membrane of SUIT2 cells, was poorly internalized at the same period (Fig. 2a). We next investigated the kinetics of entry of three mAbs into the tumors using an *in vivo* imaging system with near infrared fluorescence, which can provide deep tissue imaging with high fidelity.[27,28] Until day 3, all fluorescent-mAbs were delivered and retained both in RL-tumor, and SUIT2-tumor, indicating passive targeting (Fig. 2b). On Day 7, anti-EpCAM mAb in RL-tumor and anti-CD20 mAb in SUIT-2-tumor as a negative control, were almost eliminated, but anti-CD20 mAb in RL-tumor, anti-EpCAM mAb in PC-tumor and anti-collagen 4 mAb in both RL-tumor and SUIT-2-tumor were still retained in each tumor, indicating active targeting, that is, binding to their respective antigens within the lesion. There were also clear differences in the degree of intratumor accumulation among three mAbs. Anti-CD20 mAb accumulation was higher in RL-tumors than anti-EpCAM mAb in SUIT2-tumors (Fig. 2b). On the other hand anti-collagen 4 mAb accumulation was higher in SUIT2-tumors than in RL-tumors (Fig. 2b). We then examined the histological distribution of mAbs in each tumor. In RL tumor, fluorescent anti-CD20 mAb was distributed in the whole tumor area and bound to the cancer cells (Fig. 2c). Qdot-labeling system detecting lower fluorescent signals in SUIT-2 tumor was conducted to evaluate the biodistribution of each mAb. The quantity of anti-CD20 mAb observed in SUIT2 tumor was small (Fig. 2d). Anti-EpCAM mAb was observed mainly around the tumor cell-abundant area, in which collagen 4 was negative (Fig. 2d). In contrast, anti-collagen 4 mAb was mainly observed in collagen 4-positive stroma, and rarely in the tumor cell-abundant area (Fig. 2d). Thus, we succeeded in preparing three mAbs: anti-CD 20 mAb for cell-targeting against RL tumor, anti-EpCAM mAb for cell-targeting against SUIT2-tumor, and anti-collagen 4 mAb for stroma-targeting against both tumors. These three mAbs can selectively exit the vascular system through the leaky tumor vessels and distribute within each tumor according to the nature of tissue component.

Preparation and characterization of cell-targeting or stroma-targeting Immunoconjugate-PEG-SN-38 via a carbamate-bond or ester-bond. To specify the appropriate immunoconjugatge therapy against malignant lymphoma or pancreatic cancer, we prepared two types of the conjugates, one being mAb-PEG-SN-38 via a carbamate-bond[29] (Fig. 3a) and another being mAb-PEG-SN-38 via an ester-bond[21,22] (Fig. 3b). Consequently, six types of immunoconjugates, anti-CD20, anti-EpCAM or anti-collagen 4 mAb- SN-38 via a carbamate-bond or an ester-bond, were obtained. The average number of conjugated SN-38 per one mAb (drugs/mAb), ranging from 7.0 to 8.5, was shown in Figure 3c. There was no clear loss of antigen-binding activity of each mAb after the conjugation (Fig. 3D). An *in vitro* release experiment, both bonds can be cut by a carboxylesterase localized in cytoplasm to release SN-38 inside various cells (Fig. 3e). However, in physiological conditions (non-enzymatic hydrolysis), the immunoconjugate prepared via an ester-bond can release SN-38 gradually and effectively. In contrast, the immunoconjugate via a carbamate-bond cannot release SN-38 effectively in the conditions outside the cells (Fig. 3e). We then evaluated the release profiles of SN-38 from both types of immunoconjugate in mouse blood, which contained high amounts of carboxylesterase.[30] *In vivo* analysis of mouse plasma, the concentration of unbound SN-38 or bound and unbound of SN-38 from the immunoconjugate via an ester-bond or a carbamate-bond at 72 h after the mice tail vein injection were shown. Most of the immunoconjugates in the mouse blood were protected from the enzymatic cleavage (Fig. 3f). Next, we examined the difference between carbamate-bond and ester-bond in combination with cell-targeting or stromal-targeting antibody by the cytotoxicity assay. In RL cells, anti-CD 20 immunoconjugate via carbamate-bond showed strong cytotoxicity

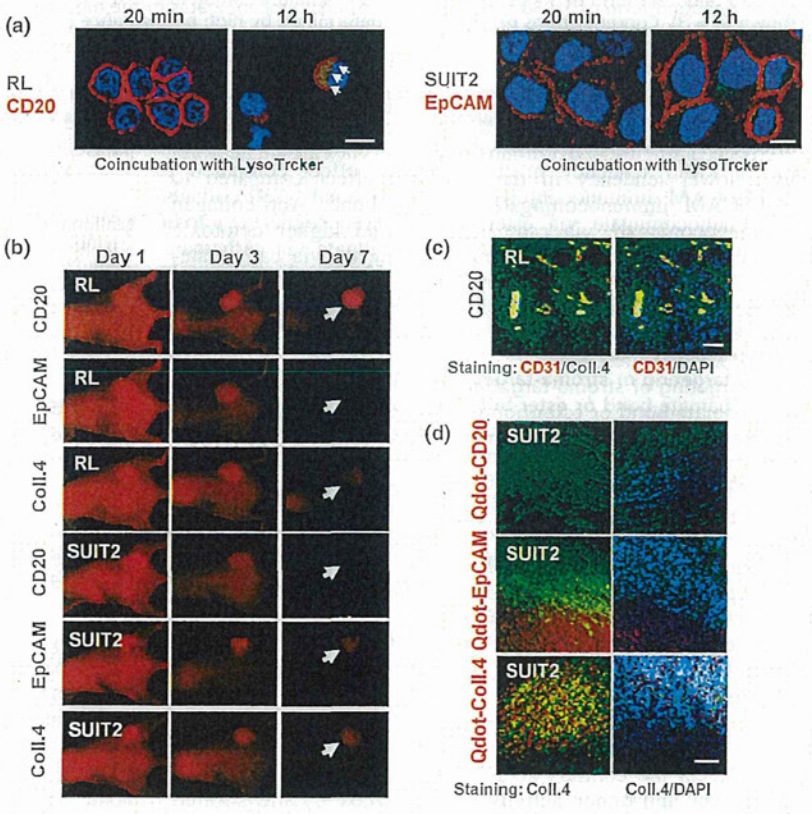

Fig. 2. Internalization and biodistribution of anti-CD20, anti-EpCAM monoclonal antibody (mAb) and anti-collagen 4 mAb. (a) Internalization of Alexafluor-633-anti-CD20 or anti-EpCAM mAb (Red) on RL or SUIT2 cells was examined at 20 min or 12 h after the incubation. Arrow shows merged yellow as co-localization with lysostracker (Green). Scale bar: 10μm. (b) *In vivo* imaging analysis of RL-, SUIT2-tumor was conducted using near-infrared labeled anti-CD20, EpCAM and collagen 4 mAbs on days 1, 3 and 7 after injection. Arrows indicate each tumor position. (c) The intra-RL-tumor distribution of Alexa 647 (green)-labeled anti-CD20 mAb was examined by confocal laser scanning microscopy at 24 h after injection. CD31 (red), Collagen 4 (blue, left panel) and nuclei (blue, right panel) were stained by immunohistochemistry or DAPI. Scale bar: 100 μm. (d) The intra-SUIT2-tumor distribution of each Qdot (red)-labeled antibody was examined by confocal laser scanning microscopy on day 7 after injection. Collagen 4 (green) or nuclei (blue) were stained by immunohisochemisty or DAPI. Scale bar: 100 μm.

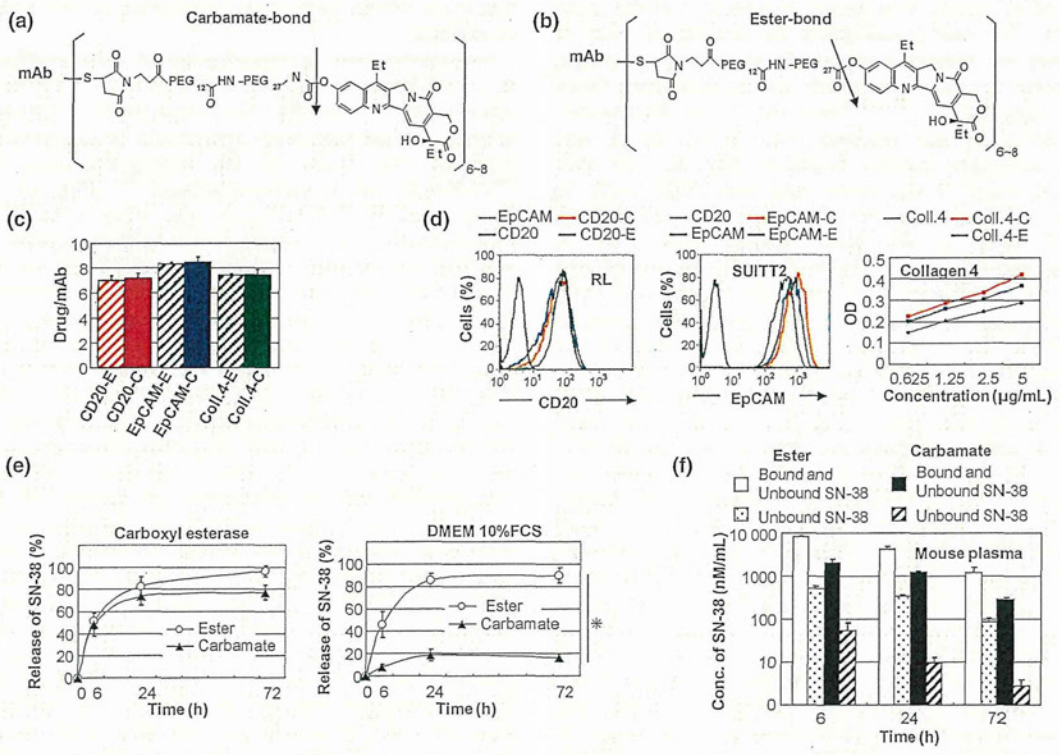

Fig. 3. Preparation and characterization of two types of immunoconjugqates-PEG-SN-38 via carbamate-bond and eser-bond. (a,b) Drug design of two types of immunoconjugates; monoclonal antibody (mAb) -PEG-SN-38 via carbamate-bond (a) and mAb-PEG-SN-38 via ester-bond (b). One antibody bears six to eight molecules of SN-38. The arrow indicates the cleavage site for releasing free active SN-38. (c)The average number of conjugated SN-38 per one mAb was shown (n = 3). Bar = standard deviation (SD). (d) Antigen-binding activity of the mAb before and after the conjugation was shown. Anti-CD 20 and EpCAM mAb were examined by fluorescence-activated cell sorting (FACS) analysis using RL cells and SUIT2 cells, respectively. Anti-collagen 4 mAb was examined by enzyme linked immunosorbent assay (ELISA) using purified protein. (e) *In vitro* release of SN-38 from two types of immunoconjugates in carboxylesterase-contained solution (left) and Dulbecco's modified eagle medium (DMEM) 10% fetal calf serum (FCS) (right) (n = 3). Bar standard deviation (SD), $*P < 0.05$. (f) Concentration of bound and unbound form of SN-38, and unbound form of SN-38 from two types of immunoconjugates in the mouse plasma at 6, 24, 72 h after the mice tail vein injection, were shown (n = 3). Concentrations of SN-38 were determined by high performance liquid chromatography (HPLC). Bar = standard deviation (SD).

compared with anti-CD 20 immunoconjugate via ester-bond significantly. In SUIT2 cells, although there was no significant difference, anti-EpCAM immunoconjugate via carbamate-bond had a lower tendency in the cytotoxic effect compared to anti-EpCAM immunoconjugate via ester-bond. Anti-collagen 4 immunoconjugate via ester-bond showed higher cytotoxic activity than anti-collagen 4 immunoconjugate via carbamate-bond in both cells significantly (Table 1). These results indicated that a carbamate-bond was useful for the immunoconjugate linker to work inside of the cells and an ester-bond to work outside the cells.

Cell-targeting or stroma-targeting immunoconjugate-PEG-SN-38 via carbamate-bond or ester-bond differs drastically in their anti-tumor effects depending on tumor stromal component in mice. Three mAbs conjugated with SN-38 via carbamate-bond or ester-bond (administered once, at an equivalent SN-38 dose of 3 mg/kg) were evaluated in order to know their anti-tumor effects in RL (CD20-positive stroma-poor human malignant lymphoma), SUIT2 (EpCAM-positive stroma-rich human pancreatic tumor). In RL lymphomas, cell-targeting anti-CD20 mAb- SN-38 via carbamate-bond showed superior anti-tumor activity compared to anti-CD20 mAb- SN-38 via ester-bond after the treatment (Fig. 4a). Stroma-targeting anti-collagen 4 mAb- SN-38 via ester-bond showed significant superior anti-tumor activity as compared to saline as control, but inferior to anti-CD20 mAb- SN-38 via carbamate-bond (Fig. 4a). On the contrary to RL tumor, in SUIT2 tumor, the most potent anti-tumor activity was obtained by the stroma-targeting anti-collagen 4 mAb- SN-38 via ester-bond (Fig. 4b). However, there was no significant difference in anti-tumor activity between anti-EpCAM mAb- SN-38 via carbamate-bond and via ester-bond, whereas the anti-tumor activity of anti-collagen 4 mAb- SN-38 via carbamate-bond was inferior to that of anti-collagen 4 mAb- SN-38 via ester-bond (Fig. 4b). These results clearly indicated that, in

Table 1. IC50 of free SN-38 and SN-38 conjugated to monoclonal antibody (mAb) (immunoconjugate) for malignant lymphoma and pancreatic cancer cell lines (WST-8 assay)

		SN-38 conjugated to mAb	
Malignant lymphoma cell lines	Free SN-38	CD20	Collagen 4
		Ester *vs* carbamate	Ester *vs* carbamate
RL	4.6 ± 3.7	8.7 ± 2.9 *vs* 2.1 ± 1.0*	34 ± 17 *vs* 90 ± 30*
		SN-38 conjugated to mAb	
Pancreatic cancer cell lines	Free SN-38	EpCAM	Collagen 4
		Ester *vs* carbamate	Ester *vs* carbamate
SUIT2	7.8 ± 3.6	24 ± 13 *vs* 15 ± 9	29 ± 15 *vs* 75 ± 22*

IC50 (50% cell survival) (nM), Mean ± standard deviation (n = 3), $*P < 0.05$.

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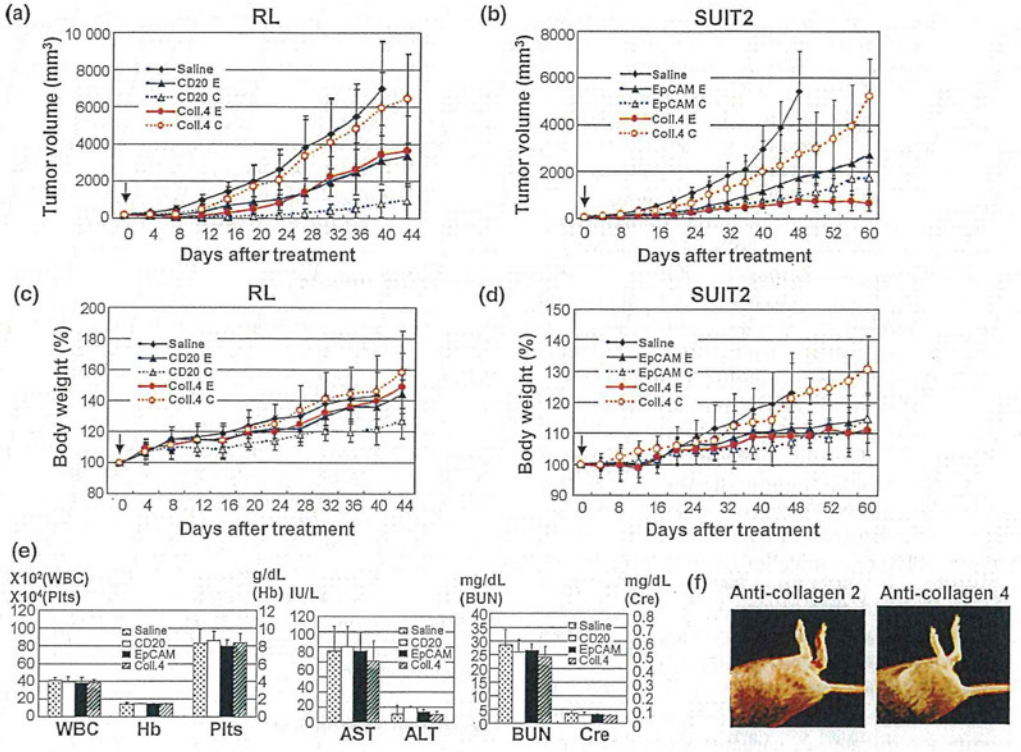

Fig. 4. Antitumor effects and toxicities of immunoconjugates-PEG-SN-38 in the combinations of anti-cell or anti-stroma targeting, carbonate-bond or ester-bond. (a,b) Anti-tumor activities and (c,d) percent changes of body weight were examined. In animal models of RL (a,c) and SUIT2 (b,d), the six types of immunoconjugates (combined anti-CD20 monoclonal antibody (mAb) =CD20, anti-EpCAM mAb=EpCAM or anti-collagen 4 mAb=Coll.4 and ester-bond=E or carbamate-bond=C), or saline as control, were administered once at an equivalent SN-38 dose of 3 mg/kg to separate groups of mice ($n = 5$) by intravenous bolus injection to the mice on day 0. Arrows indicate day of administration and the curves illustrate the effect of treatment on tumor size. $P < 0.0001$ (saline versus CD20-E or CD20-C, CD20-C versus CD20-E, Coll.4-E or Coll.4-C in RL tumor; saline versus EpCAM-E, EpCAM-C or Coll.4-E, Coll.4-E versus EpCAM-C or Coll.4-C, EpCAM-C versus Coll.4-C in SUIT2 tumor), $P < 0.001$ (Saline versus Coll.4-E in RL tumor; saline versus Coll.4-C, Coll.4-E versus EpCAM-E in SUIT2 tumor). Bar = standard deviation (SD). (e) Hematological (WBC, Hb and Plts) and biochemical (aspartate aminotransferase [AST], alanine aminotransferase [ALT], blood urea nitrogen [BUN] and creatinine [Cre]) examination were conducted at 7 days after i.v. administration of immunoconjugates via ester-bond or saline as control. Bar = standard deviation (SD). (f) Anti-collagen antibody induced arthritis in DBA/1J mice. The arthritis was admitted on day 7 only after the administration of anti-collagen 2 (left) but not anti-collagen 4 (right) antibodies.

stroma-poor solid tumors like malignant lymphoma, cytotoxic immunoconjugate should target to the tumor cell surface and ACA should be conjugated to mAb through carbamate-bond, which can be specifically cut by a carboxylesterase inside the tumor cell after the internalization. On the other hand, in stroma-rich tumors, the immunoconjugate should target to the stroma within tumor tissue and ACA should be attached to the mAb via ester-bond, which can be cut gradually outside the tumor cell following the accumulation of the cytotoxic immunoconjugate in the tumor stroma. It is remarkable that the feature of tumor stromal component influence the outcome of the two types of immunoconjugation drugs, cell-targeting mAb-PEG-SN-38 via carbamate-bond, or stroma-targeting mAb-PEG-SN-38 via ester-bond.

Regarding normal tissue distribution and elimination of antibodies and SN-38, there was no difference among immunoconjugates on day 7 after the administration. The dose in this study did not cause significant toxicity as shown by the change of mouse body weight (Fig. 4c,d). Moreover, there was no hepatotoxicity, nephrotoxicity, or bone marrow toxicity in mice treated with all three immunoconjugates as compared to controls (Fig. 4e). In addition, no autoimmune disease-like adverse effects such as arthritis and nephritis were observed in the administration of anti-collagen 4 mAb, whereas anti-collagen 2 mAb combined with lipopolysaccharide caused severe arthritis (Fig. 4f).

Discussion

Conventional immunoconjugate is composed of cell-targeting mAb, ACA as payload and linker for the conjugation. The linker technology is an important part of the immunoconjugate strategy, and various linkers have been exploited to date. Among them, acid labile hydrazine linkage, thiol reduction of disulfide linkers, and lysosomal peptidase proteolysis of peptide linkers were favorably applied to ensure stability in blood.[6–8] For these types of linkers, cell-mediated endocytosis of antibody (antibody-internalization) and intracellular biochemical (enzymatic) processing of the immunoconjugate were indispensable to make the active ACA work. Our carbamate-bond based linker, which is used in a clinically approved anticancer prodrug CPT-11 to release an active component SN-38 within the tumor cell but not in blood circulation[29,31,32] can be classified into the conventional type mentioned above. Anti-CD 20 Immunoconjugate-PEG-SN-38 via carbamate-bond showed strong anti-tumor activity against malignant lymphoma, in which the distribution within the tumor tissue and antibody-internalization into tumor cells occur effectively. Although there were negative reports concerning the internalization of anti-CD20 mAbs, several authors, recently demonstrated internalization of anti-CD20 mAbs including rituximab in malignant lymphoma and leukemia cells.[33–36] These conflicting results might reflect

the differences of cell types, mAbs or methodologies used.[35,36] In contrast to malignant lymphoma, most human solid tumors possess abundant stroma that hinders the tissue-distribution of antibodies.[17–22] Cell–cell interaction between malignant epithelial cells also inhibits the penetration of mAb besides tumor-stroma.[18,37] Moreover, heterogeneity of the cells in the tumor prevents development of immunoconjugate therapy based on cancer cell-specific antigen.[9–12] This led us to design an anti-stromal targeting immunoconjugate strategy using the tumor stroma both as a scaffold for binding and assembling immunoconjugates and as a relay base for a second attack by payload-ACA persistently released from the scaffold.[21,22] In this drug design we selected a specially selected linker using ester-bond, which can release SN-38 in physiological condition (non-enzymatic hydrolysis) outside the cells. Both ester-bond and carbamate-bond were concerned to be cleaved by plasma carboxyl-esterase in the circulation after the injection. Cleavages of our conjugates were very low in mouse plasma, which has much higher levels of carboxyl-esterase activity than in human.[30] Recently, we conducted clinical trials of NK012, a SN-38 incorporating polymeric micelle. In this formulation, SN-38 was conjugated to poly-Glu-chain via ester-bond. From these trials, we learned that human blood also contains high amounts of carboxylesterase. Nevertheless, NK012 proved good stability in human blood circulation.[38,39]

Anti-collagen 4 immunoconjugate exiting from vessel can bind to the outer vessel wall and cells surrounding the stroma. Anti-collagen 4 immunoconjugate via carbamate-bond was not useful because it can scarcely release SN-38 outside the cells. On the other hand, anti-collagen 4 immunoconjugate via ester-bond can release SN-38 on the stroma. Low molecular weight agent SN-38 can penetrate through stroma into the cells. SN-38 released from the scaffold of adjacent collagen-4-positive vascular wall also attack tumor endothelium.[21] Anti-collagen 4 mAb-SN-38 via ester-bond exerted more potent antitumor activity compared to anti-EpCAM mAb-SN-38 via carbamate-bond or ester-bond. It is too complicated to explain these data. However, we speculate that in addition to the insufficient attainability of anti-EpCAM mAb to tumor cells by stromal barrier and its low internalization into the cells, the retention of anti-EpCAM mAb within the tumor cell lesion is lower than that of anti-collagen 4 mAb within the tumor stroma. Consequently, the amount of SN-38 released inside of the cells from anti-EpCAM mAb-SN-38 via carbamate-bond or outside of the cells from anti-EpCAM mAb-SN-38 via ester-bond, may be less than that of SN-38 released outside of the cells from anti-collagen 4 mAb-SN-38 via ester-bond.

Although there had been a concern about the influence of anti-collagen 4 immunoconjugate on normal tissues having high level of collagen 4, we observed the safety of the immunoconjugate in several mouse models. We think that cancer stromal targeting (CAST) therapy is dependent on the fundamental concept that antibodies or immunoconjugates are generally too large to pass through the normal vessel walls, whereas they can extravasate from leaky tumor vessels to achieve tumor selective targeting by using EPR effect and bind to collagen 4, a plentiful component of the tumor stroma.[1–4,40] We also speculate that such a passive targeting effect is one of the reasons why recent anti-EGFR antibody therapies show no serious adverse effects in spite of high level EGFR expression in normal tissues including intestinal mucosa, dermis and others.[9,10,41]

In general, human cancer is classified into three types according to the tissue component. One is hypervascular stroma-poor tumor such as malignant lymphoma, the second is hypovascular stroma-rich tumor such as pancreatic cancer and stomach cancer, and the third is intermediated tumor between the two types such as breast cancer and colorectal cancer. We thus propose the new therapeutic strategy of immunoconjugates to the feature of individual tumor as tissue stromal component: (i) cell-targeting mAb conjugated with ACAs via carbamate-bond for hypervascular and stroma-poor tumor; (ii) stroma-targeting mAb conjugated with ACAs via ester-bond for hypovascular and stroma-rich tumor; (iii) both cell-targeting immunoconjugate via carbamate-bond and stroma-tgargeting via ester-bond for intermediated type of tumor (Fig. 5).

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Disclosure Statement

The authors have no conflict of interest.

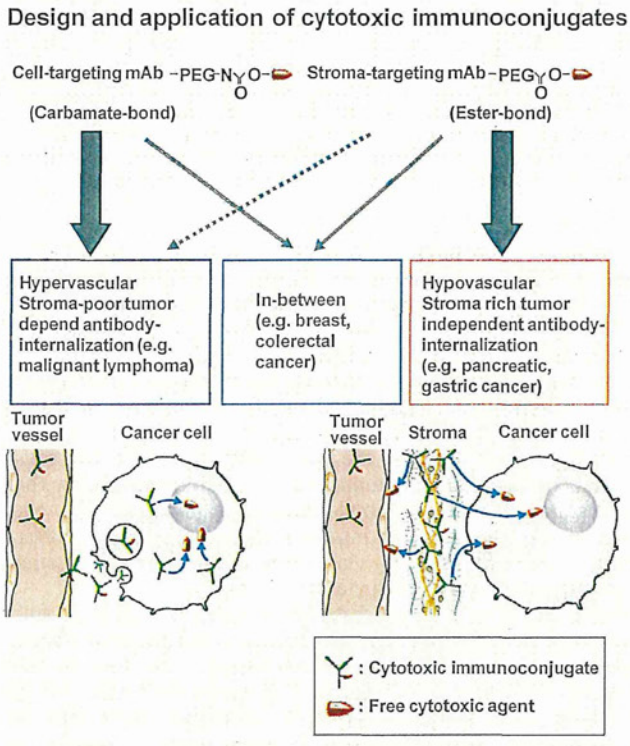

Fig. 5. Diagram of Immunoconjugate strategy to tumor tissue component and characteristic of cancer-cells. Design and application of cytotoxic immunoconjugates. SN-38 conjugated cell-targeting monoclonal antibody (mAb) via carbamate-bond is suitable for hypervascular, stroma-poor tumor dependent antibody-internalization. SN-38 conjugated stroma-targeting mAb via ester-bond is suitable for hypovascular, stroma-rich tumor independent antibody-internalization.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Detailed methods including linker-SN-38 derivative synthesis.

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The effect of CYP2C19 polymorphism on the safety, tolerability, and pharmacokinetics of tivantinib (ARQ 197): results from a phase I trial in advanced solid tumors

N. Yamamoto[1], H. Murakami[1], T. Nishina[2], T. Hirashima[3], K. Sugio[4], K. Muro[5], T. Takahashi[1], T. Naito[1], H. Yasui[6], S. Akinaga[7], Y. Koh[8] & N. Boku[6]

[1]Division of Thoracic Oncology, Shizuoka Cancer Center, Naga-izumi; [2]Department of Gastroenterology, National Shikoku Cancer Center, Matsuyama; [3]Department of Thoracic Malignancy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka; [4]Department of Thoracic Oncology, National Kyushu Cancer Center, Fukuoka; [5]Department of Clinical Oncology, Aichi Cancer Center Hospital, Nagoya; [6]Division of Gastrointestinal Oncology, Shizuoka Cancer Center, Naga-izumi; [7]Development Division, Kyowa Hakko Kirin Co., LTD., Tokyo; [8]Division of Drug Discovery and Development, Shizuoka Cancer Center Research Institute, Naga-izumi, Japan

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Background: Tivantinib (formerly ARQ 197) is a selective inhibitor of c-Met mainly metabolized by CYP2C19. CYP2C19 is known for genetic polymorphisms, and ~20% of Asians are poor metabolizers (PMs), while others are extensive metabolizers (EMs). In this study, we examined the safety, pharmacokinetics (PK), and preliminary efficacy of tivantinib as a single agent to determine recommended phase II doses (RPIIDs).

Patients and methods: Forty-seven patients (EMs, 33; PMs, 14) with solid tumors were orally treated with tivantinib, from 70 to 360 mg bid in a 3 + 3 dose-escalation scheme. EMs and PMs were separately enrolled at the doses >120 mg bid.

Results: Tivantinib was well tolerated up to 360 mg bid for EMs and 240 mg bid for PMs. Neutropenia, leukopenia, anemia, fatigue, and anorexia were the frequent adverse events related to tivantinib and were commonly observed in both EMs and PMs. PMs had 1.9-fold higher AUC_{0-12} compared with EMs at 240 mg bid. Regardless of CYP2C19 phenotype, Gr.4 neutropenia occurred in patients with relatively high exposure to tivantinib. A confirmed partial response was achieved in two non-small-cell lung cancer (NSCLC) patients.

Conclusion: Two different settings of RPIIDs, 360 mg bid for EMs and 240 mg bid for PMs, were determined.

Key words: c-Met inhibitor, CYP2C19 polymorphism, pharmacokinetics, phase I study, tivantinib

introduction

c-Met and its ligand hepatocyte growth factor (HGF) play important roles in oncogenesis [1, 2]. Aberrant activation of the HGF/c-Met signaling pathway may lead to increased tumor cell proliferation, resistance to apoptosis, invasive growth, and tumor angiogenesis. In view of critical effects of HGF/c-Met signaling on cancer progression, several biologics, and low-molecular-weight compounds are currently under clinical investigation as HGF/c-Met pathway inhibitors [3–5].

Tivantinib (also known as ARQ 197) is a low-molecular-weight compound, and is the first in class orally available selective inhibitor of c-Met [6]. Tivantinib disrupts c-Met phosphorylation in a non-adenosine triphosphate competitive manner, distinguishing it from other c-Met inhibitors in clinical trials [7]. Tivantinib has been studied in several clinical trials across multiple tumor types [8–11]. So far, all of these studies were conducted in Western countries, including the United States and Europe [12–14].

An *in vitro* study showed that recombinant cytochrome P450 2C19 (CYP2C19) most rapidly degraded tivantinib when compared with other recombinant cytochrome P450 family enzymes, thus suggesting that CYP2C19 should play a key role in drug metabolism in humans [12]. CYP2C19 is known for the genetic polymorphisms that can affect the pharmacokinetics (PK) of the drugs which are the substrates of CYP2C19. Three major single-nucleotide polymorphisms (SNPs) have been identified in the CYP2C19 gene, including the wild-type CYP2C19*1 and two functionally deficient variants, CYP2C19*2 and CYP2C19*3 [15, 16]. The prevalence of the functionally deficient variants varies among races; Asians (30%–80%) showed much higher prevalence than White and Black (12%–19%) [17]. Therefore, by taking into account the CYP2C19 polymorphism variability, this study

*Correspondence to: Dr N. Yamamoto, Division of Thoracic Oncology, Shizuoka Cancer Center, 1007 Shimonagakubo, Shizuoka, Japan. Tel: +81-055-989-5222; Fax: +81-055-989-6089; E-mail: n.yamamoto@scchr.jp

original

provided a more careful safety evaluation of tivantinib in an Asian patient population.

This is an open-label phase I dose-escalation study among Japanese patients with metastatic solid tumors. The primary objectives were safety, tolerability, and recommended phase II doses (RPIIDs) using the continuous twice-daily dosing schedules. The secondary objectives were preliminary antitumor activity and PKs, and the exploratory objectives were pharmacodynamics and predictive biomarkers. Because of the higher incidence of CYP2C19 SNPs in Japanese, all subjects were prospectively tested for their genetic background of CYP2C19 SNPs and divided into two subgroups: (i) extensive metabolizers (EMs) who possess at least one allele of wild-type CYP2C19*1; (ii) poor metabolizers (PMs) whose two alleles consist of either CYP2C19*2 or CYP2C19*3, but not any wild-type.

patients and methods

study design

This was a multicenter (eight institutes in Japan), open-label, dose-escalating phase I study. Patients were enrolled into sequential dose-escalation cohorts starting from 70 mg bid. Tivantinib was supplied by the sponsor as capsules containing formula A of tivantinib [12], and was orally administered twice a day at fasted condition. Dose escalation or cohort expansion followed a standard 3 + 3 design; cohort expansion to six patients took place if only one dose-limiting toxicity (DLT) was reported within the first 28 days of tivantinib treatment for each patient, and dose escalation stopped if two DLTs in a cohort were observed during that period. DLT was a drug-related adverse event, and was defined as Gr. ≥ 3 non-hematological toxicity, except for controllable Gr. ≥ 3 nausea, vomiting and diarrhea, or Gr. ≥ 4 hematological toxicity. Toxic effects were graded according to the Common Terminology Criteria for Adverse Events v3.0 [18]. A genetic test for CYP2C19 [Invader assay, measured by BML Inc. (Tokyo, Japan, a commercial laboratory)] was conducted for all enrolled patients. Patients were allowed to continue tivantinib treatments, as long as there was no evidence of disease progress or safety concerns.

Two major protocol amendments were implemented during the study. The first amendment was required due to the updated clinical study result: a clinical pharmacological study in healthy volunteers showed much higher plasma exposure of tivantinib in PMs than EMs at the same dose. Accordingly, the protocol was amended to determine the recommended doses of tivantinib separately for EMs and PMs. As a consequence, the first three cohorts included both EMs and PMs, and thereafter, separate registrations for EMs and PMs began with the cohorts testing 150 mg bid and 120 mg bid, respectively. The second amendment was implemented when two DLTs were observed in the cohort testing 300 mg bid in EMs. One of the two DLTs was Gr.4 neutropenia which returned to Gr.3 within 1 day with neither tivantinib interruption nor granulocyte-colony stimulating factor (G-CSF) treatment. The Safety Review Committee (SRC) concluded that the transient Gr.4 neutropenia was clinically acceptable and did not pose a grave safety concern. Accordingly, in the amended protocol, it was adopted as an alternative DLT definition that Gr.4 neutropenia lasting for <7 days was NOT defined as a DLT. Thus, dose escalation was carried out up to 360 mg bid.

This study was sponsored by Kyowa Hakko Kirin Co., Ltd., and was conducted in accordance with institutional guidelines, Good Clinical Practice guidelines and the Declaration of Helsinki. Documented approvals from the Institutional Review Boards were obtained. All patients provided written informed consent. This trial was registered in ClinicalTrials.gov as ID: NCT00609921.

eligibility criteria

Patients with cytologically or histologically confirmed solid malignancy for which no standard therapy was available were candidates for this study. The patients also met the inclusion criteria: ≥20 years of age; an Eastern Cooperative Oncology Group performance status (ECOG PS [19]) of ≤1 or Karnofsky performance status of >70% [20]; a life expectancy of ≥3 months; adequate organ functions [serum alanine aminotransferase (ALT) and aspartate aminotransferase ≤2.5 times ULN (or ≤5 times ULN in the case of liver metastases), hemoglobin concentration ≥10.0 g/dl (or ≥8.5 g/dl in case of gastric cancer), serum total bilirubin ≤1.5 times ULN, serum creatinine ≤1.5 mg/dl, neutrophil count ≥1500/μl, and platelet count ≥100 000/μl); and contraception for a designated period. Patients were excluded if they had prior anti-cancer therapies within 4 weeks, blood transfusion and/or colony stimulating factor therapy within 2 weeks, previous tivantinib treatment, familial history of QTc-prolongation syndrome, digestive organ dysfunction affecting tivantinib absorption, symptomatic CNS metastasis, and an uncontrollable complication. Pregnant or lactating women were also excluded.

patient evaluation

Baseline evaluation before the first administration of tivantinib included vital signs, blood count, and serum biochemistry, as well as genotyping and tumor evaluation. Adverse events were assessed continuously throughout the study. Vital signs, blood counts, and serum biochemistry were measured on days 8, 15, 22, 29, and thereafter, every 2 weeks. In addition, electrocardiograms were taken every 2 weeks, and tumor response was evaluated at 4 weeks after the beginning of treatment and every 6 weeks thereafter according to the Response Evaluation Criteria in Solid Tumors version 1.0 [21].

pharmacokinetics analysis

Pharmacokinetic (PK) blood samples were obtained on days 1 and 22 (pre, 1, 2, 4, 8, and 12 h after the first dose of the day), and at trough on days 15 and 29. PK evaluation was conducted in all patients during the DLT observation period [13]. Plasma samples were stored at −70°C until analysis by liquid chromatography/tandem mass spectrometry. Noncompartmental PK parameters were calculated using WinNonlin (Pharsight, Mountain View, CA).

exploratory biomarker studies

HGF, TGF-alpha and amphiregulin ELISA

Plasma samples were obtained on days 1 (baseline), 15, and 29 from 21 patients (16 EMs and 5 PMs) who consented to the exploratory biomarker study. Plasma HGF, TGF-alpha, and amphiregulin concentrations were determined using a HGF, TGF-alpha, and amphiregulin ELISA Development kit (R&D Systems) according to the manufacturer's instructions, respectively. A 50-μl aliquot of plasma was used for the analysis in duplicate and the absorbance of the samples was measured at 450 nm by a 96-well microplate reader (model 680 Microplate Reader, Bio-Rad Laboratories). Statistical analyses were carried out using JMP version 9.0 for Windows (SAS Institute Inc., Cary, NC).

antibody suspension bead array system

The plasma concentrations of angiogenesis-related factors were measured using an antibody suspension bead array, Bio-Plex Pro™ Assays (Bio-Rad Laboratories, Hercules, CA). Human Angiogenesis 9-Plex Panel consisting of angiopoietin-2, follistatin, G-CSF, HGF, interleukin-8, leptin, platelet-

derived growth factor beta polypeptide, platelet endothelial cell adhesion molecule-1 and vascular endothelial growth factor was used [22]. Data were obtained using a Bio-Plex suspension array system® (Bio-Rad Laboratories, Hercules, CA). The assay was carried out according to the manufacturer's instructions. The samples were tested in duplicate and the averages were used for analysis.

results

patient characteristics

A total of 176 Japanese patients with written consent were screened for CYP2C19 genotyping assessment, and the PM phenotype was found in 32 patients (18.2%). A total of 47 patients (EMs: $n = 33$, PMs: $n = 14$) were enrolled into this study from February 2008 to August 2010. After filling up of all cohorts for EMs, the genetic test for CYP2C19 had continued to screen the PMs, to an extent of a number of patients more than the registered 47 patients. There were no notable differences in patient characteristics between the EMs and the PMs who were administered with tivantinib (Table 1). As a result of dose escalation, EMs and PMs were finally assigned to each dose level as described in Table 2.

safety and tolerability

Table 3 presents the list of drug-related adverse events occurring <10% throughout the study, in either or both in EMs and PMs. Tivantinib was generally well tolerated. Leukopenia, neutropenia, anemia, lymphopenia, fatigue, and anorexia were commonly observed events in both EMs and PMs, experienced in >10% of overall patients. Hematologic toxic effects (leukopenia, neutropenia, and anemia) were the major Gr. ≥ 3 drug-related adverse events observed throughout the study for both EMs and PMs. The details of the toxicity, such as occurrence of AEs per dosing cohort or per grading, are provided in Supplementary Table S3, available at *Annals of Oncology* online.

Three patients experienced DLTs which occurred during the first 28 days of tivantinib treatment, to lead to the cohort expansion (Table 2). In cohort 7 of EMs, a colon cancer patient (54 years, F) developed Gr.3 γ-GTP elevation and Gr.4 neutropenia but recovered following G-CSF treatment after tivantinib discontinuation. In the same cohort, a non-small-cell lung cancer (NSCLC) patient (65 years, F) developed Gr.4 neutropenia that returned to Gr.3 within a day with neither tivantinib interruption nor G-CSF treatment. This transient Gr.4 neutropenia was regarded as clinically acceptable by the SRC, which led to the amendment of the protocol. One PM patient (63 years, F, NSCLC) treated with 240 mg bid developed Gr.4 leukopenia and Gr.4 neutropenia, and recovered following G-CSF treatment after tivantinib interruption. Finally, dose escalation was stopped at 360 mg bid for EMs and at 240 mg bid for PMs, respectively, according to the agreement with the SRC, based on the results of safety and PK analysis.

No death was observed throughout the study. A total of six drug-related serious adverse events (SAEs), which prolonged hospitalization, were observed. Two SAEs were Gr.3 febrile neutropenia and Gr.3 pneumonia, which were observed in one EM patient (62 years, M, colon cancer) treated with 360 mg bid, on days 71 and 79, respectively. Those two SAEs were ameliorated with G-CSF treatment during tivantinib interruption. The other four SAEs were the DLTs mentioned above in the EM patient (54 years, F, colon cancer) and the PM patient (63 years, F, NSCLC).

pharmacokinetics and neutropenia

PK parameters of tivantinib were highly variable in each cohort, and therefore, a relationship between the dose level and plasma exposure was not clearly demonstrated (Supplementary Table S1, available at *Annals of Oncology* online). Nonetheless, there were

Table 1. Patient characteristics

	EM	PM	Overall
Patient No.	33	14	47
Age (years; median)	61.0	59.5	61.0
Gender			
Male	22	8	30
Female	11	6	17
Primary cancer			
Non-small-cell lung cancer (NSCLC)	17	8	25
Colon	7	4	11
Gastric	3	1	4
Other	6	1	7
Eastern Cooperative Oncology Group performance status (ECOG PS)			
0	15	6	21
1	18	8	26
No. of prior chemotherapy			
1	1	0	1
2	6	1	7
3	9	5	14
≥4	17	8	25

Table 2. Patient enrollment

Cohort	Dose (mg bid)	No. of patients	
		EM	PM
1	70	3 (including 0 PM)	
2	90	3 (including 1 PM)	
3	120	5 (including 3 PMs)[a]	
4	150	3	3
5	180	4[b]	Not tested
6	240	7[b,c]	7[b,d]
7	300	6[d]	Not tested
8	360	6	Not tested
Overall	33	14	

[a]Protocol amendment required a total of three PMs at least in this cohort.
[b]One patient was replaced due to tivantinib-unrelated adverse events that led to an insufficient compliance to evaluate the safety of the dose level.
[c]Cohort 6 was expanded after the development of two DLTs in cohort 7. Thereafter, amendment of DLT definition allowed the dose escalation up to cohort 8.
[d]Cohort expansion due to DLT.

Table 3. Drug-related adverse events occurring >10% of either or both of EM and PM, through the study

Drug-related adverse events	EM ($n = 33$)		PM ($n = 14$)		Overall ($n = 47$)	
	All grade n (%)	≥Gr.3 n (%)	All grade n (%)	≥Gr.3 n (%)	All grade n (%)	≥Gr.3 n (%)
Hematological						
Leukocytopenia	13 (39.4)	4 (12.1)	9 (64.3)	4 (28.6)	22 (46.8)	8 (17.0)
Neutropenia	7 (21.2)	4 (12.1)	8 (57.1)	5 (35.7)	15 (31.9)	9 (19.1)
Anemia	8 (24.2)	3 (9.1)	4 (28.6)	2 (14.3)	12 (25.5)	5 (10.6)
Lymphopenia	5 (15.2)	1 (3.0)	2 (14.3)	0 (0.0)	7 (14.9)	1 (2.1)
Non-hematological						
Fatigue	16 (48.5)	1 (3.0)	3 (21.4)	0 (0.0)	19 (40.4)	1 (2.1)
Anorexia	6 (18.2)	1 (3.0)	2 (14.3)	1 (7.1)	8 (17.0)	2 (4.3)
Nausea	4 (12.1)	0 (0.0)	0 (0.0)	0 (0.0)	4 (8.5)	0 (0.0)
Alopecia	1 (3.0)	0 (0.0)	3 (21.4)	0 (0.0)	4 (8.5)	0 (0.0)
Prolonged QTc	1 (3.0)	0 (0.0)	2 (14.3)	0 (0.0)	3 (6.4)	0 (0.0)
Alanine aminotransferase (ALT)	1 (3.0)	0 (0.0)	2 (14.3)	0 (0.0)	3 (6.4)	0 (0.0)
Rash	1 (3.0)	0 (0.0)	2 (14.3)	0 (0.0)	3 (6.4)	0 (0.0)
Sinus bradycardia	0 (0.0)	0 (0.0)	2 (14.3)	0 (0.0)	2 (4.3)	0 (0.0)

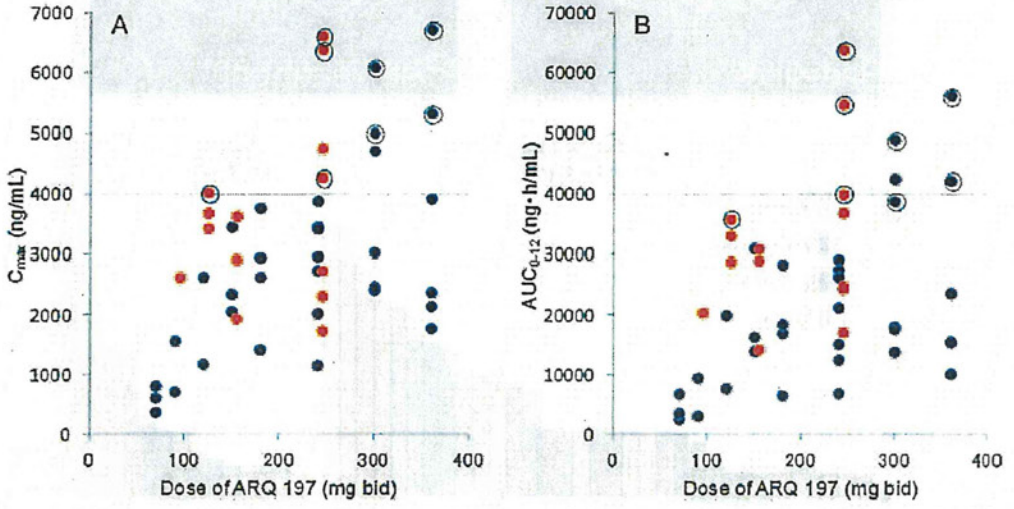

Figure 1. Plasma exposures to tivantinib on day 1. C_{max} (A) or AUC_{0-12} (B) is dotted for each individual (blue dots: EMs, red dots: PMs) treated with the indicated doses. Some circle-surrounded dots indicate a subject who developed Gr. ≥ 4 neutropenia or febrile neutropenia. The lines on the dot blots were putative threshold implying the possible occurrence of neutropenia above the line.

some important findings in the PK analysis. In EMs, mean exposures of tivantinib (AUC_{0-12}) on day 1 were likely to increase dose-dependently up to 300–360 mg. On the other hand, PMs received 240 mg had the mean AUC_{0-12} approximately twofold higher than the AUC_{0-12} in EMs receiving the same dose, which was comparable with or slightly higher than that in EMs at the plateau dose of 300–360 mg bid (Figure 1).

In Figure 1, the patients who experienced Gr.4 neutropenia or febrile neutropenia throughout the study are presented as a surrounded symbol. Most of these subjects were found to distribute above the certain level of tivantinib exposure on day 1, ~>4000 ng/ml of C_{max} (Figure 1A) or >40 000 ng h/ml of AUC_{0-12} (Figure 1B), regardless of CYP2C19 phenotypes.

efficacy

The best overall response included: SD:25 (76%) and PD:8 (24%) in 33 EMs; PR:2 (14%), SD:7 (50%) and PD:5 (36%) in 14 PMs; PR:2 (4%), SD:32 (68%) and PD:13 (28%) in overall patients. A total of eight NSCLC patients experienced a benefit, including two patients with confirmed PR (Figure 2A), and six with long-term SD (>28 weeks, Figure 2B). One of the PR patients possessed wild-type EGFR, and the other possessed mutated EGFR (Supplementary Table S2, available at *Annals of Oncology* online).

PD assessment

We measured the plasma concentrations of HGF from each patient on days 1 (baseline), 15, and 29. Statistical analysis

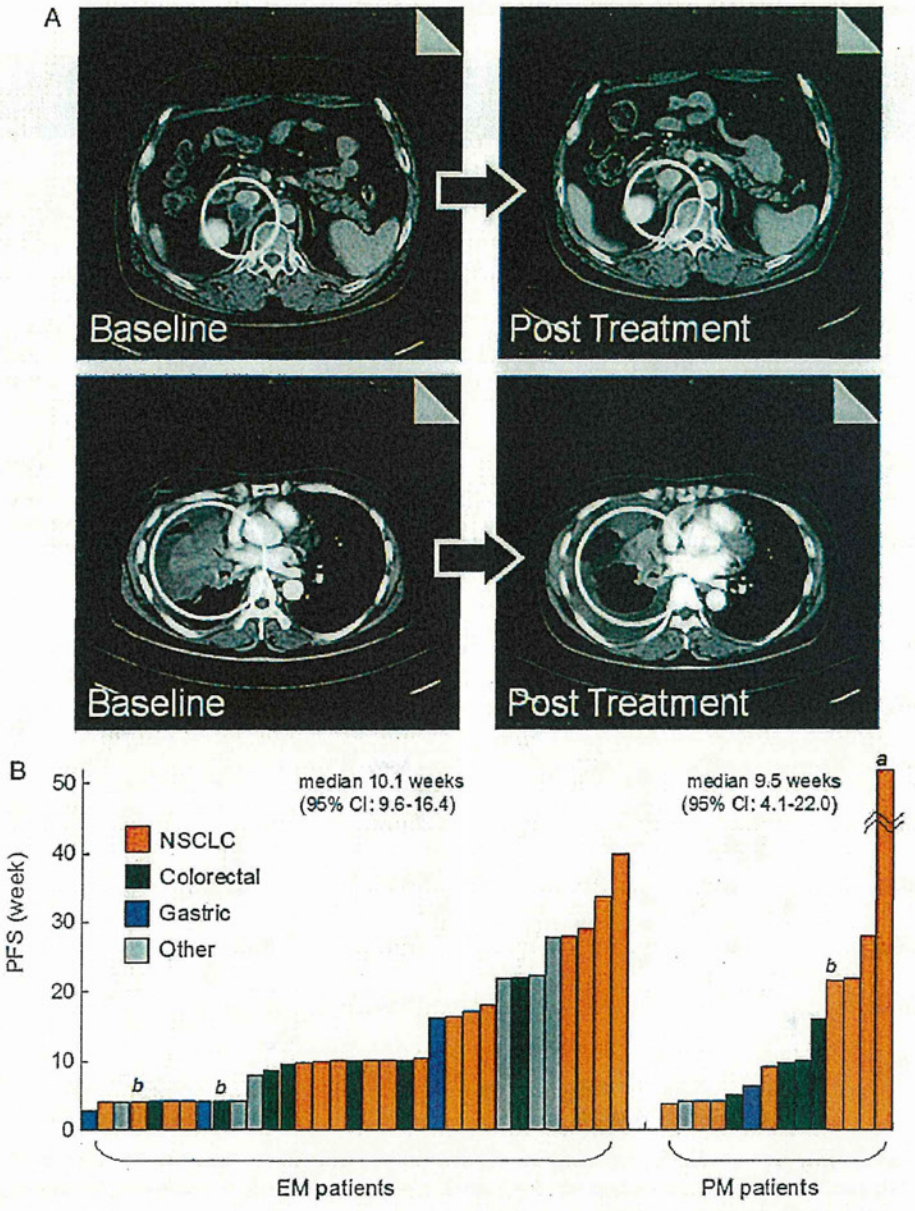

Figure 2. (A) CT scan images of the patients who experienced a confirmed PR. A circle in the images indicates a target lesion. (B) Progression-free survival for individual patient. EM and PM are, respectively, sorted for low and high. Color of the bars indicates his/her cancer type. '*a*' means a patient who was censored for data cut-off at 71.0 weeks with PFS, '*b*' means patients who was censored for treatment discontinuation due to adverse events.

showed that the plasma concentration of HGF after the treatment with tivantinib was significantly lower than that of HGF before the treatment (ANOVA, $P = 0.0339$) (Figure 3). We did not find any significant difference between EMs and PMs regarding the change of plasma HGF level (Supplementary Figure S4, available at *Annals of Oncology* online). On the other hand, there were no significant correlations between the measured biomarkers and tivantinib treatments.

discussion

This is the first clinical trial of tivantinib in Asia. At the commencement of this phase I trial, patients were enrolled without distinguishing between EMs and PMs, similar to the previous clinical trials in Western countries [12, 13]. However, during the course of this trial, we were informed that a clinical pharmacological study in healthy volunteers demonstrated much higher plasma exposure of tivantinib in PMs than EMs at the same dose [12]. Accordingly, considering the fact that ~20% of Asians are PMs [17], the protocol in this Japanese trial was amended to determine the recommended doses of tivantinib separately for EMs and PMs. As a result, tivantinib treatment in Japanese EMs was well tolerated with manageable toxic effects up to 360 mg bid, which is the same dose used in phase II/III studies in Western countries where EMs comprise

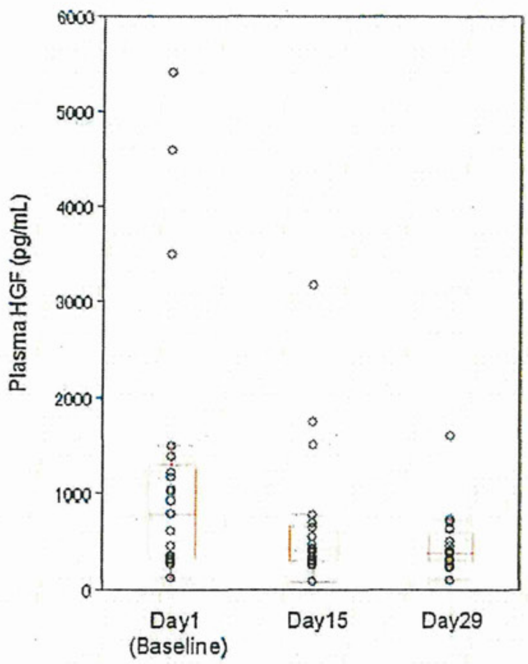

Figure 3. Each dot represents a plasma hepatocyte growth factor (HGF) level on days 1 (baseline), 15, and 29 for individual patients. The box plot shows the median ± confident interval in each day [$n = 21$, except for day 15 ($n = 20$)]. The difference in plasma HGF levels among indicated sampling points was assessed by ANOVA ($P = 0.0339$, 0.1003, respectively).

the large majority of patients. Tivantinib was also well tolerated with manageable toxicit effects in Japanese PMs, up to 240 mg bid. PK analysis demonstrated that not only in EMs at 360 mg but also in PMs at 240 mg, the AUC_{0-12} values were almost the same as or slightly higher than that at 360 mg in Western countries [12, 13], suggesting that 360 mg for EMs and 240 mg for PMs would be sufficient dosages to achieve the plasma exposure level seen in the clinical trials in Western countries [8–11].

Although plasma exposures of tivantinib among Japanese patients were quite variable, it was suggested that CYP2C19 polymorphism may have an impact on the pharmacokinetics of tivantinib. Roughly 1.3–1.8 fold higher AUC_{0-12} was found in PMs than EMs, when administered with tivantinib at the same dose. The increased exposure of tivantinib in PMs was relatively small when compared with other CYP2C19 substrates such as omeprazole, where four- to eightfold higher AUC was found in omeprazole-treated PMs as compared withEMs [23, 24]. The reason for the difference in the fold increase between tivantinib and omeprazole is still unclear, but one possibility would be a change of metabolic conditions in cancer patients. A pharmacokinetics study comparing the omeprazole exposure between cancer patients and non-cancer patients has demonstrated that the CYP2C19 activity was severely compromised in advanced cancer patients with EM genotypes [25]. Another possible explanation for the difference would be involvement of other cytochrome P450 family and/or other nonenzymatical degradation mechanisms in metabolizing CYP2C19 substrates [25]. On the other hand, no clear difference in plasma exposure of tivantinib was found between wild-type CYP2C19 homozygote and wild-type/functionally deficient CYP2C19 homozygote (data not shown).

A notable toxicity related to tivantinib in this Japanese phase I trial was hematologic toxic effects. This finding is consistent with the previous phase I studies conducted in Western countries [12, 13]. Hematologic toxic were commonly found in both EMs and PMs. Our findings demonstrated a good accordance between the occurrence of severe neutropenia and the high plasma level of the parental tivantinib, regardless of CYP2C19 phenotypes. This possible correlation led us to conclude that tivantinib could be administered to both EMs and PMs with a similar profile of adverse events by properly modifying the dose level of tivantinib.

To our knowledge, this is the first report showing that tivantinib administered as a single agent resulted in partial responses in NSCLC patients. It was interesting that PRs were found in NSCLC with both mutated and wild-type EGFR, and this result may suggest that the contribution of c-Met in aggravating NSCLC would be independent of EGFR signaling. In fact, it has been reported that tivantinib in combination with erlotinib showed higher efficacy than erlotinib alone in a phase II trial in NSCLC conducted in Western countries [8]. These results strongly suggested an additive or synergistic effect in NSCLC patients resulting from simultaneous inhibition of EGFR and c-Met. In Asia, a phase III study was recently initiated to evaluate the efficacy of tivantinib in combination with erlotinib in NSCLC patients. In this study, the dose of tivantinib was established following genetic testing for CYP2C19.

Despite the small number of patients, a significant decrease in plasma HGF from baseline was observed in tivantinib-treated patients. Our result indicated a possibility that the decrease of HGF would be a pharmacodynamic biomarker for c-Met inhibitors including tivantinib. In addition, the effect on the plasma HGF level would potentiate the tivantinib-induced inhibition on HGF/c-Met signaling by both inhibiting the kinase activity and reducing the plasma level of HGF. It is reported that higher level of plasma HGF is associated with poor prognosis [26, 27].

In conclusion, the RPIID of tivantinib was similar in both Western countries and Asian countries, when the Asian patient shows an EM phenotype based on CYP2C19 SNPs assessment. However, a lesser dose was recommended for the Asian patient with a PM phenotype of CYP2C19. Hematological toxicity was the most prevalent toxicity for tivantinib in both EMs and PMs, and was well manageable. In further Asian trials, the dose of tivantinib should be individually determined on the basis of pre-treatment testing for CYP2C19 SNPs.

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disclosure

SA is an employee of Kyowa Hakko Kirin Co., Ltd. Other authors have declared no conflicts of interest.

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TAS-102 monotherapy for pretreated metastatic colorectal cancer: a double-blind, randomised, placebo-controlled phase 2 trial

Takayuki Yoshino, Nobuyuki Mizunuma, Kentaro Yamazaki, Tomohiro Nishina, Yoshito Komatsu, Hideo Baba, Akihito Tsuji, Kensei Yamaguchi, Kei Muro, Naotoshi Sugimoto, Yasushi Tsuji, Toshikazu Moriwaki, Taito Esaki, Chikuma Hamada, Takanori Tanase, Atsushi Ohtsu

Summary

Background Treatments that confer survival benefit are needed in patients with heavily pretreated metastatic colorectal cancer. The aim of this trial was to investigate the efficacy and safety of TAS-102—a novel oral nucleoside antitumour agent.

Methods Between August 25, 2009, and April 12, 2010, we undertook a multicentre, double-blind, randomised, placebo-controlled phase 2 trial in Japan. Eligible patients were 20 years or older; had confirmed colorectal adenocarcinoma; had a treatment history of two or more regimens of standard chemotherapy; and were refractory or intolerant to fluoropyrimidine, irinotecan, and oxaliplatin. Patients had to be able to take oral drugs; have measurable lesions; have an Eastern Cooperative Oncology Group performance status of between 0 and 2; and have adequate bone-marrow, hepatic, and renal functions within 7 days of enrolment. Patients were randomly assigned (2:1) to either TAS-102 (35 mg/m^2 given orally twice a day in a 28-day cycle [2-week cycle of 5 days of treatment followed by a 2-day rest period, and then a 14-day rest period]) or placebo; all patients received best supportive care. Randomisation was done with minimisation methods, with performance status as the allocation factor. The randomisation sequence was generated with a validated computer system by an independent team from the trial sponsor. Investigators, patients, data analysts, and the trial sponsor were masked to treatment assignment. The primary endpoint was overall survival in the intention-to-treat population. Safety analyses were done in the per-protocol population. The study is in progress and is registered with Japan Pharmaceutical Information Center, number JapicCTI-090880.

Findings 112 patients allocated to TAS-102 and 57 allocated to placebo made up the intention-to-treat population. Median follow-up was 11·3 months (IQR 10·7–14·0). Median overall survival was 9·0 months (95% CI 7·3–11·3) in the TAS-102 group and 6·6 months (4·9–8·0) in the placebo group (hazard ratio for death 0·56, 80% CI 0·44–0·71, 95% CI 0·39–0·81; p=0·0011). 57 (50%) of 113 patients given TAS-102 in the safety population had neutropenia of grade 3 or 4, 32 (28%) leucopenia, and 19 (17%) anaemia. No patient given placebo had grade 3 or worse neutropenia or leucopenia; three (5%) of 57 had grade 3 or worse anaemia. Serious adverse events occurred in 21 (19%) patients in the TAS-102 group and in five (9%) in the placebo group. No treatment-related deaths occurred.

Interpretation TAS-102 has promising efficacy and a manageable safety profile in patients with metastatic colorectal cancer who are refractory or intolerant to standard chemotherapies.

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National Cancer Center Hospital East, Chiba, Japan (T Yoshino MD, A Ohtsu MD); The Cancer Institute Hospital of the Japanese Foundation for Cancer Research, Tokyo, Japan (N Mizunuma MD); Shizuoka Cancer Center, Shizuoka, Japan (K Yamazaki MD); National Hospital Organization Shikoku Cancer Center, Matsuyama, Japan (T Nishina MD); Hokkaido University Hospital, Hokkaido, Japan (Y Komatsu MD); Kumamoto University Hospital, Kumamoto, Japan (Prof H Baba MD); Kochi Health Sciences Center, Kochi, Japan (A Tsuji MD); Saitama Cancer Center, Saitama, Japan (K Yamaguchi MD); Aichi Cancer Center Hospital, Nagoya, Aichi, Japan (K Muro MD); Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan (N Sugimoto MD); KKR Sapporo Medical Center Tonan Hospital, Hokkaido, Japan (Y Tsuji MD); University of Tsukuba, Tsukuba, Japan (T Moriwaki MD); National Kyusyu Cancer Center, Fukuoka, Japan (T Esaki MD); Tokyo University of Science, Tokyo, Japan (Prof C Hamada PhD); and Taiho Pharmaceutical, Tokyo, Japan (T Tanase MSc)

Correspondence to: Dr Takayuki Yoshino, Department of Gastroenterology and Gastrointestinal Oncology, National Cancer Center Hospital East, Kashiwa, Chiba 277-8577, Japan tyoshino@east.ncc.go.jp

Introduction

Colorectal cancer accounts for about 10% of all cancer cases and is the fourth leading cause of cancer-related deaths worldwide.[1] Cytotoxic agents such as a fluoropyrimidine, irinotecan, and oxaliplatin, and antibodies such as bevacizumab (an anti-VEGF monoclonal antibody) and cetuximab and panitumumab (anti-EGFR monoclonal antibodies) significantly improve the survival of patients with unresectable metastatic colorectal cancer.[2–5] Although many patients have a good long-term performance status, a standard treatment for those who are refractory to or unable to tolerate these agents does not exist.

TAS-102 (Taiho Pharmaceutical, Tokyo, Japan) is a novel oral nucleoside antitumour agent consisting of α,α,α-trifluorothymidine (FTD) and 5-chloro-6-(2-iminopyrrolidin-1-yl) methyl-2,4 (1*H*,3*H*)-pyrimidinedione hydrochloride (TPI) at a molar ratio of 1:0·5. FTD is the active antitumour component of TAS-102: its monophosphate form inhibits thymidylate synthase and its triphosphate form is incorporated into DNA in tumour cells. The incorporation into DNA is known to have antitumour effects, because inhibition of thymidylate synthase caused by oral FTD rapidly disappears after the drug's elimination.[6] TPI is a potent inhibitor of thymidine phosphorylase, which is the enzyme that degrades FTD. After intravenous injection of FTD alone, sufficient concentrations have been recorded in plasma.[7] However, when monkeys are given oral FTD alone, it is rapidly degraded to its inactive

form in the intestines and liver (first-pass effect). Therefore, TPI is necessary to maintain adequate plasma concentrations of FTD that has been taken orally.[8]

Preclinical studies[9,10] have shown that TAS-102 exerts an antitumour effect against cancer cells irrespective of their sensitivity to fluoropyrimidines. TAS-102 has a mechanism of action different from that of other antitumour agents such as a fluoropyrimidine, irinotecan, and oxaliplatin. As a result, TAS-102 is expected to be effective against tumours refractory to the various antitumour agents available.

The results of several independent phase 1 clinical trials[11–13] of patients with solid tumours in the USA showed that the optimum dosage of TAS-102 was a 28-day cycle: a 2-week cycle of 5 days of treatment followed by a 2-day rest period, and then a 14-day rest period. The maximum tolerated dose was 25 mg/m² given orally twice daily to patients with heavily pretreated breast cancer.[14]

Subsequently, a phase 1 clinical trial[15] was done in Japan; the recommended dose was 35 mg/m² twice daily given orally, with the same treatment cycle. 21 patients were enrolled in the Japanese phase 1 study,[15] 18 of whom had colorectal cancer. Clinical benefit was achieved in 11 patients, including one with a partial response; eight were able to continue treatment for 12 weeks. These results suggested that TAS-102 could further improve the outcomes of patients with unresectable metastatic colorectal cancer who have already received conventional chemotherapy with a fluoropyrimidine, irinotecan, and oxaliplatin. Thus, we further investigated the efficacy and safety of TAS-102.

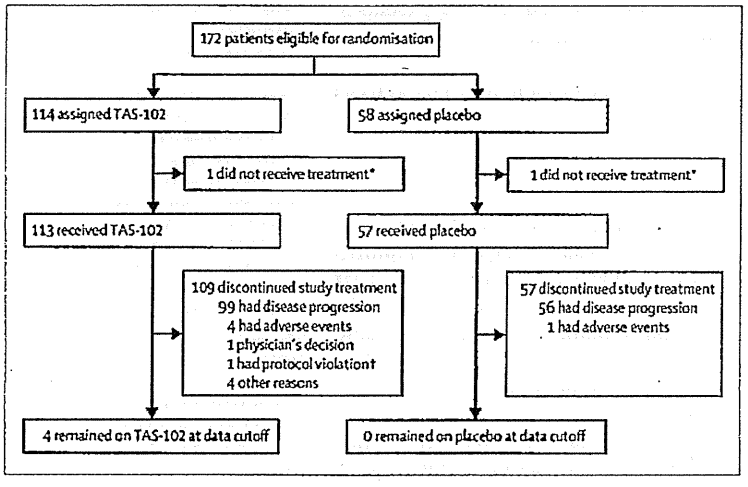

Figure 1: **Trial profile**
*One patient was randomly allocated to TAS-102 did not receive treatment because of aggravation of a rash related to previous chemotherapy and one patient allocated to placebo did not receive treatment because of occurrence of pulmonary thromboembolism; these patients were excluded from the efficacy and safety populations. †One patient received TAS-102 but was concomitantly taking a prohibited treatment, so was excluded from the efficacy population, but included in the safety population.

Methods

Study design and participants

Between Aug 25, 2009, and April 12, 2010, we undertook a multicentre, double-blind, randomised, placebo-controlled phase 2 trial of TAS-102 in Japan. Eligible patients were 20 years or older; had histologically or cytologically confirmed unresectable metastatic colorectal adenocarcinoma; had a previous treatment history of two or more regimens of standard chemotherapy; and were refractory or intolerant to a fluoropyrimidine, irinotecan, and oxaliplatin. Patients had to be able to take oral drugs; and to have measurable lesions as per the Response Evaluation Criteria In Solid Tumors (RECIST; version 1.0)[16] and an Eastern Cooperative Oncology Group (ECOG) performance status of between 0 and 2. Adequate bone-marrow, hepatic, and renal functions were established by tests within the 7 days before enrolment. Patients could have no serious comorbidities.

Previous treatments were discussed by the investigators in charge and study monitors before enrolment to confirm eligibility—ie, whether progression of disease as documented in medical records could be reasonably interpreted as refractory, and whether discontinuation due to unacceptable toxic effects could be reasonably interpreted as intolerance. Whether patients of doubtful eligibility could be enrolled was assessed by the steering committee (AO, TD, IH, and HB) at a central review meeting.

The study was done in accordance with the Declaration of Helsinki and the Japanese Good Clinical Practice guideline. The protocol was approved by the institutional review boards of participating hospitals. Written informed consent was obtained from all patients.

Randomisation and masking

Patients were randomly assigned in a 2:1 ratio to either TAS-102 plus best supportive care or placebo plus best supportive care through central registration. Randomisation was done with minimisation methods, with baseline ECOG performance status (0 *vs* 1 or 2) as the allocation factor. The randomisation sequence was generated by an independent team from the trial sponsor who used a validated computer system. Assignment of patients was initiated via fax. The investigators, patients, data analysts, and the trial sponsor were masked to the randomisation sequence and treatment assignment.

Procedures

A dose of 35 mg/m² TAS-102 was taken orally twice a day after meals (ie, 70 mg/m² per day). Two tablets (15 mg and 20 mg) were used to achieve the correct dose. TAS-102 or placebo was taken in a 28-day cycle: a 2-week cycle of 5 days of treatment followed by a 2-day rest period, and then a 14-day rest period. Placebo was matched to TAS-102 tablets for taste, colour, and size, and contained lactose, partly pregelatinised starch, stearic acid, hydroxypropyl methyl cellulose, polyethylene glycol, and

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

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

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

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

Statistical analysis

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

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

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

Table 1: Demographics and baseline characteristics of the efficacy population

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

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

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

See Online for appendix

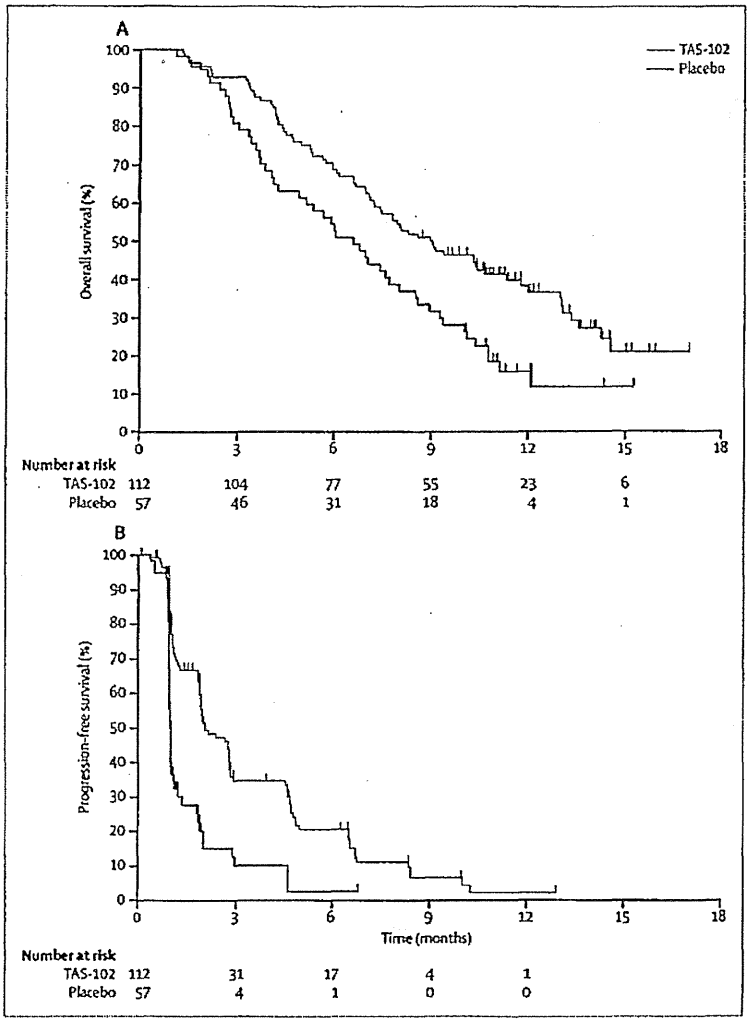

Figure 2: Kaplan-Meier curves of overall survival (A) and progression-free survival (B) as assessed by independent review committee

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

Role of the funding source

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

Results

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

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

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

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

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

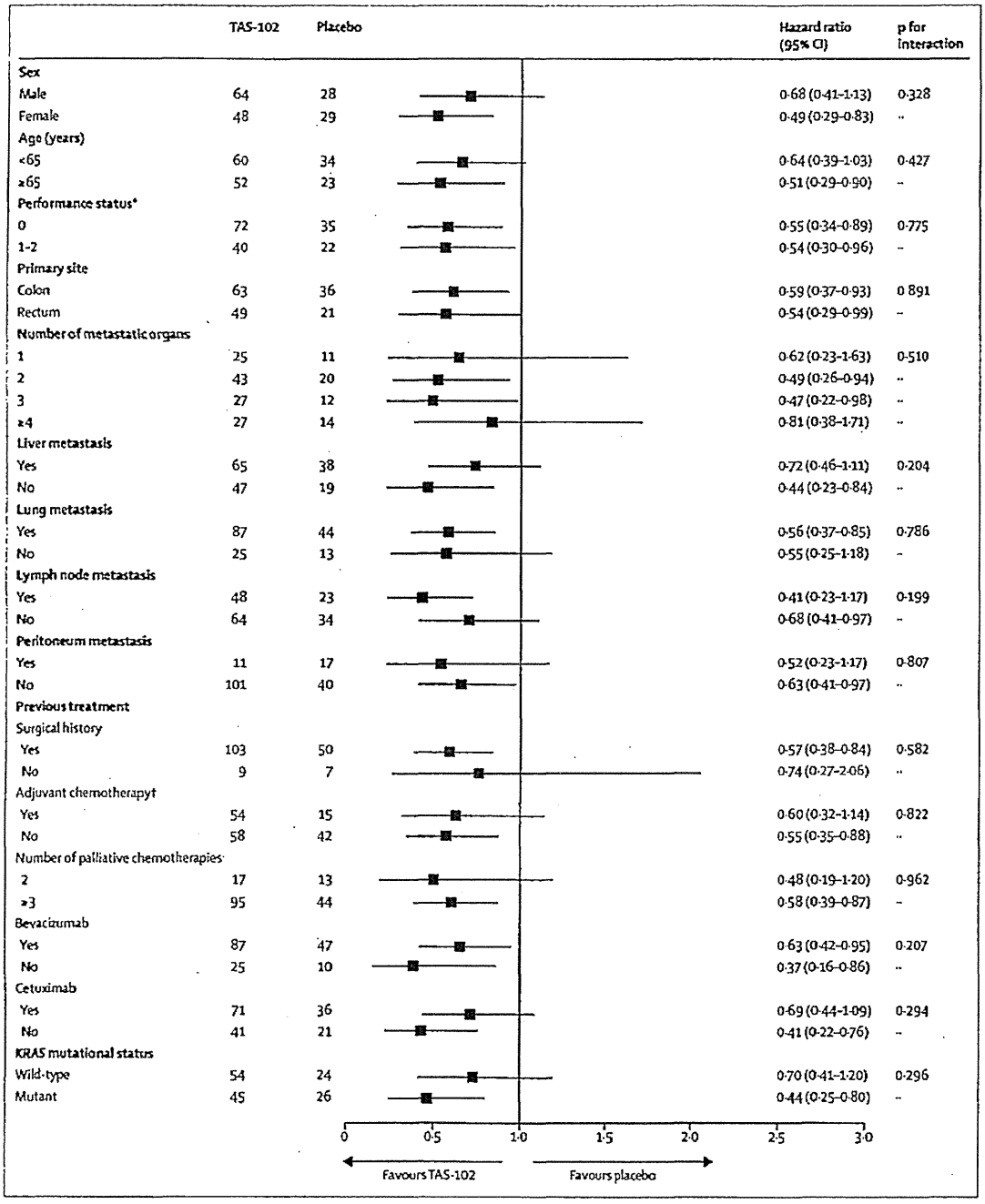

Figure 3: Overall survival in prespecified subgroups

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

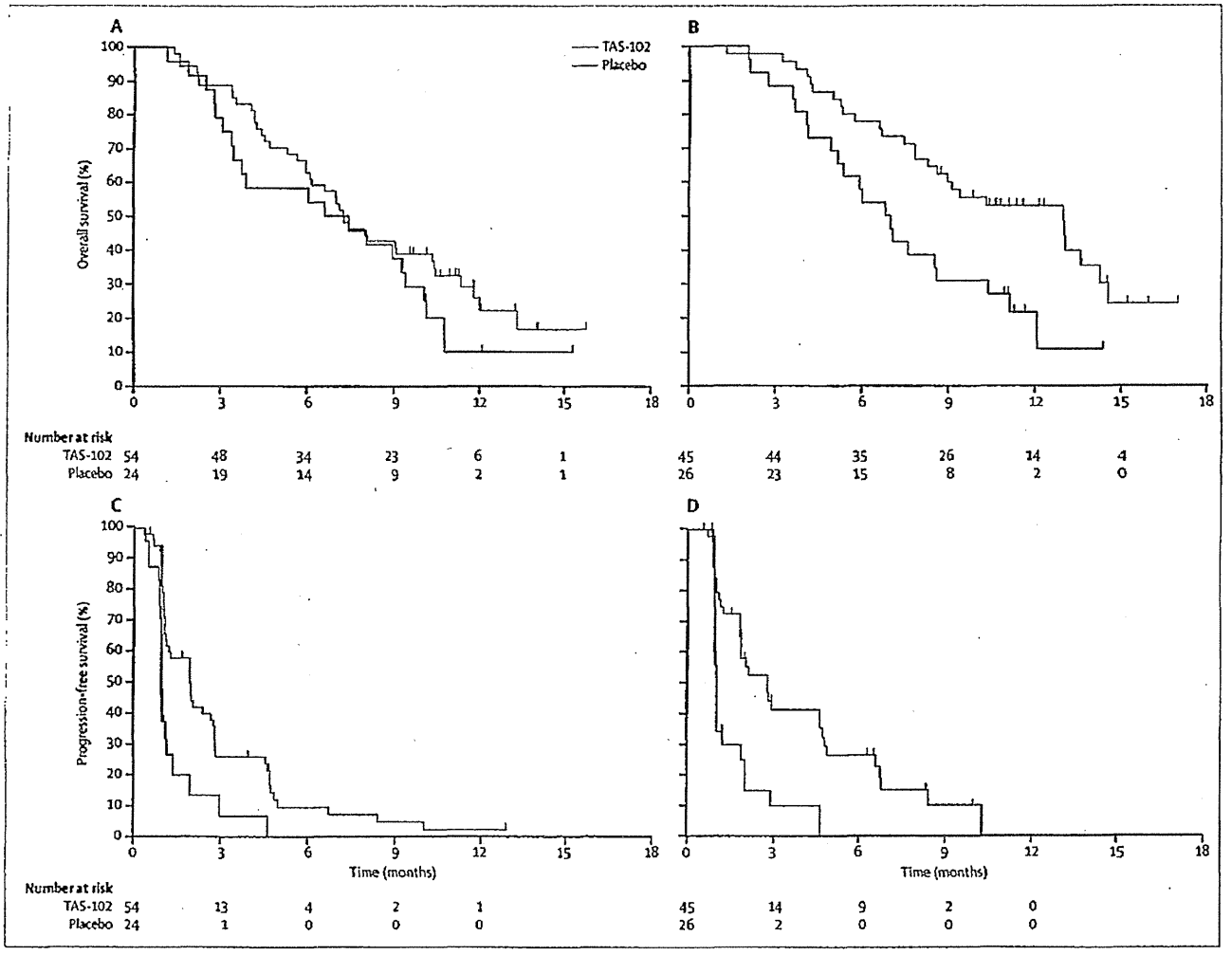

Figure 4: Kaplan-Meier curves of overall survival and progression-free survival in patients with wild-type and mutant *KRAS*
(A) Overall survival of patients with wild-type *KRAS*. (B) Overall survival of patients with mutant *KRAS*. (C) Progression-free survival of patients with wild-type *KRAS*, as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

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

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

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

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

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

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

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

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

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

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

Table 3: Cancer treatment after discontinuation of study treatment

Discussion

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

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

Panel: Research in context

Systematic review

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

Interpretation

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

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

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

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

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

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

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

Contributors

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

Conflicts of interest

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