marketed, as well as S-1, and the survival time was prolonged from 7–8 months to one year. Subsequently, in the 21st century, a more specifically targeted drug, trastuzumab, has been developed for human epidermal growth factor receptor 2 (HER-2)-positive subgroup, constituting the only recent progress in biological therapies in the gastric cancer field.

Data of two late Phase II studies conducted in patients with advanced gastric cancer reported response rate for S-1 monotherapy of 49.0 and 44.2 %, respectively.[11,12] S-1 was very effective at the primary disease site for 39 and 28.9 %, respectively. The frequency of side effects was generally very low. The most commonly observed grade 3/4 toxicities were neutropenia and diarrhoea, with only a 2 % incidence rate. Moreover, the median OS were 250 and 207 days, whereas the one year OS rates were 37 and 36 %, respectively.

In the Japanese Nationwide Post-Marketing Survey of S-1, involving 3,808 Japanese patients, the toxicity profile of S-1 was shown to be similar to that reported in the late Phase II studies. Diarrhoea of grade ≥3 was only 2 %, but the incidence of neutropenia was 6 %. The toxicity of S-1, especially haematological toxicity, was related to creatinine clearance. This was not surprising given that CDHP, which is a DPD inhibitor, is excreted from the kidney. In patients with renal failure, the incidence of haematological toxicities was higher: 40 % (8/20) for a standard initial dose versus 23.5 % (4/17) for a reduced initial dose (for patients with creatinine clearance values <30 mL/min).[8] Therefore, when administering S-1, renal function must be checked and dose adjustments must be made according to age and gender.

Compared with other Japanese Phase II clinical trials of other fluoropyrimidines or cytotoxic agents, S-1 monotherapy was extremely high (see *Table 2*). In the first Japanese Phase III clinical trial conducted by the Japan Clinical Oncologist Group (JCOG) in patients with unresectable advanced/recurrent gastric cancer (JCOG 9205), 5-FU continuous infusion (800 mg/m^2 24 h continuous infusion (CI), D1–5, q4w) was the standard treatment. The test arms were tegafur/uracil (375 mg/m^2 daily) plus mitomycin C (5 mg/m^2 weekly) and 5-FU (800 mg/m^2, 24 h CI, D1-5) plus cisplatin (20 mg/m^2, D1-5, q4w). 5-FU plus cisplatin did not show superiority to 5-FU continuous infusion.[13] Therefore, the control arm with 5-FU continuous infusion (800 mg/m^2 24 h CI, D1–5, q4w) was selected in the next Phase III clinical trial, JCOG 9912. In this study, the test arms were S-1 monotherapy (80, 100 and 120 mg/body/day for body surface area <1.25 m^2, 1.25 - < 1.5 m^2 and ≥ 1.5, respectively, D1-28, q6w) and irinotecan (70 mg/m^2, D1, 15) plus cisplatin (80 mg/m^2, D1, q4w) with a primary endpoint of overall survival (OS). S-1 monotherapy was shown to be non-inferior to 5-FU continuous infusion; the median survival time for S-1 was 11.4 months and the hazard ratio (HR) relative to 5-FU continuous infusion was 0.832 (95 % CI: 0.68–1.01, p=0.0005).[14]

Interestingly, in JCOG 9912 study, the expression of DPD in patients with diffuse type gastric cancer receiving S-1 (poorly differentiated carcinoma, signet-ring cell carcinoma, mucinous adenocarcinoma, n=82) was significantly higher than in patients with intestinal type (papillary or tubular adenocarcinoma, n=86) following the Lauren classification (p<0.001). Hence, 5-FU may not be sufficient for this group of patients. The median progression-free survival (PFS) among 5-FU- or S-1-treated patients with tumours expressing higher levels of DPD was 2.1 and 4.2 months, respectively (HR=2.05; 95 % CI: 1.13–3.71; p=0.016) and S-1 maintained its efficacy in patients with both high and low DPD expression.[15]

The S-1 Plus cisplatin versus S-1 In RCT In the Treatment for Stomach cancer (SPIRITS) study investigated the efficacy of S-1 (80–120 mg/day, D1–21) plus cisplatin (60 mg/m^2, D8, q5w) compared with S-1 monotherapy (80–120 mg/day, D1–28, q6w).[16] This was the first trial to demonstrate the direct survival benefit of S-1 plus cisplatin compared with S-1 monotherapy. S-1 plus cisplatin showed statistically significant survival benefits. The survival rates were 54 % for S-1 in combination with cisplatin versus 47 % for S-1 monotherapy at one year and 24 % for the combination regimen versus 15 % for monotherapy at two years (p=0.04, HR=0.77, 95 % CI; 0.61–0.98).

On the basis of results from these two pivotal trials, S-1 monotherapy appears to be non-inferior to 5-FU. The S-1 plus cisplatin combination however, is superior to S-1 monotherapy. The S-1 plus cisplatin combination regimen has therefore been considered as a standard chemotherapy for unresectable (advanced or metastatic) gastric cancer patients. In the Japanese gastric cancer treatment guidelines 2010,[17] S-1 plus cisplatin is recommended for gastric cancer as a first-line treatment. When S-1 plus cisplatin is considered inappropriate, either S-1 or 5-FU should be delivered as a single agent, depending on the condition of the patients.

In a Phase III trial of gastric cancer, S-1 was compared with S-1 plus irinotecan. Irinotecan was administered at a dose of 80 mg/m^2 on day 1 and day 15, every five weeks.[18] The primary endpoint was also OS. The HR was 0.856 (p=0.233), the median OS were 10.5 months for S-1 alone and 12.8 months for S-1 with irinotecan and the

Table 2: The Response Rate of Japanese Phase II Studies in Advanced Gastric Cancer

Agent	Number of Patients	Response Rate (%)
Doxifluridine (Niitani et al., 1985)[19]	140	14.3
Tegafur/uracil (Ota et al., 1988)[20]	188	27.7
S-1 (Sakata et al., 1998)[11]	51	49.0
S-1 (Koizumi et al., 2000)[12]	43	44.2
Epirubicin (Sakata and Yoshida, 1986)[21]	31	16.1
Cisplatin (Ishibiki et al., 1989)[22]	68	19.1
Irinotecan (Futatsuki et al., 1994)[23]	60	23.3
Docetaxel (Taguchi et al., 1998[24]	66	23.7
and Mai et al., 1999[25])	63	23.7
Paclitaxel (Yamada et al., 2001)[26]	60	23.3

Source: Data presented by Y Yamada.

one-year survival rates were 44.9 and 52.0 %, respectively; however, these were not significantly different. In a further trial, S-1 alone was compared with S-1 plus docetaxel in which the primary endpoint was OS. Docetaxel (40 mg/m^2, D1) plus S-1 (80–120 mg/day, D1–14, q4w) were administrated in the patients. The HR was 0.88. As presented during the last ESMO meeting, the follow up of this trial showed a statistically significant improvement of the overall survival in favour of the S-1 + docetaxel regimen (12.5 versus 10.8 months, p=0.0319).[27b] In recent Phase III trials, S-1 monotherapy showed very similar outcomes with median PFS of four months and median OS of 11 months.[16,18,27]

In Japan, S-1 plus cisplatin is currently the standard first-line treatment and 70 to 80 % of patients will eventually receive second-line treatment with irinotecan, docetaxel or paclitaxel monotherapy. More than 50 % of these patients will receive chemotherapy that includes paclitaxel.

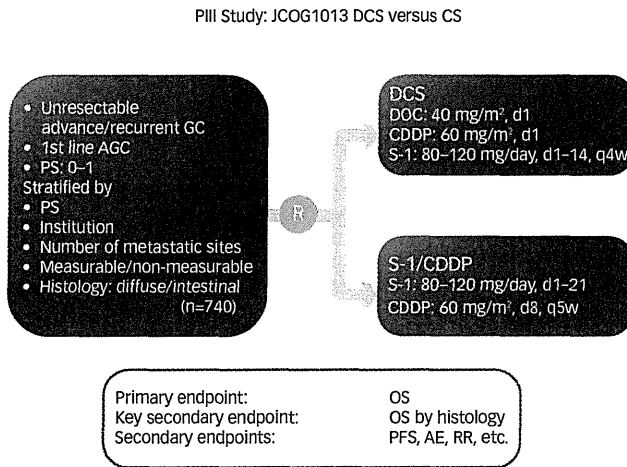
Another promising gastric cancer treatment regimen is S-1 plus oxaliplatin (SOX). In a Phase II study investigating this combination, Oxaliplatin (100 mg/m^2, D1) plus S-1 (80–120 mg/m^2, D1–14, q3w) were administrated in the patients.[28] The response rate was 59 %. The most commonly observed grade 3 or 4 toxicities were neutropenia in 22 % and thrombocytopenia in 13 % of patients. The median OS was 16.5 months. This trial led to an ongoing Phase III trial (n=680) in Japan that is to evaluate non-inferiority of PFS and OS comparing SOX versus S-1 plus cisplatin.

An alternative approach to gastric cancer treatment was evaluated in a Phase I/II trial in which patients received a triplet of docetaxel, cisplatin and S-1 (DCS). Docetaxel (40 mg/m^2) and cisplatin (60 mg/m^2) were given on day 1 of 28-day cycle; S-1 (40 mg/m^2) was given twice daily on days 1–14.[29] The most commonly observed grade 3/4 toxicity was neutropenia in 70 % of patients. The median PFS was 8.7 months and the median OS was 18.5 months. This DCS regimen showed marked efficacy against intestinal and diffuse types of gastric cancer. In this study, down-staging of gastric cancer was achieved in nine (19 %) of 48 patients who responded to DCS.

The DCS is also being compared with a S-1 and cisplatin and combination in another ongoing Phase III trial in patients with unresectable, recurrent or advanced gastric cancer in Japan (JCOG 1013) (see *Figure 2*). The aim of this study is to evaluate superiority of OS (n=740).

For HER2-positive gastric cancer patients, there are currently three Phase II clinical trials with S-1 in progress in Japan and Southeast Asia

Figure 2: Schema of Phase III Trial Comparing the DCS with Cisplatin and S-1 Combination in Patients with Unresectable, Recurrent or Advanced Gastric Cancer in Japan



AE = adverse event; AGC = advanced gastric cancer; CDDP = cisplatin; CS = cisplatin and S-1; DCS = docetaxel, cisplatin and S-1; DOC = docetaxel; GC = gastric cancer; JCOG = Japan Clinical Oncology Group; OS = overall survival; PFS = progression-free survival; PS = performance status; RR = response rate.

(n=25–60). In two of them, a combination of S-1 plus cisplatin plus trastuzumab is being evaluated and in the other S-1 plus trastuzumab is being investigated in elderly patients.

While S-1 plus cisplatin is the current standard regimen for gastric cancer in Japan, SOX and DCS regimens are likely to become more widely used in gastric cancer once Phase III clinical trial results become available in the near future. Phase III data supporting the use of these promising combinations in gastric cancer are now awaited with interest. In clinical use, combination regimens that include S-1 are likely to extend the lives of many patients with advanced gastric cancer.

Established Benefits of S-1 Confirmed in Western Populations

Asian patients, particularly the Japanese, have a markedly different metabolism of anti-cancer drugs compared with Western populations. This phenomenon has been observed with drugs such as 5-FU for which the doses used in Asian patients are much higher than are normally used in Caucasians.

The combination of S-1 and cisplatin is highly active in Japanese patients with advanced gastric cancer. Following the discovery of regional dose variations of one of the S-1 components, it was necessary early in drug development to define the optimal dosing of both S-1 and cisplatin in combination for use in a Caucasian patient population. This necessitated a Phase I pharmacokinetic study in which a combination of S-1 and cisplatin were used to treat advanced gastric carcinoma.[30] In the Japanese population, given an S-1 dose of 32 to 40 mg/m², the exposure, as expressed as the AUC, is around 700 ng x h/mL.

In the Caucasian population, to reach a similar AUC with an acceptable tolerability, it is necessary to decrease to a dose of 25 mg/m² in combination with cisplatin and it is necessary to decrease to 30 mg/m² in monotherapy.[30b]

The design of this Phase I trial allowed various levels of dose escalation, and concluded that the recommended doses were 25 mg/m² for S-1 and 75 mg/m² for cisplatin. At these doses, the incidence of grade 3/4 toxicities was very low although this was a small Phase I study; 6 patients receiving 25/75 mg/m²/dose S-1/cisplatin, six receiving 30/60 mg/m²/dose and three receiving 30/75 mg/m². At the highest doses the incidence of grade 3/4 toxicities was greater. These findings led to the initiation of a single-arm Phase II study that investigated the efficacy of the recommended dose based on the Phase I trial: 25 mg/m² of S-1 twice daily (BID) for three weeks and cisplatin on day 1 at 75 mg/m².[31,32] This treatment improved the time to disease progression to one year; one-year survival rate was 42 % and the two-year survival rate was 21 %. The efficacy data, as reviewed by an independent committee, showed that there was a response rate of 55 % and this was confirmed by time to progression and duration of response. The results of 25 mg/m² of S-1 BID plus cisplatin 75 mg/m² combination confirmed the efficacy of this treatment.

The S-1/cisplatin combination showed an advantageous tolerability profile in which grade 3/4 neutropenia in 19 % of the patients was the main finding. The remaining toxicities were non-haematologic types. These consisted of fatigue/asthenia (grade 3/4) in 24 %, diarrhoea (grade 3 /4) in 13 %, a low incidence of stomatitis, and a low incidence of febrile neutropenia. This is considered to be an acceptable toxicity profile.

The findings of this study led to the FLAGS study, a multi-centre, international Phase III trial focussing on a Caucasian population with advanced metastatic gastric cancer.[3] This study used the recommended doses that were identified in the Phase I study and confirmed in the Phase II: S-1 at 25 mg/m² orally BID for three weeks, with one week of rest plus cisplatin at 75 mg/m². This was compared with the conventional 5-FU/cisplatin regimen in which 5-FU was given at 1,000 mg/m²/day over five days and cisplatin was given at a dose of 100 mg/m² on day 1 every four weeks. A total of 1,053 patients were randomised and the patient population was stratified according to the extent of disease, prior adjuvant chemotherapy and measurable versus non-measurable disease; the primary endpoint was overall survival. The statistical analysis plan aimed to demonstrate improvement in median survival from 8.5 months in the cisplatin/5-FU arm to 10.5 months in the experimental arm of cisplatin S-1, corresponding to a HR of 0.81. There was also a delayed endpoint of non-inferiority on OS for which the upper limit was 1.10 to reach statistical significance and to retain potentially 74 % of the effect of cisplatin/5-FU. In parallel, the secondary endpoints of the FLAGS study were PFS, safety, time to treatment failure (TTF), response rate, duration of response, time to tumour response, time to tumour progression, clinical benefit and the quality of life using the FACT gastric scale. The patient population was well balanced in both arms. The majority of patients were male and importantly, 86 % were Caucasian.

The cancers were primarily of the stomach, a few subjects had gastro-oesophageal junction disease, and the diffuse histological type was slightly more frequently than the non-diffuse type. Metastatic disease was present in almost all the patients with two-thirds of the patients having more than two metastatic sites and most were measurable. Very few patients had received prior adjuvant chemotherapy and approximately one-third had prior stomach resection.

There was no major difference in treatment compliance between the two arms and the median number of cycles per patient was four, ranging from one to 28 or one to 24. The dose intensities of S-1 and

5-FU were similar (92 and 95 %, respectively). The duration of treatment was also similar to the planned regimen in both arms.

Toxic death under treatment was three times higher in the cisplatin/5-FU arm as compared with S-1/cisplatin. For OS, the first primary objective of superiority was not met (HR= 0.92, *Figure 3*). However, the calculated level of OS non-inferiority was met and showed that S-1/cisplatin is non-inferior to 5-FU/cisplatin (p=0.0068). For the secondary endpoints, PFS was not superior (HR=0.99) but TTF was (HR=0.87, p=0.032) (see *Figure 3*). The Forest plot in Figure 4 shows that no patient subgroups clearly benefited from either treatment regimen. There are slight advantages for both S-1 and 5-FU but no overall trend towards either treatment for any subgroup. There were similar overall response rates for S-1/cisplatin and 5-FU/cisplatin of 29.1 and 31.9 %, respectively. The duration of response was slightly longer for S-1/cisplatin compared with 5-FU/cisplatin.

The incidence of severe neutropenia was significantly different between the two treatment groups: approximately 35 % for S-1/cisplatin compared with almost 70 % for 5-FU/cisplatin. There was also a clear advantage in terms of myelosuppression, reflected in a significant reduction of thrombocytopenia and a lower incidence of febrile neutropenia in patients receiving S-1/cisplatin. These results therefore clearly confirm the low myelotoxicity profile of S-1 that was observed in the initial Phase I trial.

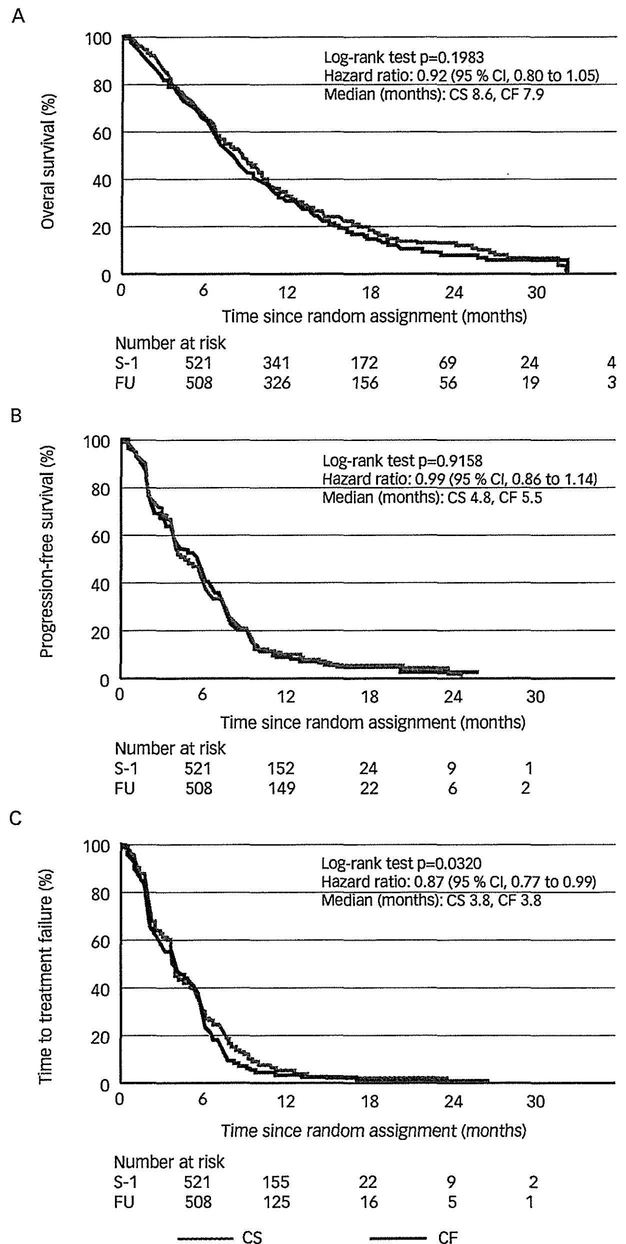
In terms of non-haematological toxicity, there was a significant difference in the incidences of diarrhoea (all grades) and the use of anti-diarrhoeal medication was significantly reduced in the cisplatin/S-1 arm compared with the 5-FU/cisplatin arm. There was also a reduction in the incidence of dehydration which is quite often associated with diarrhoea and interestingly, a very low incidence of stomatitis, mucosal inflammation (mostly conjunctivitis), hypophosphatemia and hypomagnesemia with S-1/cisplatin compared with the 5-FU/cisplatin. Therefore the tolerance profile of cisplatin/S-1 appears to be significantly better than that of 5-FU/cisplatin.

In the FLAGS study, renal toxicity appeared to be reduced in the S-1/cisplatin arm compared with 5-FU/cisplatin. The lower dose of cisplatin used in the S-1 arm (75 mg/m^2) versus the IV 5-FU arm (100 mg/m^2) seems to be the main driver of this advantage. Liver function tests showed slight increases in bilirubin as well as all grades of liver-related adverse events for S-1/cisplatin compared with cisplatin/5-FU. Liver impairment showed no statistical difference between treatments and most of this was not associated with symptoms. Thus there was a significantly reduced incidence of serious adverse events with S-1/cisplatin compared with 5-FU/cisplatin. Death related to treatment was significantly reduced by half with S-1/cisplatin. S-1/cisplatin therefore clearly has a superior tolerability profile than 5-FU/cisplatin but with similar efficacy.

It has been observed in previous studies and in general clinical experience that patients prefer oral fluoropyrimidine to the IV form, mostly as a result of improved quality of life. In the FLAGS study, three quality of life parameters were significantly improved in the S-1/cisplatin group.[3] These included the time to more than 5 % weight loss and the physical well being subscale within the FACT Gastric Scale. In addition, the use of anti-diarrhoeal medication was significantly reduced by 40 % with S-1/cisplatin and the use of colony stimulating factors which are associated with neutropenia was reduced by 49 %. S-1/cisplatin therefore clearly provides advantages in terms of quality of life, the need for supportive care, anti-diarrhoeal drugs and colony stimulating factor.[33,33b]

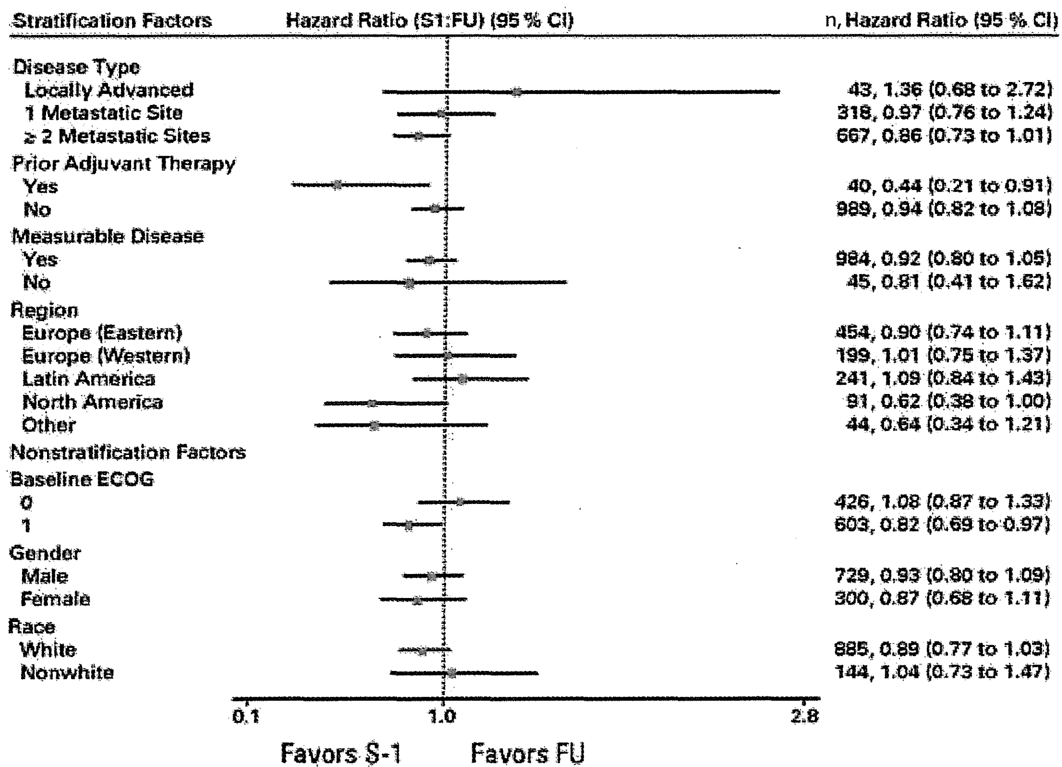
The number of hospitalisations also differed between the treatment groups in the FLAGS study. Patients receiving oral drug treatments spent fewer days in hospital and needed to attend only to receive cisplatin. This is reflected in the number of patients that needed to be hospitalised to receive the S-1/cisplatin combination (67.4 %) compared with those receiving 5-FU/cisplatin (80.7 %). Overall, the median number of days patients were hospitalised was twice as high in the 5-FU/cisplatin group (24 days) compared with S-1/cisplatin, (11 days).

Figure 3: Kaplan-Meier Plot of Overall Survival by the Two Treatment Arms in the FLAGS Study



A: Overall survival; B: progression-free survival; C: time without treatment failure. CF = cisplatin/5-FU; CS =cisplatin/S-1; FU = 5-fluorouracil; S-1 = tegafur/gimeracil/oteracil combination (Teysuno®). Source: Ajani et al., 2010.[3]

Figure 4: Forest Plot of Pre-planned Stratification Factors for Overall Survival in the FLAGS Study



ECOG = Eastern Cooperative Oncology Group; FU = 5-fluorouracil; S-1 = tegafur/gimeracil/oteracil combination (Teysuno®). Source: Ajani et al 2010.[3]

In summary, in the FLAGS study, the primary endpoint of superiority in OS was not met, but the comparison of the non-inferiority calculated level of the upper HR limit 1.10 is highly statistically significant when compared with the upper HR limit obtained in FlAGS for OS 1.05 (p=0.0068).[33]

This approach was considered by the Committee for Medicinal Products for Human Use (CHMP) of the European Medical Agency (EMA), who concluded that the benefits of S-1 are greater than its risk and recommended that it be given marketing authorisation in March 2011. Approval was therefore granted for S-1 for the treatment of advanced unresectable metastatic gastric cancer in combination with cisplatin.

Committed to Filling Knowledge Gaps – The S-1 Development Programme

The FLAGS study provided evidence to support the view that S-1 should be incorporated into treatment strategies for gastrointestinal cancers in Europe. Following an EMA Market Authorisation, S-1 is currently registered as Teysuno® and is already available in Northern European countries including, Denmark, Finland, Norway, Sweden and the UK. It became available in Austria and Germany on the July 1 2012 and other European countries will follow soon.

The projected next step in S-1 development in the US will be the Diffuse Gastric and Esophagogastric Junction Cancer S-1 Trial (DIGEST, NCT 01285557), which uses a similar strategy as the FLAGS study but will look into the superiority of OS for S-1/cisplatin in patients with diffuse type histology. Approximately 60 % of patients in the FLAGS study had the diffuse type of cancers and OS in this group was reported to be better than in those with non-diffuse histology, especially in individuals with lower weight loss. The OS in patients with this histology was 9.0 months for S-1/cisplatin compared with 7.1 months for FU/cisplatin.[34] The trial is being conducted in numerous treatment centres across 20 countries worldwide and plans to recruit at total of 500 patients between 2010 and 2013 with a one-year follow-up.

S-1 may also be used as part of a triplet treatment regimen for gastric cancer but at present there are no data available for Caucasian populations. It remains controversial whether a triplet regimen is needed. However, a meta-analysis demonstrated significant benefit from adding an anthracycline to a platinum and fluoropyrimidine doublet, and ECF (epirubicin plus cisplatin plus protracted infusion 5-FU) is among the most active and well-tolerated regimens.

Docetaxel increases the activity of 5-FU/cisplatin, but is also more toxic when used in a three-weekly regimen, with 29 % complicated neutropenia reported.[35] A randomised Phase II study demonstrated maintained activity with reduced toxicity when a weekly docetaxel schedule was employed in combination with cisplatin and infused 5-FU or capecitabine.[36]

The substitution of capecitabine (X) for 5-fluorouracil (F) and oxaliplatin (O) for cisplatin (C), in the ECF regimen was examined in the recent UK National Cancer Research Institute (NCRI) Randomised ECF for Advanced and Locally Advanced Esophagogastric Cancer 2 (REAL-2) trial, which demonstrated non-inferiority between ECF, ECX, EOF and EOX. The EOX regimen was associated with a longer OS (11.2 versus 9.9 months, HR 0.80, 95 % CI 0.66–0.97; p=0.02) than the reference ECF regimen and the rate of thromboembolism was also significantly reduced by the oxaliplatin substitution (7.6 % compared with 15.1 %, p=0.0003).[37]

These data prompted a Phase I study to evaluate an epirubicin/oxaliplatin/S-1 (EOS) combination. To date, this has recruited eight patients and aims to determine the maximum tolerated dose of S-1, either 20 mg/m^2 (dose level 1) or 25 mg/m^2 (dose level 2) combined with epirubicin at 50 mg/m^2 and oxaliplatin at 130 mg/m^2. This trial will allow a recommended dose of S-1 in an EOS regimen to be established and it will be possible therefore to analyse this EOS regimen in a later Phase III trial and compare it directly with EOX.

A recent study combined S-1 with oxaliplatin in a Caucasian population with advanced solid tumours.[38] In this study, patients received one of two treatment schedules. Schedule A consisted of S-1 25 mg/m^2 BID for 14 consecutive days then a seven-day recovery period in a 21-day cycle and bevacizumab 7.5 mg/kg IV on day 1 of each three-week cycle and oxaliplatin 130 mg/m^2 IV on day 1 of each three-week cycle. Schedule B consisted of S-1 35 mg/m^2 BID on day 1 for seven consecutive days then a seven-day recovery period in a 14-day cycle and bevacizumab 5 mg/kg IV on day 1 of each two-week cycle and oxaliplatin 85 mg/m^2 IV on day 1 of each two-week cycle. The toxicity data show that higher dosages of oxaliplatin also increase grade 1 and 2 peripheral sensory neuropathy. From this trial, therefore, it can be concluded that S-1 and oxaliplatin can be administered with the biological bevacizumab and perhaps other biologics, with acceptable safety and tolerability without evidence of pharmacokinetic interactions.

In another randomised trial conducted in Asia, SOX was compared with CAPOX in the treatment of gastric cancer.[6] Both the SOX and CAPOX regimens were equally active and well tolerated in advanced gastric cancer patients. Grades 3/4 neuropathy, nausea, vomiting and asthenia were less frequent with SOX and as anticipated, HFS at any grade was more frequent for CAPOX (SOX = 3 %; CAPOX = 25 %, p=0.001). Therefore two viable treatments may reduce the risk of HFS. One is the cisplatin/S-1 regimen in a four-weekly regimen and the other is the SOX regimen which is the 130 mg/m^2 oxaliplatin with 25 mg/m^2 S-1 BID regimen in a three-weekly cycle.

Finally, S-1 is also being developed for use in colorectal cancer and ongoing studies in Asia are comparing SOX with other combinations such as folinic acid/fluorouracil/oxaliplatin (FOLFOX).

Conclusion

The 13 years of clinical experience of S-1 in advanced gastric cancer in Japan has shown that the use of this new oral formulation of FU in combination therapy consistently improves survival whilst reducing toxicities and improving tolerability. Non-haematological and haematological adverse events such as diarrhoea and neutropenia are substantially reduced with S-1 compared with other treatments. Pharmacokinetic studies in Western populations showed a marked contrast in the metabolism of S-1 between Japanese and Caucasian populations necessitating substantial dose reductions in Europe to achieve the same AUC values as seen in Japan. This emphasises the importance of independent development programmes for chemotherapy in both Asian and Caucasian patients.

The FLAGS study demonstrated non-inferiority of the S-1 combination versus the 5-FU combination for OS. However, the most important finding from this study was the superior safety profile of the S-1 treatment, making S-1 a suitable replacement for 5-FU in gastric cancer treatment. The results of the DIGEST and other studies are awaited with interest and will help define whether S-1 is suitable for the treatment of more diffuse type tumours and whether it is appropriate as part of a triplet regimen. S-1 has also been investigated in the treatment of solid tumours and in combination with oxaliplatin in which it also shows advantageous safety performance compared with capecitabine regimens. It is therefore likely that fluoropyrimidine-containing combinations, and especially S-1-containing treatments, will continue to be used in gastric cancer treatment for the foreseeable future. ■

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MET amplification as a potential therapeutic target in gastric cancer

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ABSTRACT:

Our aim was to investigate both the prevalence of MET amplification in gastric cancer as well as the potential of this genetic alteration to serve as a therapeutic target in gastric cancer. MET amplification was assessed by initial screening with a PCR-based copy number assay followed by confirmatory FISH analysis in formalin-fixed, paraffin-embedded specimens of gastric cancer obtained at surgery. The effects of MET tyrosine kinase inhibitors (MET-TKIs) in gastric cancer cells with or without MET amplification were also examined. The median MET copy number in 266 cases of gastric cancer was 1.7, with a range of 0.41 to 21.3. We performed FISH analysis for the 15 cases with the highest MET copy numbers. MET amplification was confirmed in the four assessable cases with a MET copy number of at least 4, whereas MET amplification was not detected in those with a gene copy number of <4. The prevalence of MET amplification was thus 1.5% (4 out of 266 cases). Inhibition of MET by MET-TKIs resulted in the induction of apoptosis accompanied by attenuation of downstream MET signaling in gastric cancer cell lines with MET amplification but not in those without this genetic change. MET amplification identifies a small but clinically important subgroup of gastric cancer patients who are likely to respond to MET-TKIs. Furthermore, screening with a PCR-based copy number assay is an efficient way to reduce the number of patients requiring confirmation of MET amplification by FISH analysis.

INTRODUCTION

Gastric cancer is the third most common cause of death from malignant disease in men (fifth in women) worldwide [1]. The prognosis for patients with unresectable advanced or recurrent gastric cancer remains poor, with a median survival time of less than 1 year in individuals receiving conventional therapy [2-8]. The combination of trastuzumab, an antibody targeted to HER2, with chemotherapy has yielded a survival benefit for patients with HER2-positive gastric or gastro-esophageal junction cancer [7], with HER2-positive tumors accounting for 7 to 17% of all gastric cancers [9-11]. Further research is thus warranted to identify new therapeutic targets for gastric cancer patients.

The *MET* proto-oncogene encodes the receptor tyrosine kinase c-MET. The binding of its ligand, hepatocyte growth factor, to MET results in tyrosine phosphorylation of the receptor and activation of downstream signaling molecules. Oncogenic activation of *MET* suppresses apoptosis and promotes cell survival, proliferation, migration, and differentiation as well as gene transcription and angiogenesis [12]. In gastric cancer, such activation of *MET* has been attributed to

gene amplification [13-15]. However, the prevalence of *MET* amplification has varied among studies [13-21], possibly as a result of differences in the methods applied. This uncertainty led us to determine the prevalence of *MET* amplification in 266 formalin-fixed, paraffin-embedded (FFPE) specimens of gastric cancer obtained during surgery. To ensure the efficient detection of *MET* amplification, we adopted a sequential approach involving PCR-based determination of gene copy number followed by confirmatory FISH analysis. Moreover, to assess the potential of *MET* amplification as a therapeutic target in gastric cancer, we investigated its impact on cell survival and signal transduction.

RESULTS

MET amplification in gastric cancer cell lines

We first applied FISH (Figure 1A) and a real-time PCR–based method (Figure 1B) to examine *MET* copy number in gastric cancer cell lines whose *MET* amplification status was previously determined [22]. In gastric cancer cell lines negative for *MET* amplification, including KATO III, SNU1, SNU216, MKN1, MKN7, HSC39, MKN28, and NUGC3, the copy number of *MET* as determined by the PCR-based assay ranged between 1.3 and 3.3. In contrast, cell lines positive for *MET* amplification, including Hs746T, MKN45, and SNU5, showed *MET* copy numbers of 21.3, 21.3, and 17.9, respectively. The PCR-based assay thus revealed a high copy number for *MET* only in gastric cancer cell lines previously shown to be positive for *MET* amplification by FISH.

MET amplification in gastric cancer specimens

To determine the prevalence of *MET* amplification in advanced gastric cancer, we examined 266 FFPE specimens of surgically resected primary gastric tumors. Most of the patients were male (68.8%) and had undifferentiated-type gastric cancer (62.8%), including mucinous adenocarcinoma, signet ring cell adenocarcinoma, and poorly differentiated

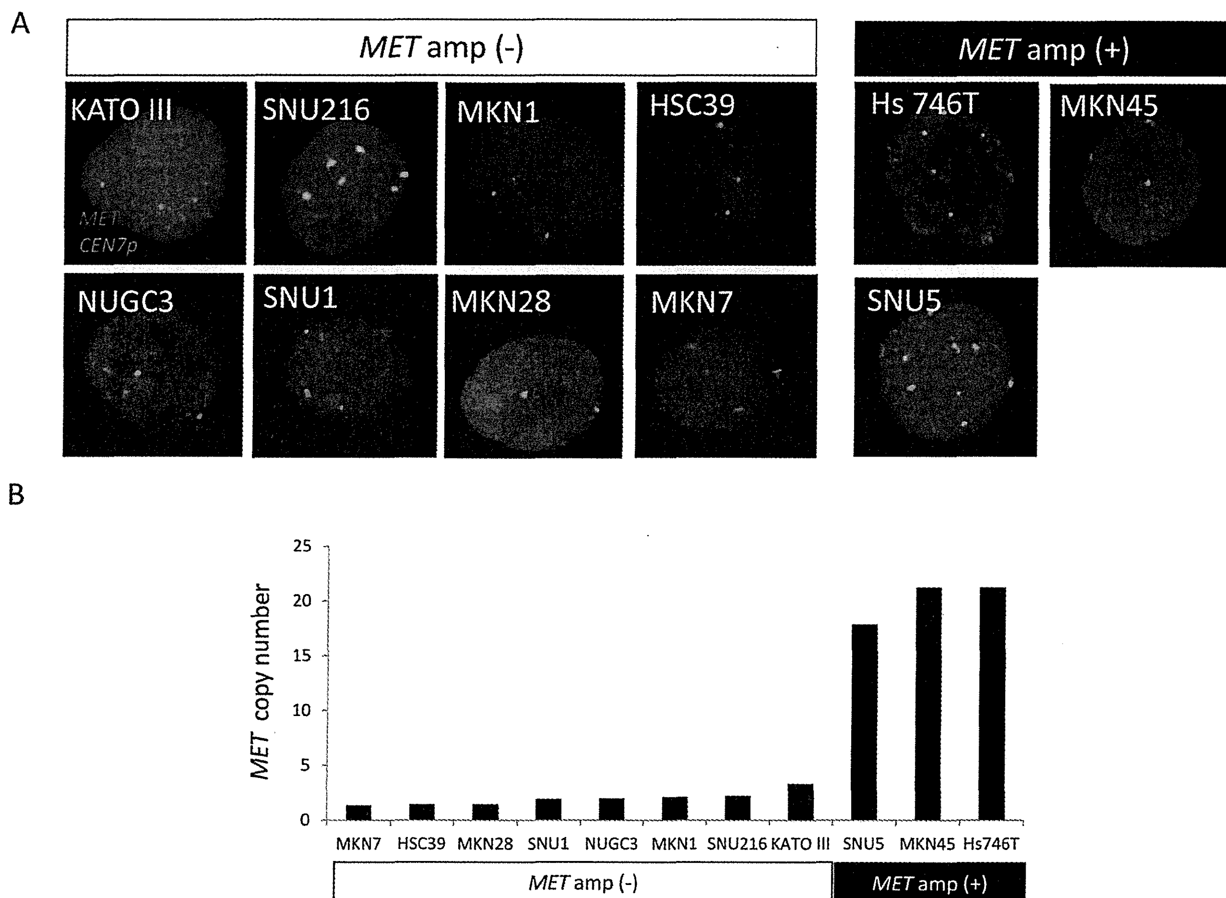

Figure 1: Amplification of *MET* in gastric cancer cell lines. *A*, FISH analysis of cell lines positive or negative for *MET* amplification (amp). Each image shows a single cancer cell, with green and red signals corresponding to CEN7p and the *MET* locus, respectively. *B*, Evaluation of *MET* copy number in gastric cancer cell lines with a PCR-based assay.

Table 1: Characteristics of the 266 study patients

Characteristic	n
Sex	
Male	183 (68.8%)
Female	83 (31.2%)
Pathological stage	
I	25 (9.4%)
II	31 (11.7%)
III	77 (28.9%)
IV	133 (50.0%)
Histology	
Differentiated type	99 (37.2%)
Undifferentiated type	167 (62.8%)

The patients had a median age of 63 years (range, 31 to 91 years).

adenocarcinoma (Table 1). The median age was 63 years, with a range of 31 to 91 years.

The PCR-based assay revealed that the median *MET* copy number for the 266 cases was 1.7, with a range of 0.41 to 21.3 copies (Figure 2A). Given that gastric cancer cell lines with *MET* amplification have been found to have a high copy number for *MET* [23], we arranged all cases in the order of *MET* copy number and performed FISH analysis for the 15 cases with the highest copy numbers (Table 2). *MET* amplification was detected by FISH in four of these cases (G72, G289, G322, and G181), which had a *MET* copy number of at least 4, whereas six cases

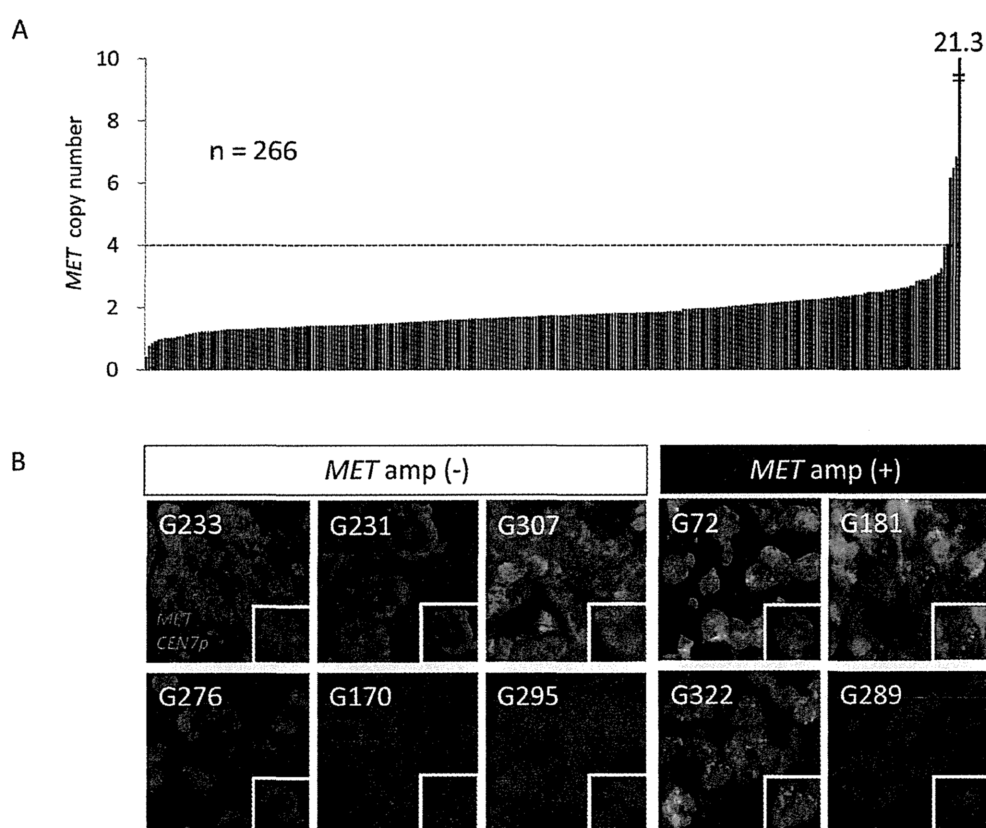

Figure 2: Amplification of *MET* in surgical specimens of gastric cancer. *A*, *MET* copy number determined with a PCR-based assay for 266 FFPE surgical specimens of gastric cancer. A *MET* copy number of >4 was observed in five cases. *B*, FISH analysis of gastric cancer specimens among the 15 samples with the highest *MET* copy numbers as determined with the PCR-based assay. Green and red signals correspond to CEN7p and the *MET* locus, respectively. Higher magnification images of individual cancer cells are shown in the insets. The specimens are grouped into those determined to be positive of negative for *MET* amplification by FISH.

(G276, G233, G295, G170, G307, and G231) with a copy number of less than 4 did not exhibit *MET* amplification (Figure 2B, Table 2). The remaining five cases (G331, G223, G217, G118, and G42) were not assessable by FISH analysis because of a lack of hybridization signals.

We thus identified four out of 266 gastric cancer patients (1.5%) as having *MET* amplification. The clinical features of patients with or without *MET* amplification are shown in Tables 2 and 3. All four patients with *MET* amplification had undifferentiated-type gastric cancer. We further examined the prognostic impact of *MET* amplification for all 266 patients but found that OS after surgery did not differ significantly between those with or without *MET* amplification (log-rank test, $P = 0.3$).

MET amplification is associated with increased sensitivity to MET-TKIs in gastric cancer cell lines

To investigate the biological impact of *MET* amplification in gastric cancer, we first examined the effects of two highly selective MET receptor tyrosine kinase inhibitors (MET-TKIs), JNJ38877605 and SGX523, on the growth of gastric cancer cell lines positive or negative for *MET* amplification. The IC_{50} values of JNJ3887605 and SGX523 for inhibition of cell growth were 0.02 to 0.05 μM and 0.06 to 0.07 μM, respectively, for cells positive for *MET* amplification, whereas they were >10 μM for *MET* amplification–negative cells (Figure 3A). An annexin V binding assay revealed that both MET-TKIs induced a substantial level of apoptosis in *MET* amplification–positive cells but were largely without

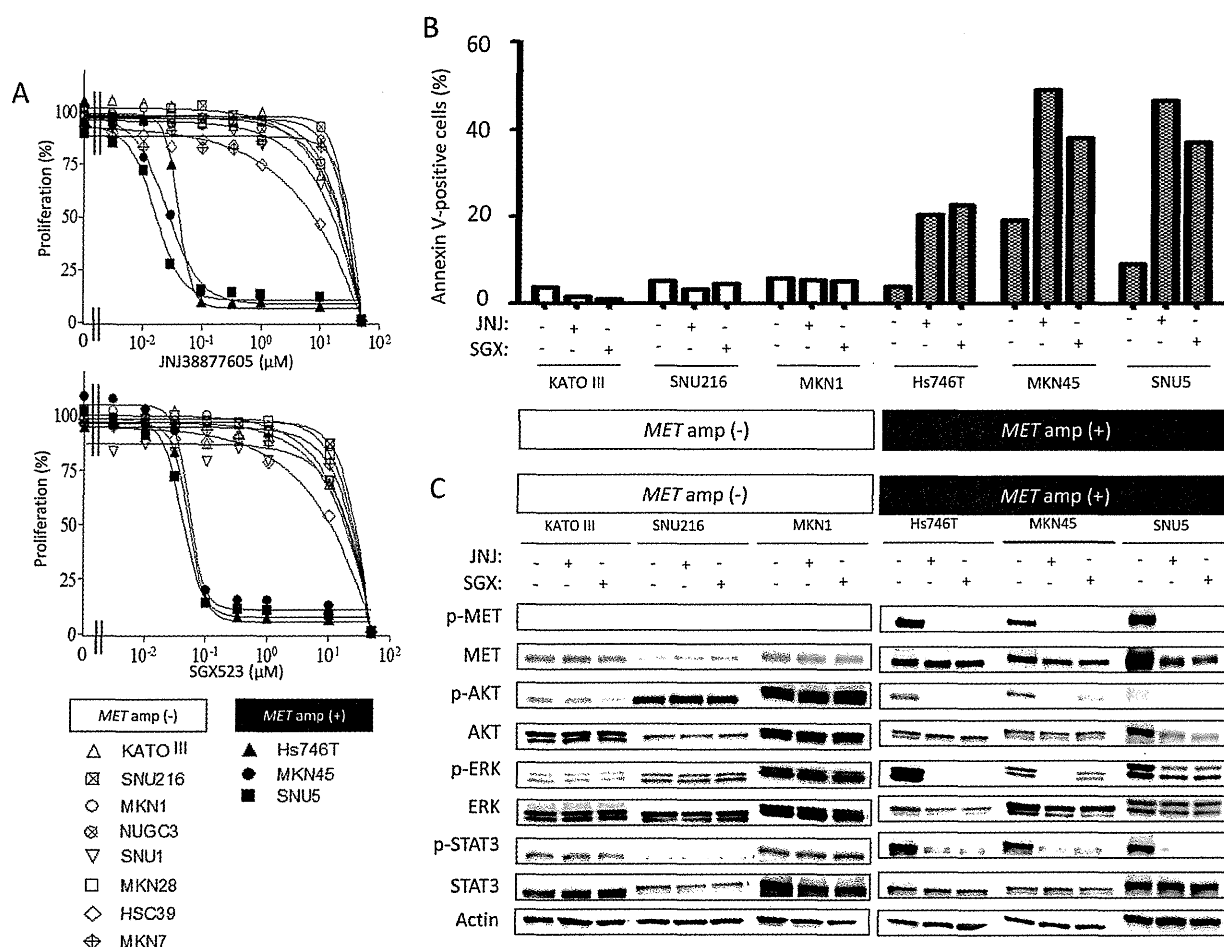

Figure 3: Effects of MET-TKIs in human gastric cancer cell lines classified according to *MET* amplification status. *A*, Effects of JNJ38877605 and SGX523 on cell growth as determined with the MTT assay. Data are means of triplicates from representative experiments. *B*, Cells were incubated in the absence or presence of 0.10 μM JNJ38877605 or 0.10 μM SGX523 for 48 h, after which the number of apoptotic cells was determined by staining with annexin V followed by flow cytometry. *C*, Cells were incubated in the absence or presence of 0.10 μM JNJ38877605 or 0.10 μM SGX523 for 48 h, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to phosphorylated (p) or total forms of MET, AKT, ERK, or STAT3 or with those to β-actin (loading control).

Table 2: Characteristics of the 15 patients with the highest MET copy numbers

Case no.	MET copy number	MET/ CEN7p	Sex	Age (years)	Histology	Stage	OS (days)
G72	21.3	5.9 (+)	M	66	U	IV	157
G289	6.84	5.2 (+)	F	48	U	IV	483
G322	6.45	7.2 (+)	F	70	U	IV	84
G331	6.14	ND	M	76	U	II	>2764
G181	4.02	6.6 (+)	F	52	U	IIIb	>1977
G223	3.92	ND	M	62	U	IIIa	>3340
G276	3.23	1.2 (–)	M	70	U	Ib	1089
G233	3.09	1.2 (–)	F	63	U	IIIb	>4732
G217	3.02	ND	F	69	U	IIIa	>3827
G118	2.97	ND	M	66	U	IIIa	2650
G295	2.89	2.0 (–)	M	74	U	IV	539
G170	2.88	1.7 (–)	M	53	D	Ia	>2088
G42	2.87	ND	M	64	D	II	1907
G307	2.85	1.3 (–)	M	60	U	IV	824
G231	2.83	1.9 (–)	M	67	D	IIIa	>2921

Abbreviations: ND, signals not detected; U, undifferentiated type; D, differentiated type. (+) or (–) denote positive or negative for MET amplification on the basis of the MET/CEN7p ratio; > for OS indicates the patient was still alive.

effect in cell lines without *MET* amplification (Figure 3B). Immunoblot analysis showed that the MET-TKIs inhibited the phosphorylation of MET, AKT, ERK, and STAT3 in gastric cancer cells with *MET* amplification, whereas they had no effect on signaling events in those negative for *MET* amplification (Figure 3C). These findings thus indicated that gastric cancer cells with *MET* amplification are predominantly dependent on MET signaling for their growth and survival and are therefore rendered hypersensitive to MET-TKIs.

DISCUSSION

Activation of MET signaling promotes tumor cell growth, survival, migration, and invasion as well as tumor angiogenesis [24]. In gastric cancer, gain-of-function mutations of *MET* are exceedingly rare [25-27], with MET activation having been attributed mostly to gene amplification [13-15]. Previous studies based on FISH analysis have detected *MET* amplification in up to 4% of patients with gastric cancer [14, 16, 20]. On the other hand, an increase in *MET* copy number was found in 10 to 20% of gastric cancer patients by Southern blot analysis [17-19] or with a PCR-based assay [28, 29]. An increase in gene copy number in malignant tumors can result from at least two genetic mechanisms, gene amplification and polysomy. Gene amplification refers to a copy number gain for a specific gene (or group of genes) on a given chromosome arm without a change in copy number for genes located in other regions of the chromosome [30], whereas polysomy gives rise to a copy number gain for a given gene as a result of the presence of extra copies of the entire chromosome. Southern blot analysis and PCR-based copy number assays recognize a gain in gene copy number regardless of the underlying cause and are thus unable to distinguish gene amplification from polysomy, a limitation that is sometimes overlooked, with consequences for determination of the true prevalence of *MET* amplification in gastric cancer.

FISH analysis is a semiquantitative method that can be performed with two probes for determination of the number of signals for a target gene and for the centromeric

Table 3: Clinical and pathological characteristics of gastric cancer patients classified according to MET amplification status

Characteristic	MET amplification(+) (n = 4)	MET amplification(–) (n = 262)	P
Median age (range), years	59 (48–70)	63 (31–91)	0.976
Sex, n			
Male	1 (25.0%)	182 (69. 5%)	0.091
Female	3 (75.0%)	80 (30. 5%)	
Pathological stage, n			
I	0	25 (9.5%)	0.582[a]
II	0	31 (11.8%)	
III	1 (25.0%)	76 (29.0%)	
IV	3 (75.0%)	130 (49.6%)	
Histology, n			
Differentiated type	0	99 (37.8%)	0.300[b]
Undifferentiated type	4 (100%)	163 (62.2%)	

[a]Comparison between stages I + II and III + IV. [b]Comparison between intestinal-type and diffuse-type gastric cancer. P values were calculated with Student's two-tailed t test for age and the chi-square test for the other variables.

portion of the corresponding chromosome. Given that the number of centromeric signals directly reflects the copy number of the chromosome, FISH analysis yields the copy number gain for the target gene from the ratio of the copy number of the gene to that of the chromosome. FISH is thus the gold standard for detection of gene amplification. However, the identification and counting of FISH signals are hampered by many factors including cutting artifacts, nuclear overlap, and heterogeneity of tumor specimens. Moreover, FISH is expensive and time-consuming, and it requires technical expertise [31]. The efficient determination of gene amplification in a large number of tumor specimens would thus benefit from the availability of a high-throughput screening assay. In this regard, PCR-based assays for determination of gene copy number are simple to perform and rapidly yield quantitative and reproducible results. Given that, among tumors showing a gain in gene copy number, those confirmed to be positive for gene amplification show the greatest increases in gene copy number [21, 23, 32, 33], we investigated the potential of a PCR-based assay for screening in order to select cases of gastric cancer for confirmation of *MET* amplification by FISH. We thus performed PCR-based screening for *MET* copy number in 266 surgically resected specimens of gastric cancer and then applied FISH analysis to the 15 cases showing the highest gene copy numbers. *MET* amplification was confirmed by FISH in four cases among the five with a *MET* copy number of at least 4; the remaining case (G331) was not assessable by FISH because of a lack of hybridization signals. *MET* amplification was not detected in the cases with a gene copy number of <4. We therefore identified *MET* amplification at a frequency of 1.5% (4 out of 266 cases), consistent with values determined by FISH analysis in recent studies of gastric cancer [16, 20]. Our results thus suggest that screening for *MET* amplification with a PCR-based assay is an efficient means with which to reduce the number of specimens requiring evaluation by FISH analysis. As mentioned above, one specimen (G331) in the present study showed a high *MET* copy number (6.14 copies) but could not be confirmed positive for *MET* amplification because of the lack of a FISH result. The issue of how to identify *MET* amplification status in such cases remains to be resolved.

We examined the biological impact of *MET* amplification in gastric cancer cells by comparing the effects of the MET-TKIs JNJ38877605 and SGX523 between gastric cancer cell lines positive for *MET* amplification and those negative for this genetic alteration. In gastric cancer cells with *MET* amplification, the MET-TKIs markedly inhibited AKT, ERK, and STAT3 signaling and triggered apoptosis, whereas such effects were not evident in cells without *MET* amplification. To investigate whether attenuation of MET signaling by the MET-TKIs is related to the induction of apoptosis, we transfected gastric cancer cell lines with an siRNA specific for MET mRNA. Such transfection inhibited MET signal transduction as well as induced apoptosis in gastric cancer cell lines with *MET* amplification but not in those without it (data not shown). Our observations thus indicate that gastric cancer cell lines positive for *MET* amplification depend predominantly on constitutive activation of the encoded growth factor receptor for their survival and thus show high sensitivity to cell killing by MET-TKIs. Targeting of MET signaling by MET-TKIs is therefore a potentially valuable therapeutic approach for patients with *MET* amplification–positive gastric cancer. Indeed, the MET-TKI crizotinib (PF-02341066) was recently found to induce a radiographic response (partial response) and rapid clinical improvement in patients with advanced

gastric cancer who were found to be positive for *MET* amplification by FISH [16]. Further investigation of the efficacy of MET-TKIs in patients with advanced gastric cancer positive for *MET* amplification is thus warranted.

Given the potential of MET-targeted therapy for gastric cancer with *MET* amplification, it is important to determine the prevalence of such gene amplification in patients with unresectable advanced gastric cancer, most of whom are currently treated with systemic chemotherapy. Our present study was limited to gastric cancer patients who underwent gastrectomy, and so further studies will be needed for patients with unresectable advanced tumors. Given the apparent low prevalence of *MET* amplification in gastric cancer, implementation of a sequential approach including screening with a PCR-based copy number assay followed by confirmatory FISH analysis should facilitate the identification of *MET* amplification in a large cohort of patients with unresectable advanced gastric cancer.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cell lines SNU1, SNU5 and Hs746T were obtained from American Type Culture Collection (Manassas, VA); MKN1, MKN7, MKN45, and NUGC3 were from the Health Science Research Resources Bank (Japan Health Sciences Foundation, Tokyo, Japan); KATO III, MKN28, and HSC39 were from Immuno-Biological Laboratories (Gunma, Japan); and SNU216 was from the Korean Cell Line Bank (Seoul National University, Seoul, Korea). All of the cell lines were maintained under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Gibco BRL, Grand Island, NY), penicillin, and streptomycin.

Patients

A total of 267 patients with histologically confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital (Tokyo, Japan) between 1996 and 2006 were included in the study. All the patients had an Eastern Cooperative Oncology Group performance status of 0 to 2. One patient was subsequently excluded as a result of an insufficient quantity of DNA extracted from the corresponding tissue specimen. The specimens from the remaining 266 patients were thus analyzed. The present study was approved by the Institutional Review Board of the National Cancer Center Hospital, and informed consent was obtained from all subjects.

Isolation of genomic DNA

Macrodissection of the surgical specimens preserved as FFPE tissue was performed after removal of paraffin in order to select a region of cancer tissue. Genomic DNA was extracted from the cancer tissue with the use of a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany). The DNA concentration of the extracts was determined with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA).

PCR-based determination of MET copy number

The copy number of *MET* was determined with the use of a TaqMan Copy Number Assay [32] and the Hs05005660_cn (intron 16) primer (Applied Biosystems, Foster City, CA). The *TERT* locus was used as the internal reference, and DNA from noncancerous FFPE tissue was used as a normal control. Real-time PCR was performed in a total volume of 20 μL per well containing 10 μL of TaqMan genotyping master mix, 20 ng of genomic DNA, and each primer. The amplification protocol included an initial incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The resulting products were detected with the use of ABI Prism 7900HT Sequence Detection System (Applied Biosystems). Data were analyzed with SDS 2.2 software and Copy Caller software (Applied Biosystems).

FISH

MET copy number per cell was determined by FISH with the use of the c-met / CEN7p Dual Color FISH Probe (GSP Laboratory, Kawasaki, Japan) [22], where CEN7p is the centromeric region of chromosome 7p. The signals were detected by fluorescence microscopy and were evaluated by independent observers (H.K. and I.O.). After screening all entire sections, images of tumor cells were captured and recorded and the signals for 60 random nuclei were counted for an area where individual cells were recognized in at least 10 representative images. Nuclei with a disrupted boundary were excluded from the analysis. Gene amplification was strictly defined by a mean *MET*/CEN7p copy number ratio of >2.2, corresponding to a previous definition of *MET* amplification [16]. The presence of polysomy or an equivocal *MET*/CEN7p ratio (1.8 to 2.2) were thus scored as negative for amplification.

Immunoblot analysis

Immunoblot analysis was performed as described previously [22]. Rabbit polyclonal antibodies to phosphorylated human MET (pY1234/pY1235), to total AKT, to phosphorylated AKT, to phosphorylated

extracellular signal–regulated kinase (ERK), to phosphorylated or total forms of STAT3 were obtained from Cell Signaling Technology (Danvers, MA); those to total ERK were from Santa Cruz Biotechnology (Santa Cruz, CA); those to total MET were from Zymed/ Invitrogen (Carlsbad, CA); and those to β-actin were from Sigma. All antibodies were used at a 1:1000 dilution, with the exception of those to β-actin (1:200).

Cell growth inhibition assay

Cells were transferred to 96-well flat-bottomed plates and cultured for 24 h before exposure to various concentrations of JNJ38877605 (Janssen Pharmaceutica NV, Beerse, Belgium) or SGX523 (SGX Pharmaceuticals, San Diego, CA) for 72 h. Tetra Color One (5 mmol/L tetrazolium monosodium salt and 0.2 mmol/L 1-methoxy-5-methyl phenazinium methylsulfate; Seikagaku Kogyo, Tokyo, Japan) was then added to each well, and the cells were incubated for 3 h at 37°C before measurement of absorbance at 490 nm with a Multiskan Spectrum instrument (Thermo Labsystems, Boston, MA). Absorbance values were expressed as a percentage of that for nontreated cells, and the IC_{50} values of JNJ38877605 and SGX523 for inhibition of cell growth were determined.

Annexin V binding assay

The binding of annexin V to cells was measured with the use of an Annexin-V-FLUOS Staining Kit (Roche, Basel, Switzerland). Cells were harvested by exposure to trypsin-EDTA, washed with PBS, and centrifuged at 200 × *g* for 5 min. The cell pellets were resuspended in 100 μL of Annexin-V-FLUOS labeling solution, incubated for 10 to 15 min at 15° to 25°C, and then analyzed for fluorescence with a flow cytometer (FACSCalibur) and Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

Statistical analysis

Overall survival (OS) curves were estimated with the Kaplan-Meier method and compared with the log-rank test. Other statistical analysis was performed with Student's two-tailed t test or the chi-square test. A P value of <0.05 was considered statistically significant.

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Conflict of interest statement

The authors declare no conflict of interest.

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Gene Amplification of Ribosomal Protein S6 Kinase-1 and -2 in Gastric Cancer

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Abstract. *Background: The gene amplification of ribosomal protein S6 kinase 1 and 2 (S6K1 and S6K2) and its clinical relevance in gastric cancer remain unclear. Materials and Methods: A comparative genomic hybridization analysis and DNA copy number assay were performed for nine cancer cell lines. The gene amplification of S6K1 and S6K2 were determined using a DNA copy number assay of 213 gastric cancer tissues. Results: S6K1 and S6K2 amplifications were observed in one and three cancer cell lines, respectively. No amplification of S6K1 was detected in the gastric cancer tissues, while S6K2 amplification was observed in 4.7% of the gastric carcinoma tissues. Patients with stage IV gastric cancer whose tumors exhibited amplification had a significantly shorter overall survival. Conclusion: S6K2 amplification was frequently observed in gastric cancer and was related to a poor prognosis. Our findings may provide novel insight into the dysregulation of mammalian target of rapamycin signaling by S6K2 amplification in gastric cancer.*

Many investigations on treatment of gastric cancer have been performed over the past decades; however, the prognosis for patients with advanced gastric cancer remains poor (1, 2). Thus, detailed information on genomic alterations in clinical samples is needed in order for new treatment modalities of molecular-targeted drugs to be developed.

The serine/threonine kinase mammalian target of rapamycin (mTOR) is a downstream effector of the phosphatidylinositol 3-kinase (PI3K)/ v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway and regulates transcription, mRNA translation, cellular growth, proliferation, and survival of cells in response to various stimuli such as growth factors, nutrients, energy, and stress signals (3). mTOR forms two complexes, which are known as mTORC1 and mTORC2, and these multiple-protein complexes are activated by phosphorylated AKT (4). Activated mTORC1 further activates downstream ribosomal protein S6 kinases by phosphorylation (5). Ribosomal protein S6 kinase-1 (*S6K1*, also known as *p70-S6K/RPS6KB1*) and ribosomal protein S6 kinase-2 (*S6K2*, also known as *p70S6Kbeta/ RPS6KB2*) are key target molecules of mTORC1 and belong to the AGC [protein kinase, cAMP-dependent (PKA), protein kinase, cGMP-dependent (PKG) and protein kinase C (PKC)] kinase family (6). S6 kinases possess consensus domains, including a TOR signaling motif in the *N*-terminal domain, a kinase domain and a C-terminal domain (7). S6K1 phosphorylates numerous downstream molecules and regulates many cellular process including mRNA processing, translation initiation, translational elongation, protein folding, cell growth, motility, and survival (6). Therefore, mTORC1-S6K1 signaling is considered to be important for cellular physiology, and its deregulation often leads to disease.

In oncology, both cancerous and non-cancerous cells that contribute to the formation of tumor tissue, such as leukocytes, endothelial cells, and fibroblasts, depend on the mTORC1-S6K1 signaling pathway, and frequent dysregulation of this signaling has been associated with the development of cancer (8). Thus, many mTOR inhibitors are now under clinical development, and some mTOR inhibitors have shown clinical benefits as molecular-targeted drugs (9). However, very limited information on *S6K1* and *S6K2* amplification and its clinical relevance to gastric cancer is available.

In this study, we retrospectively studied the DNA copy numbers of *S6K1* and *S6K2* using formalin-fixed, paraffin-embedded (FFPE) samples from patients with gastric cancer who had undergone surgery, and evaluated the clinical significance of these amplifications.

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Key Words: Ribosomal protein S6 kinase, RPS6KB2, gastric cancer, gene amplification, S6K2 mTOR signaling.

Materials and Methods

Cell cultures. Gastric cancer cell lines (44As3, 58As1, HSC43 and OKAJIMA) were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) except for IM95, which was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). The esophageal cancer cell lines KYSE170 and KYSE180 were maintained in a 1:1 mixture of Ham's F12 medium and RPMI-1640 with 2% heat-inactivated FBS. KYSE70 was maintained in DMEM with 2% FBS. KYSE150 was maintained in Ham's F12 with 2% FBS. The cell lines were maintained in a 5% CO_2-humidified atmosphere at 37˚C. The IM95 and KYSE series were obtained from the Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan), while the others were provided by the National Cancer Center Research Institute (Tsukiji, Tokyo, Japan).

Patients and samples. A total of two-hundred thirteen patients with histologically-confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital were included in this study, as previously described (10). All the patients in this series had an Eastern Cooperative Oncology Group performance status of 0 to 2. The present study was approved by the Institutional Review Board of the National Cancer Center Hospital.

Genomic DNA samples were extracted from surgical specimens preserved as FFPE tissues using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany), as previously described (10). Macro dissection of the FFPE samples was performed to select for a cancer region, which was marked by a pathologist. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA, USA).

DNA copy number assay for S6K1 and S6K2. The copy numbers for S6K1 and S6K2 were determined using commercially available and pre-designed TaqMan Copy Number Assays, as previously described (10). The primer IDs used were as follows: S6K1, Hs03958357_cn; S6K2, Hs05250093_cn. The telomerase reverse transcriptase (*TERT*) locus was used for the internal reference copy number. Human Genomic DNA (TaKaRa, Otsu, Japan) and DNA from non-cancerous FFPE tissue were used as normal controls. Real-time genomic PCR was performed in a total volume of 20 μl in each well, containing 10 μl of TaqMan genotyping master mix, 20 ng of genomic DNA, and each primer. The PCR conditions were 95˚C for 10 min, 40 cycles of 95˚C for 15 s and 60˚C for 1 min; the resulting products were detected using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the SDS 2.2 software and CopyCaller software (Applied Biosystems).

Real-time reverse-transcription PCR. cDNA was prepared from the total RNA of each cultured cell line using a GeneAmp® RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed as described previously (11). The primers used for the real-time RT-PCR were as follows: *S6K1*, forward 5'-CAC ATA ACC TGT GGT CTG TTG CTG-3' and reverse 5'-AGA TGC AAA GCG AAC TTG GGA TA-3'; *S6K2*, forward 5'-CTT CCA GAC TGG TGG CAA ACT CTA-3' and reverse 5'-CAG CGT GAT CTC AGC CAG GTA-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-ATG GTG GTG AAG ACG CCA GT-3'. *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses.

Immunoblotting. A western blot analysis was performed as described previously (11). The following antibodies were used: anti-S6K1 (Cell Signaling Technology, Beverly, MA, USA), anti-RPS6KB2 (Sigma), anti-β-actin and horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology).

Array-based comparative genomic hybridization (CGH). The array-based CGH analysis was performed as previously described (12). The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) was used for each of the esophageal cancer cell lines. The GeneChip Human Mapping 250K Nsp Array (Affymetrix) was used for the gastric cancer cell lines. A total of 250 ng of genomic DNA was digested with *Nsp*I (250K) or both *Nsp*I and *Sty*I in independent parallel reactions (SNP6.0), with the restriction enzymes ligated to the adaptor, and then amplified using PCR with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA, USA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a GeneChip Human Mapping 250K Nsp Array or a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and the GeneChip Operating Software Ver.1.4. In the array-CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek, St. Louis, MO, USA).

Statistical analysis. The statistical analyses of the clinico-pathological features were performed using the Student's *t*-test and the χ^2 test using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan). The overall survival (OS) curves were estimated using the Kaplan-Meier method.

Results

Gene amplification of S6K1 and S6K2 in cancer cell lines. An array-CGH analysis was performed to detect the gene amplifications of *S6K1* and *S6K2* in five gastric cancer and four esophageal cancer cell lines. Among the nine cell lines that were examined, the chromosomal region of 17q23 around *S6K1* was amplified in the 44As3 cell line (Figure 1A). The chromosomal region of 11q13 around *S6K2* was amplified in the Okajima, KYSE170, and KYSE180 cell lines (Figure 1B).

To evaluate the high-throughput method for detecting the gene amplification of *S6K1* and *S6K2*, real-time PCR-based DNA copy number assays were performed using DNA from the cancer cell lines (Figure 1C and 1D). The DNA copy number assay demonstrated that copy numbers greater than four copies were observed for *S6K1* in 44As3 cells (4.7±0.3 copies) and for S6K2 in Okajima, KYSE170, and KYSE180 cells (4.4±0.3, 4.0±0.5, and 4.6±1.0 copies, respectively). Collectively, similar results were observed for the gene amplifications of *S6K1* and *S6K2* between the array-CGH and DNA copy number assay. These results indicated that *S6K1* and *S6K2* were amplified in gastric and esophageal cancer cell lines.

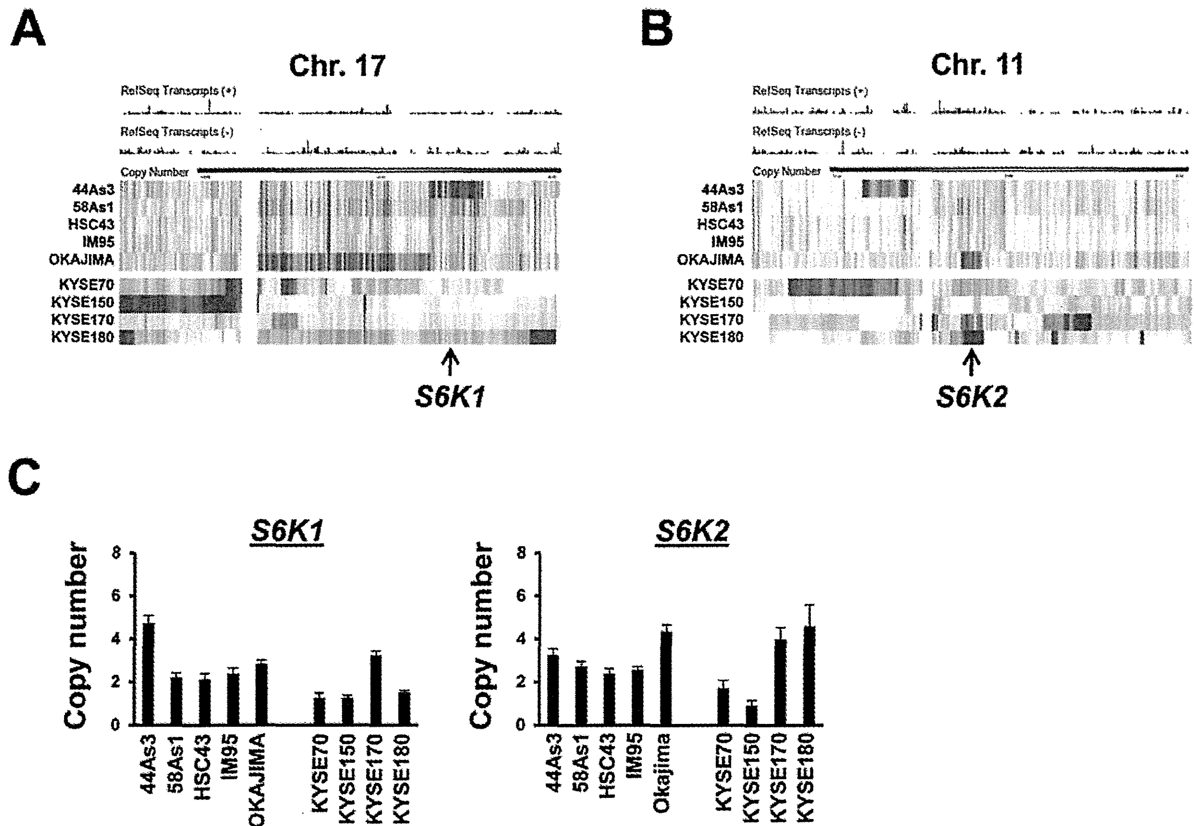

Figure 1. *Gene amplification of ribosomal protein S6 kinase-1 and -2 (S6K1 and S6K2) in gastric cancer and esophageal cancer cell lines. The DNA copy numbers of S6K1 on chromosomal 17 (A) and of S6K2 on chromosomal 11 (B) were evaluated using an array-comparative genomic hybridization analysis. The arrows indicate the genomic loci of the S6K1 and S6K2 genes. (C) Evaluation of DNA copy numbers in cancer cell lines. A DNA copy number assay was performed to determine the copy numbers of the S6K1 and S6K2 genes.*

mRNA and protein expression levels of S6K1 and S6K2 in cancer cell lines. To investigate the correlation between gene amplification and mRNA and protein expression, we examined the mRNA expression levels of *S6K1* and *S6K2* using real-time RT-PCR for gastric and esophageal cancer cell lines. Real-time RT-PCR demonstrated that the *S6K1* mRNA was up-regulated in Okajima cells and that *S6K2* mRNA was up-regulated in Okajima and KYSE180 cells (Figure 2A). A western blot analysis revealed that S6K1 protein expression was observed in all the cell lines, with differences in expression being relatively small, while a high S6K2 protein expression level was observed in Okajima and KYSE180 cells (Figure 2B). In *S6K2*-amplified cell lines, although the mRNA and protein expression levels of S6K2 were not increased in KYSE170 cells, those of Okajima and KYSE180 cells were clearly increased. When compared with the results for the DNA copy numbers, *S6K2* amplification seemed to mediate the mRNA and protein up-regulation, but the effects of *S6K1* were unclear.

Gene amplification of S6K1 and S6K2 in clinical gastric cancer samples. S6K1 and *S6K2* amplification was evaluated using a DNA copy number assay of 213 FFPE samples of primary gastric cancer. The *S6K1* copy number ranged from 0.4 to 3.8 copies, and no obvious *S6K1* amplification was observed in gastric cancer samples (copies ≥4, Figure 3A). The *S6K2* copy number ranged from 0.5 to 9.5 copies. Overall, 4.7% (10/213) of gastric carcinomas harbored the *S6K2* amplification at a level of more than four copies (copies ≥4, Figure 3B). The copy numbers of S6K2 amplification were 4.1, 4.2, 4.3, 4.4, 4.8, 4.9, 5.5, 7.1, 7.3 and 9.5. The mean copy number in the non-amplified *S6K1* and *S6K2* cases was 1.6±0.5 and 2.5±0.6, respectively. The results indicate that *S6K2* was actually amplified in the clinical gastric cancer samples.

Clinicopathological features of S6K2-amplified gastric cancer. The patient characteristics according to *S6K2* amplification are shown in Table I. The median age was 63 years, and 69% (147/213) of this cohort were male. The