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Activin A inhibits vascular endothelial cell growth and suppresses tumour angiogenesis in gastric cancer

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BACKGROUND: Activin A is a multi-functional cytokine belonging to the transforming growth factor-β (TGF-β) superfamily; however, the effect of activin A on angiogenesis remains largely unclear. We found that *inhibin β A subunit* (*INHBA*) mRNA is overexpressed in gastric cancer (GC) specimens and investigated the effect of activin A, a homodimer of INHBA, on angiogenesis in GC.

METHODS: Anti-angiogenic effects of activin A via p21 induction were evaluated using human umbilical vein endothelial cells (HUVECs) *in vitro* and a stable *INHBA*-introduced GC cell line *in vivo*.

RESULTS: Compared with TGF-β, activin A potently inhibited the cellular proliferation and tube formation of HUVECs with induction of p21. A promoter assay and a chromatin immunoprecipitation assay revealed that activin A directly regulates p21 transcriptional activity through Smads. Stable p21-knockdown significantly enhanced the cellular proliferation of HUVECs. Notably, stable p21-knockdown exhibited a resistance to activin-mediated growth inhibition in HUVECs, indicating that p21 induction has a key role on activin A-mediated growth inhibition in vascular endothelial cells. Finally, a stable *INHBA*-introduced GC cell line exhibited a decrease in tumour growth and angiogenesis *in vivo*.

CONCLUSION: Our findings highlight the suppressive role of activin A, unlike TGF-β, on tumour growth and angiogenesis in GC.

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Activins are homodimers formed by the assembly of two closely related inhibin β subunits, βA and βB, which generate three isoforms, activin A (βA–βA), activin B (βB–βB), and activin AB (βA–βB) (Chen *et al*, 2002). Activin A is a member of the transforming growth factor (TGF-β) superfamily and shares the Smad intracellular signalling proteins with TGF-β (Shi and Massague, 2003). Activin A binds to activin type II receptors, ActR-II and ActR-IIB, and the ligand/type II receptor complex then recruits, binds, and phosphorylates the type I receptor ActR-IB, also known as activin receptor-like kinase 4 (ALK4), resulting in the propagation of the signal downstream. The activation of ALK4 kinase phosphorylates and activates the cytoplasmic signalling molecules Smad2 or Smad3, and a specific activated Smad complex then translocates and accumulates in the nucleus, where it is involved in the transcriptional regulation of target genes (Shi and Massague, 2003).

Activins have been found to control a wide spectrum of biological effects, such as cellular growth and developmental differentiation in many cell types, although it was originally described as a regulator of follicle-stimulating hormone release from the anterior pituitary (Ying, 1988; Dawid *et al*, 1992; Munz *et al*, 2001; Shav-Tal and Zipori, 2002). Recently, activin A has been reported as an essential growth factor involved in embryonic stem cell renewal and pluripotency (Xiao *et al*, 2006; Jiang *et al*, 2007). In general, activin A causes growth inhibition in epithelial cells ranging from many normal mesenchymal and haematopoietic cells to a variety of cancer cells. In addition, activin A not only inhibits cell proliferation, but it also induces apoptosis in multiple cells and tissues. For example, activin A inhibits the cellular proliferation of breast cancer T47D cells by enhancing the expression of p15 cyclin-dependent kinase (cdk) inhibitors, and the overexpression of activin A in human prostate cancer LNCaP cells inhibited proliferation, induced apoptosis, and decreased the tumourigenicity of these cells (Zhang *et al*, 1997; Burdette *et al*, 2005). In addition, activin A also reportedly exerts a tumour suppressor function in human neuroblastoma cells (Panopoulou *et al*, 2005). In angiogenetic roles, emerging evidence has demonstrated that TGF-β, the other superfamily member, may definitely stimulate angiogenesis during the late stage of cancer (Jakowlew, 2006), while neuroblastoma cells with restored activin A expression exhibit a decreased tumour growth and reduced vascularity (Panopoulou *et al*, 2005). Collectively, whether activin A inhibits angiogenesis in other cancers in addition to its underlying mechanism resulting in the growth inhibition of vascular endothelial cells remains unclear.

We previously performed a microarray analysis of paired gastric cancer (GC) and non-cancerous gastric mucosa samples and

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identified the overexpression of *INHBA* in the GC samples (Yamada *et al*, 2008 and unpublished data). Based on this finding of *INHBA* overexpression and accumulating evidence of the role of TGF-β in angiogenesis, we focused on the role of activin A in angiogenesis in GC in the present study.

MATERIALS AND METHODS

Antibodies and ligands

The following antibodies were used: anti-p21, anti-cdk2, anti-cyclin D, anti-phospho-Rb, anti-Smad2, anti-phospho-Smad2, anti-Smad3, anti-Smad4, and secondary antibodies (Cell Signaling, Beverly, MA, USA); anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and a mouse anti-CD31 monoclonal antibody (BD Biosciences, San Jose, CA, USA). Recombinant human activin A and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). The Alk4/Alk5/Alk7-specific inhibitor SB341542 was purchased from Sigma (St Louis, MO, USA).

Cell lines and cultures

58As1, 44As3, Okajima, KATOIII, TU-KATOIII, MKN1, MKN7, MKN28, and MKN74 cell lines were cultured in RPMI-1640 medium (Sigma) with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL). The HEK293 (Human Embryonic Kidney cell line 293) cell lines were cultured in DMEM medium (Sigma) with 10% heat-inactivated FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Kurabo (Osaka, Japan) and maintained in Humedia (Kurabo) supplemented with 2% FBS, 5 ng ml^{-1} FGF-2, 10 ng ml^{-1} EGF, 10 μg ml^{-1} heparin, 1 μg ml^{-1} hydrocortisone, and antibiotics. The cell lines were maintained in a 5% CO_2-humidified atmosphere at 37°C.

Patients and samples

The methods were described previously (Yamada *et al*, 2008). This study was approved by the institutional review board, and written informed consent was obtained from all the patients.

Plasmid construction, viral production, and stable transfectants

The methods used in this section have been previously described (Kaneda *et al*, 2010). Briefly, the cDNA fragment encoding human full-length INHBA was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with 5′-GGG AAT TCG CCA GGA TGC CCT TGC TTT GGC TGA GAC-3′ and 5′-GCC CTC GAG GGC AAC TCT ATG AGC ACC CAC ACT CC-3′ sense and antisense primers, respectively. Stable transfectants expressing EGFP or INHBA in TU-KATOIII cells were designated as TK3/EGFP and TK3/INHBA. Short hairpin RNA (shRNA)-targeting p21 was constructed using oligonucleotides encoding small interfering RNA directed against p21 and a non-specific target as follows: 5′-CTA AGA GTG CTG GGC ATT TTT-3′ for p21 shRNA and 5′-TGT TCG CAG TAC GGT AAT GTT-3′ for control shRNA. They were then cloned into an RNAi-Ready pSIREN-RetroQZsGreen vector (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. The stable transfectants expressing shRNA-p21 or shRNA-scramble in HUVECs were designated as HUVEC/sh-p21 and HUVEC/sh-Scr, respectively.

Real-time reverse transcription PCR and western blot analysis

The methods used in this section have been previously described (Matsumoto *et al*, 2009). The primers used for real-time RT–PCR were as follows: *INHBA* forward, 5′-CAT TGC TCC CTC TGG CTA TCA T-3′ and reverse, 5′-GCA CAC AGC ACG ATT TGA GGT T-3′; GAPD forward, 5′-GCA CCG TCA AGG CTG AGA AC-3′ and reverse, 5′-ATG GTG GTG AAG ACG CCA GT-3′. The densitometry data from the western blot analysis were quantified automatically using Multigauge Ver. 3.0 (Fujifilm, Tokyo Japan). The densitometry data were normalised by β-actin and is shown above the western blot as a ratio of that in the control sample.

ELISA

A total of 10^6 cells from each of the GC cell lines TK3/EGFP and TK3/INHBA were cultured in normal medium for 12 h and the medium was replaced with a serum-free medium. After 12 h of culture, the medium was collected, centrifuged to remove floating cells, and used for analysis. The concentration of activin A described above was determined using a human activin A DuoSet ELISA Development kit (R&D Systems), according to the manufacture's instructions.

Cell proliferation assay

HUVECs were plated at a density of 3×10^3 cells in 96 wells in growth medium overnight. The cells were then stimulated with the vehicle, activin A, or TGF-β at the indicated concentrations for 72 h (Figures 2A and 5C) or the indicated time (Figure 5B). The experiment was performed using an MTT assay in triplicate. The methods have been previously described (Kaneda *et al*, 2010).

Tube formation assay

A 96-well plate was coated with Matrigel (BD Biosciences) avoiding bubble formation and was incubated at 37°C for 30 min to allow the Matrigel to solidify. HUVECs (2×10^4 cells per well) were pretreated with 10 ng ml^{-1} of activin A or 1 ng ml^{-1} of TGF-β for 48 h and then were plated onto the Matrigel-coated plate. After 16 h of incubation, the HUVECs were photographed using fluorescence microscopy (IX71; Olympus, Tokyo, Japan).

Luciferase reporter assay

The human p21 promoter-containing reporter vector was constructed according to a previously described method (Kaneda *et al*, 2010). Briefly, a 2.4-kb section of the p21 promoter region was subcloned into a luciferase reporter vector, pGL4.14 (Promega, Madison, WI, USA). All the sequences were verified using DNA sequencing. The empty and p21 promoter-containing reporter vectors were designated as pGL4.14-mock and pGL4.14-p21, respectively. The results were normalised to co-transfected β-galactosidase activity and are representative of at least three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology) according to the manufacturer's protocol. The Smad-binding region (SBR) of the p21 promoter was amplified using the following primers: forward 5′-TTC ATT GTG AAG CTC AGT ACC AC-3′ and reverse 5′-TCA AAT GTC CAG CAG AGG ACA G-3′. As a negative control, the GAPDH second intron promoter was amplified using the following primers: (forward) 5′-AAT GAA TGG GCA GCC GTT AG-3′ and (reverse) 5′-AGC TAG CCT CGC TCC ACCTGA C-3′.

Xenograft studies and immunohistochemical staining

Nude mice (*BALB/c nu/nu*; 6-week-old females; CLEA Japan Inc., Tokyo, Japan) were used for the *in vivo* studies and were cared for

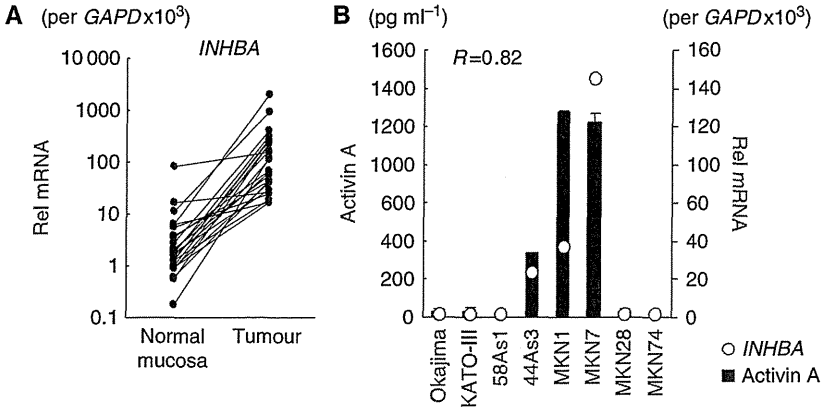

Figure 1 Overexpression of *INHBA* (inhibin β A) mRNA in GC specimens and secretion of its homodimer form, activin A, in GC cell lines. (**A**) The mRNA expressions of *INHBA* in 24 GC and paired non-cancerous gastric mucosa samples were determined using real-time RT–PCR. (**B**) A strong correlation between the expressions of *INHBA* mRNA and activin A protein was observed in GC cell lines, as determined using real-time RT–PCR and an ELISA, respectively. Rel mRNA: normalised mRNA expression levels (*INHBA/GAPD* × 10^3). The correlation coefficient is shown in the figure.

in accordance with the recommendations for the Handling of Laboratory Animals for Biomedical Research, as compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animals Experiments, Kinki University. The ethical procedures followed and met the requirements of the UKCCCR guidelines. To evaluate tumour growth and tumour angiogenesis, the cell suspensions of 1×10^7 TK3/EGFP or TK3/INHBA cells in 0.1 ml PBS were subcutaneously injected into the left or right flanks of nude mice ($n = 5$), respectively. The tumour volume was calculated as the length × width^2 × 0.5. The tumour volume was assessed every week. At the end of the experiment, the mice were killed and the xenografts were resected, fixed in 10% buffered formalin for 10 h, and processed for histological analysis. The immunohistochemical staining methods have been previously described (Kaneda *et al*, 2010).

Statistical analysis

The statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) to calculate the average, s.d., and results of a Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

RESULTS

INHBA mRNA is overexpressed in GC

A real-time RT–PCR analysis revealed that *INHBA* mRNA was overexpressed an average of 37-fold higher in 24 GC specimens than in paired non-cancerous mucosa samples ($P = 0.014$; Figure 1A). The average levels of *INHBA* mRNA expression in the GC and paired non-cancerous mucosa samples were 236 ± 422 and 6.0 ± 16.0 (× 10^3/*GAPD*), respectively. Since INHBA/activins proteins are multi-functional ligands and its superfamily member, TGF-β, is closely involved in angiogenesis, we speculated that the overexpression of *INHBA* may have some role in tumour biology. Thus, we focused on the effect of activin A on tumour angiogenesis.

INHBA overexpression likely leads to the overexpression of its homodimer form, activin A; therefore, we evaluated the correlation between mRNA expression and secreted activin A protein expression in nine GC cell lines. The expressions of both *INHBA* mRNA and activin A protein were increased in 44As3, MKN1, and MKN7 cells but were very low in the other cell lines (Figure 1B). These mRNA and protein expressions were strongly correlated ($R = 0.82$), indicating that *INHBA* overexpression in GC leads to the overexpression of activin A.

Activin A potently inhibits cellular proliferation in vascular endothelial cells

We examined the effect of activin A, compared with TGF-β, on cellular proliferation using HUVECs. TGF-β slightly decreased cellular proliferation at a dose of 1 ng ml^{-1}, while a higher dose of TGF-β (10 ng ml^{-1}) tended to increase proliferation (Figure 2A). In contrast, activin A potently and dose-dependently decreased cellular proliferation at a dose of 10–100 ng ml^{-1} (Figure 2A). In addition, a tube formation assay showed that activin A, but not TGF-β, inhibited tube formation in HUVECs (Figure 2B). These results indicate that activin A and TGF-β have quite different effects on the cellular proliferation of vascular endothelial cells.

Activin A mediates the persistent phosphorylation of Smad2 and p21 induction in HUVECs

Activin A and TGF-β inhibited the cellular growth of HUVECs in different manners; therefore, we examined the downstream signalling under activin A or TGF-β stimulation. Based on the many previous studies and our data for cellular growth inhibition (Figure 2A), we used 10 ng ml^{-1} of activin A and 1 ng ml^{-1} of TGF-β as moderate doses to compare the effects of activin A and TGF-β. Activin A (10 ng ml^{-1}) strongly increased the phosphorylation levels of Smad2, compared with TGF-β (1 ng ml^{-1}), and the effects were cancelled by the Alk4/Alk5/Alk7 inhibitor SB341542 (Figure 3A). Interestingly, activin A persistently increased the phosphorylation of Smad2 from 5 min to over 3 h, while TGF-β increased the phosphorylation during a shorter period of from 15 to 60 min (Figure 3B). In addition, activin A strongly induced the nuclear translocation of phosphorylated-Smad2 and Smad2, while TGF-β induced a milder effect (Figure 3C). These results suggest that activin A activates Smad signalling more potently than TGF-β in HUVECs.

Next, we evaluated the expression levels of cell cycle-related proteins to investigate the difference in the growth inhibitory effects. A western blot analysis revealed that the expression of cyclin D1 and the phosphorylation levels of Rb were decreased by activin A stimulation after 48 h of stimulation, while TGF-β showed a weak effect consistent with the results for growth inhibition (Figure 3D).

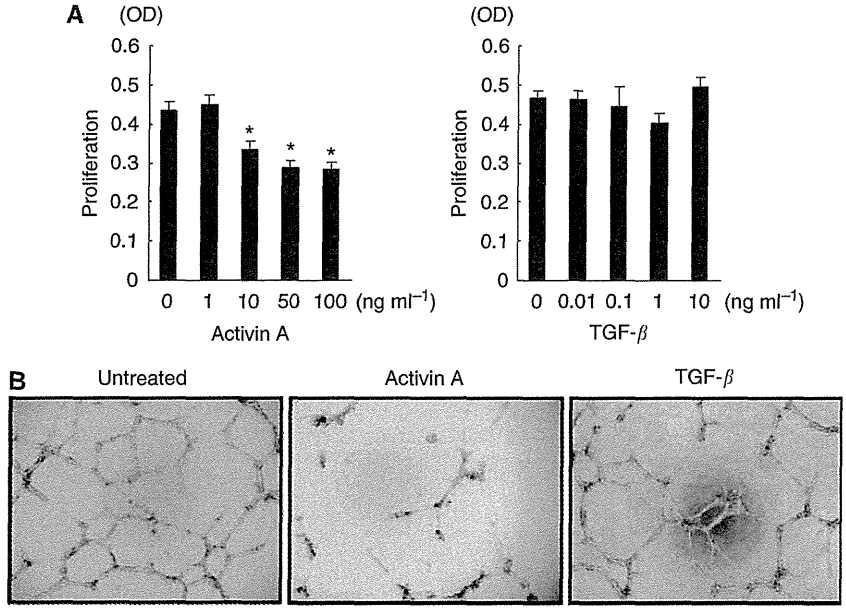

Figure 2 Activin A potently inhibits the proliferation and tube formation of HUVECs. (**A**) HUVECs were cultured in 96-well plates and stimulated with the indicated doses of activin A or TGF-β for 72 h. The cell proliferation was assayed using an MTT assay. Columns: mean of independent triplicate experiments. Bars: s.d. *$P<0.05$. (**B**) Effect of activin A on tube formation in HUVECs. HUVECs were cultured with normal medium (untreated) or activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) containing medium for 48 h and the cells were seeded in 96-well culture plates (1.5×10^4 cells per well) precoated with 80 μl Matrigel and cultured with normal medium (untreated) or activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}). After 16 h of incubation, the wells were photographed using a microscope.

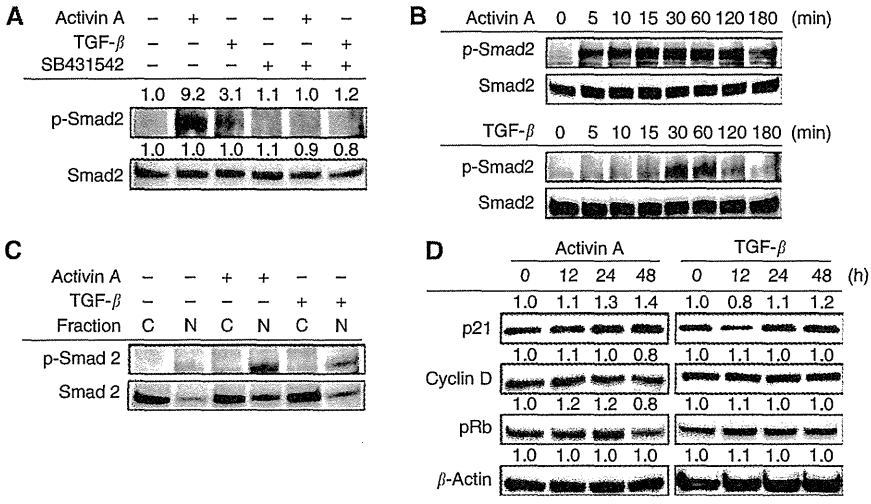

Figure 3 Activin A mediates the persistent phosphorylation of Smad2 and p21 induction in HUVECs. (**A**) HUVECs were treated with or without 2 μM of SB341542 for 30 min, then stimulated with activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) for 1 h. The phosphorylation and expression levels of Smad2 were evaluated using western blot. (**B**) Time-course analysis with activin A or TGF-β-induced Smad2 phosphorylation. HUVECs were stimulated with 10 ng ml^{-1} activin A or 1 ng ml^{-1} TGF-β for the indicated time periods. (**C**) Activin A or TGF-β-induced nuclear translocation of Smad2. HUVECs were stimulated with or without activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) for 1 h. Nuclear and cytosolic protein fractions were then analysed using a western blot analysis. C = cytosolic fraction; N = nuclear fraction. (**D**) Expression changes of cell cycle-related proteins by stimulation with activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) for the indicated time period in HUVECs. A western blot analysis was performed using anti-p21, cyclin D, and phosphorylated Rb antibodies. β-Actin was used as an internal control.

p21$^{CIP1/WAF1}$ is a major cdk inhibitor and the hallmark of the cytostatic role of TGF-β (Weiss, 2003). TGF-β is known to increase p21 expression (Datto *et al*, 1995; Hu *et al*, 1998; Pardali *et al*, 2000), but the regulation of p21 by activin A remains largely unclear, especially in vascular endothelial cells. We found that p21 expression was increased by both activin A and TGF-β stimulation at 12–48 h in HUVECs (Figure 3D); therefore, we speculated that p21 may have a role in activin A-mediated growth inhibition and cell-cycle progression in vascular endothelial cells.

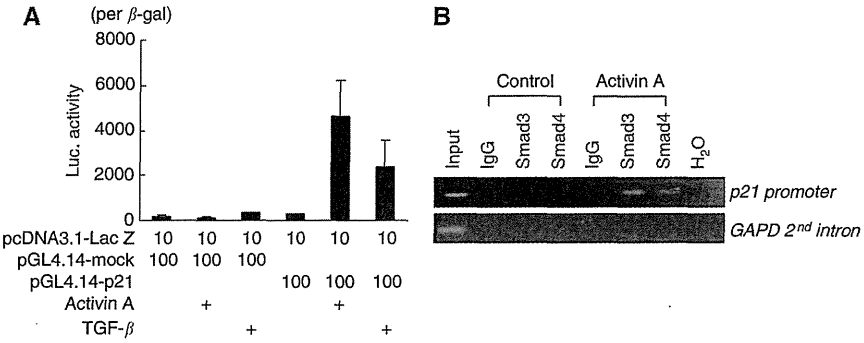

Figure 4 Activin A directly increases *p21* promoter activity. (**A**) *p21* promoter activity was determined using a luciferase assay. HEK293 cells were transiently transfected with luciferase vectors containing empty or p21 promoters (pGL4.14-mock or pGL4.14-p21) and then stimulated with activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) for 24 h. The data were normalised by β-galactosidase activity of co-transfected with the Lac Z vector in at least three independent experiments. Columns: mean of experiments. Bars: s.d. (**B**) ChIP of activin A-induced Smads on the promoter of *p21*. HUVECs were treated with 10 ng ml^{-1} of activin A for 1 h and collected for analysis. The data show the PCR amplification of the *p21* promoter using inputs (1% of chromatin used for ChIP) or ChIPs using smad3 or smad4 antibodies as templates. Primers to the *GAPDH* promoter were used as a negative control. IgG: non-specific IgG as a control.

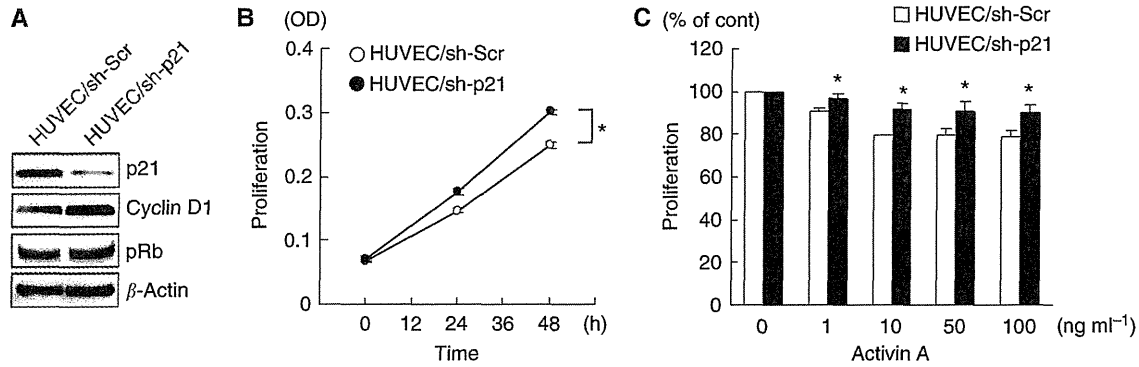

Figure 5 p21 induction has a key role in activin A-mediated growth inhibition in vascular endothelial cells. (**A**) Stable HUVECs transfected with control or p21-knockdown viral shRNA vector (HUVEC/sh-Scr and HUVEC/sh-p21) were evaluated using a western blot analysis using p21, cyclin D, and phospho-Rb antibodies. β-Actin was used as an internal control. (**B**) The cell growth curves of HUVEC/sh-Scr and HUVEC/sh-p21 cells were evaluated using an MTT assay in triplicate experiments. *$P<0.05$. (**C**) Growth inhibition of HUVEC/sh-Scr and HUVEC/sh-p21 cells by activin A stimulation. The cells were stimulated with the indicated doses of activin A for 48 h. Cell growth was determined using an MTT assay. Data were shown as the percentage of activin A-untreated controls and are shown as the mean ± s.d. of at least three independent experiments. *$P<0.05$.

Activin A directly regulates p21 transcriptional activity through Smads

A recent report demonstrated that TGF-β increases the binding of Smad2/3 and Smad4 to a distal portion of the p21 promoter, in which a SBR contains four Smad-binding elements (Seoane *et al*, 2004). To determine whether activin A regulates p21 expression at the transcriptional level, we performed a luciferase reporter assay. Activin A stimulation markedly increased the p21 promoter activity to 16.9-fold, compared with a control (Figure 4A). TGF-β also increased the promoter activity to 8.6-fold, but the effect was about half of that of activin A. A ChIP assay showed that activin A increased the binding of Smad2/3 and Smad4 to the SBR on the p21 promoter (Figure 4B). These results indicate that activin A directly regulates the p21 transcriptional activity through Smads.

p21 induction has a key role on activin A-mediated growth inhibition in vascular endothelial cells

To evaluate the role of p21 induction on activin A-mediated growth inhibition, we examined the cellular growth of stable p21-knockdown (HUVEC/sh-p21) or control (HUVEC/sh-Scr) HUVECs using viral shRNA-targeting p21 or shRNA-scramble vectors (Kaneda *et al*, 2010). Interestingly, the knockdown of p21 significantly increased the cellular proliferation, compared with a control, in HUVECs, indicating that p21 has a growth inhibitory role in HUVECs (Figure 5B). Furthermore, stable p21-knockdown HUVECs were significantly resistant to activin A-mediated growth inhibition, compared with a control, although not completely resistant (Figure 5C). Our new findings demonstrate that p21 induction at least partially has a key role in activin A-mediated growth inhibition in vascular endothelial cells.

Effect of activin A activity on cellular proliferation in GC cell lines

Since whether activin A stimulation inhibits the cellular proliferation of GC cell lines as well as vascular endothelial cells remains unclear, we evaluated this effect. Activin A stimulation strongly upregulated the expression levels of p-smad2 in KATOIII cells, while it slightly upregulated them in MKN7 cells (Figure 6A). TGF-β potently upregulated the expression levels of p-smad2 in both cell lines. Regarding cellular proliferation, activin A inhibited cellular proliferation in KATOIII cells but did not inhibit

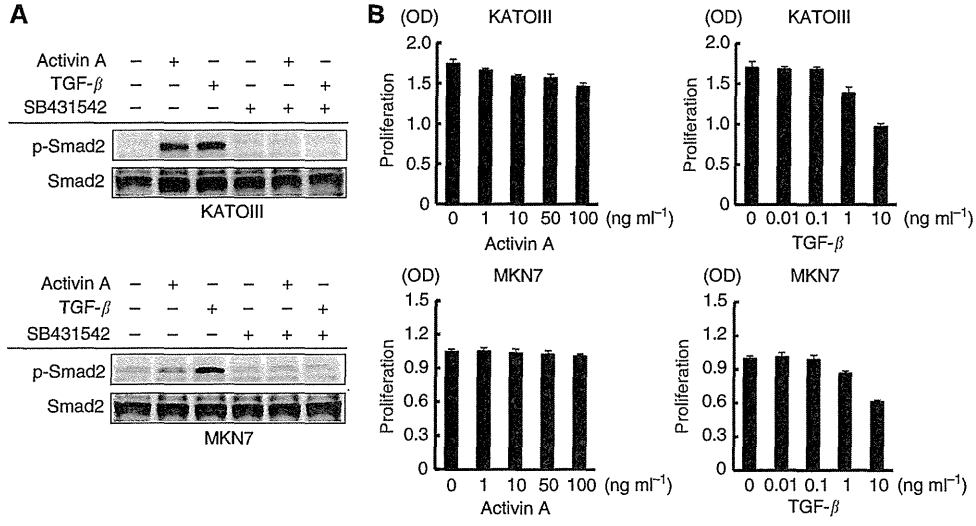

Figure 6 Activin A inhibits the cellular proliferation of KATOIII cells but does not inhibit the proliferation of MKN7 cells. (**A**) GC cell lines KATOIII and MKN7 were treated with or without 2 μM of SB431542 for 30 min, then stimulated with activin A (10 ng ml^{-1}) or TGF-β (1 ng ml^{-1}) for 1 h. The phosphorylation and expression levels of Smad2 were evaluated using a western blot. (**B**) KATOIII and MKN7 cells were cultured in 96-well plates and stimulated with the indicated doses of activin A or TGF-β for 72 h. Cellular proliferation was assayed using an MTT assay. Columns: mean of independent triplicate experiments. Bars: s.d.

proliferation in MKN7 cells, while TGF-β potently inhibited cellular proliferation when administered at a dose of 1–10 ng ml^{-1} (Figure 6B). These results indicate that activin A has a weak or no effect on the cellular proliferation of GC cells, compared with that of vascular endothelial cells.

Overexpression of activin A inhibits tumour growth and angiogenesis *in vivo*

In vitro experiments showed that activin A inhibits the cellular growth of vascular endothelial cells. Next, we evaluated the effects of activin A overexpression in GC using an *in vivo* experiment. The *INHBA* or *EGFP* genes were stably introduced to TU-KATOIII, a low activin A-expressing GC cell line, to produce cell lines that were designated as TK3/INHBA and TK3/EGFP, respectively. The TK3/INHBA cells markedly secreted activin A (12.1 ng ml^{-1}) protein into the culture medium, compared with a control cell line (Figure 7A). The conditioned medium from the TK3/INHBA cells, but not from the TK3/EGFP cells, induced the phosphorylation of Smad2 on HUVECs (Figure 7B). These results indicate that exogenous *INHBA* actually functions as activin A.

TK3/INHBA and TK3/EGFP cells were inoculated into mice, and tumour growth and angiogenesis were evaluated. The tumour volume of the TK3/INHBA cells on day 39 was significantly smaller (104.9 ± 86.2 mm^3) than that of the TK3/EGFP cells (245.1 ± 34.7 mm^3; Figure 7C). These results clearly indicated that the overexpression of activin A in GC significantly inhibited tumour growth. Next, we evaluated angiogenesis in these tumour xenografts using CD31 staining. The microvessel density was significantly reduced in the tumours of TK3/INHBA cells, compared with those of TK3/EGFP cells (Figure 7D and E). Meanwhile, the expressions of p-smad2 and p21 were clearly elevated in cancer cells in TK3/INHBA-inoculated tumours, compared with the expression levels in TK3/EGFP cells. These results indicate that the overexpression of activin A upregulated the expression levels of p-smad2 and p21 in cancer cells *in vivo* (Figure 7D). These results show that the overexpression of activin A suppresses angiogenesis *in vivo* as it does *in vitro*. In conclusion, we found that activin A is overexpressed in GC, inhibiting the cellular proliferation of vascular endothelial cells via direct p21 induction and suppressing tumour growth and angiogenesis *in vivo* (Figure 7F). Our results provide insight into activin A and angiogenesis in GC.

DISCUSSION

TGF-β is almost definitively considered to be an angiogenic factor (Wakefield and Roberts, 2002), but the role of activin A remains largely unclear. Our results showed that activin A and TGF-β have different effects on the proliferation of vascular endothelial cells *in vitro*. In line with this difference, previous knockout mice studies have demonstrated that embryos lacking any one of the TGF-β signalling components die during mid-gestation as a result of impaired vascular development, exhibiting hyper-dilated, leaky vessels, and highlighting the importance of TGF-β signalling in the vascular system (Goumans *et al*, 2009). Meanwhile, activin-β A-deficient mice did not develop any defects in angiogenesis (Matzuk *et al*, 1995). Although we did not directly compare the effect on angiogenesis between activin A and TGF-β *in vivo*, our findings support the inhibitory role of activin A on tumour angiogenesis, unlike TGF-β.

The relation between activin A expression and clinical outcome remains controversial. A high expression of *INHBA* was associated with a favourable prognostic outcome, exerting a tumour suppressor and anti-angiogenic role in neuroblastoma patients (Schramm *et al*, 2005). Meanwhile, its expression was correlated with tumour aggressiveness and a poor clinical outcome in patients with oesophageal carcinoma (Yoshinaga *et al*, 2004). This discrepancy may be explained by the dual role of TGF-β signalling as a tumour suppressor and pro-oncogenic factor. The TGF-β signalling pathway has a complicated role in cancer cells, mediating the ability of the cells to participate negatively or positively in growth inhibition, proliferation, replication, invasion, metastasis, apoptosis, immune surveillance, and angiogenesis (Jakowlew, 2006). For example, a defect of function in the TGF-β signalling component leads to carcinogenesis by acting as a definite tumour suppressor during the early phase, but it may exhibit an oncogenic function during later clinical stages

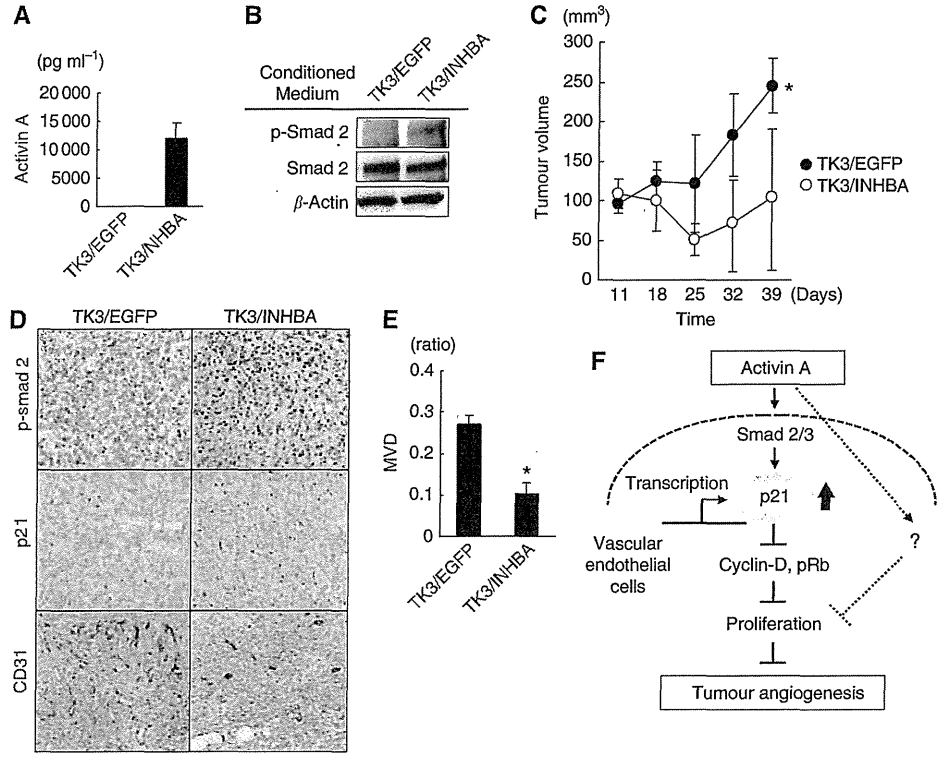

Figure 7 Overexpression of *INHBA* potently inhibited tumour growth and angiogenesis in GC *in vivo*. (**A**) TU-KATOIII GC cells were stably transfected with an *INHBA* (TK3/INHBA cells) or control vector (TK3/EGFP cells). Activin A secretion in the conditioned medium was analysed using an ELISA. (**B**) Activity of exogenous activin A on phosphorylation of smad2. Conditioned media from TK3/INHBA or /EGFP cells were exposed to HUVECs for 1 h and the phosphorylation of Smad2 in the HUVECs was assessed using a western blot analysis. β-Actin was used as an internal control. (**C**) Effect of overexpression of *INHBA* on tumour growth. TK3/EGFP and TK3/INHBA cells (1×10^7 cells) were subcutaneously inoculated into mice and evaluated for tumour growth *in vivo*. The data indicate the mean ± s.d. $^*P<0.05$. (**D** and **E**) CD31 staining for tumour specimens. Microvessel density (MVD) was evaluated using CD31-positive endothelial cells in tumour specimens using a computer-assisted image analysis. $^*P<0.05$. (**F**) Diagram of the proposed mechanism of activin A on vascular endothelial cells and in angiogenesis.

(Jakowlew, 2006). This context dependency of TGF-β signalling and differences in the origins of the cancer tissue may lead to such discrepancies. We plan to clarify the clinical meaning of activin A expression in GC in a future study.

Our findings and other reported data indicate that activin A inhibits the cellular proliferation of vascular endothelial cells (Panopoulou *et al*, 2005; Schramm *et al*, 2005): however, the underlying mechanism in endothelial cells has not been fully elucidated. p21 is a member of the cip/kip family of cyclin kinase inhibitors, and initial reports have demonstrated that p21 functions as a G1 cyclin kinase inhibitor and a downstream molecule of p53 (el-Deiry *et al*, 1993). p21 possesses a variety of cellular functions, including the negative modulation of cell-cycle progression, cellular differentiation, and the regulation of p53-dependent anti-apoptosis (reviewed in Garner and Raj, 2008). We demonstrated that activin A directly regulates p21 expression at the transcriptional level, and the knockdown of p21 increased the cellular proliferation and mediated the resistance to activin A-mediated growth inhibition. Our findings have thus shed light on p21 as an activin A-mediated growth inhibitor in vascular endothelial cells.

In conclusion, our findings indicate that activin A inhibits vascular endothelial cell growth via the direct induction of p21 and highlight the suppressive role of activin A in tumour growth and angiogenesis in GC.

ACKNOWLEDGEMENTS

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Genotype-directed, dose-finding study of irinotecan in cancer patients with *UGT1A1*28* and/or *UGT1A1*6* polymorphisms

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Irinotecan-induced severe neutropenia is associated with homozygosity for the *UGT1A1*28* or *UGT1A1*6* alleles. In this study, we determined the maximum-tolerated dose (MTD) of irinotecan in patients with *UGT1A1* polymorphisms. Patients who had received chemotherapy other than irinotecan for metastatic gastrointestinal cancer were enrolled. Patients were divided into three groups according to *UGT1A1* genotypes: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). Irinotecan was given every 2 weeks for two cycles. The wild-type group received a fixed dose of irinotecan (150 mg/m^2) to serve as a reference. The MTD was guided from 75 to 150 mg/m^2 by the continual reassessment method in the heterozygous and homozygous groups. Dose-limiting toxicity (DLT) and pharmacokinetics were evaluated during cycle 1. Of 82 patients enrolled, DLT was assessable in 79 patients (wild-type, 40; heterozygous, 20; and homozygous, 19). Dose-limiting toxicity occurred in one patient in the wild-type group, none in the heterozygous group, and six patients (grade 4 neutropenia) in the homozygous group. In the homozygous group, the MTD was 150 mg/m^2 and the probability of DLT was 37.4%. The second cycle was delayed because of neutropenia in 56.3% of the patients given the MTD. The $AUC_{0-24\ h}$ of SN-38 was significantly greater ($P < 0.001$) and more widely distributed in the homozygous group. Patients homozygous for the *UGT1A1*28* or *UGT1A1*6* allele can receive irinotecan in a starting dose of 150 mg/m^2, but many required dose reductions or delayed treatment in subsequent cycles. UMIN Clinical Trial Registration number: UMIN000000618. (*Cancer Sci* 2011; 102: 1868–1873)

Irinotecan, a semisynthetic camptothecin derivative with topoisomerase I-inhibiting activity,[1–3] entered clinical studies in the early 1990s and has been shown to be an effective anticancer drug against several malignancies. Irinotecan is a prodrug that is converted to its active metabolite, SN-38, by carboxylesterase. SN-38 is converted to an inactive metabolite, SN-38G, by UGTs. Irinotecan-associated adverse events, such as myelosuppression and diarrhea, are significantly correlated with the AUC of irinotecan, SN-38, and SN-38G.[1–3]

One of the isoforms of UGT, UGT1A1, is the main enzyme involved in SN-38 glucuronidation. Several studies have reported correlations between *UGT1A1* polymorphisms and irinotecan-associated adverse events,[4–6] and the efficiency of SN-38 glucuronidation is decreased in individuals homozygous for the *UGT1A1*28* allele.[7] In 2005, the US Food and Drug Administration revised the package insert for irinotecan (Camptosar, Yakult Honsha Co. Ltd., Tokyo, Japan), recommending that a reduced dose should be used in these individuals.[8] A subsequent meta-analysis suggested that genetic testing might not be necessary unless the dose of irinotecan is ≤150 mg/m^2; however, there was insufficient evidence for doses of approximately 150 mg/m^2,[9] and the recommendations for dose adjustments remain unclear.

Although *UGT1A1*28* is considered an important predictor of irinotecan-related toxicity, ethnic differences have been reported.[10,11] The allele frequency of *UGT1A1*28* is lower in Asians than in Caucasians, and grade 3–4 hematologic toxicity is associated with *UGT1A1*6* polymorphisms in Asians.[10] In addition, a recent Japanese study revealed that severe adverse events were associated with double heterozygosity (*UGT1A1*28/*6*).[11] Adverse events are related to the pharmacokinetic properties of the drug, and the AUC ratio of SN-38G to SN-38 ($AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$) was significantly reduced in *UGT1A1*28/*6* patients.[12,13] Thus, *UGT1A1*6* appears to be another important predictor of irinotecan-induced adverse events.

In this context, this study was designed to determine the MTD of irinotecan in patients with gastrointestinal cancer whose *UGT1A1* genotypes were *UGT1A1*28/*28, *6/*6*, or **28/*6*. The CRM[14,15] was used to determine dose escalation and reductions. Compared with the traditional phase I design, the CRM can incorporate the actual drug doses into the analytical model and evaluate the frequency of adverse events more accurately, particularly in small groups of patients, such as those who are homozygous for *UGT1A1*28* or *UGT1A1*6*. Thus, the CRM was considered appropriate for our study objectives. We also investigated the pharmacokinetics and toxicity of irinotecan in patients with the *UGT1A1*28* and *UGT1A1*6* alleles.

Patients and Methods

Patients. Patients meeting the following criteria were eligible for the study: histologically confirmed and inoperable gastrointestinal cancer; ≥20 years old; Eastern Cooperative Oncology

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Group performance status of 0–1; no prior treatment with irinotecan; a wash-out period of 21 days after previous chemotherapy; adequate bone marrow function (leukocyte count 3000–12 000/mm^3; hemoglobin concentration ≥8.5 g/dL; platelet count ≥ 100 000/mm^3); serum creatinine ≤ 1.5 mg/dL; total bilirubin ≤2.0 mg/dL; and aspartate aminotransferase and alanine aminotransferase ≤100 IU/L. Written informed consent was obtained from all participants. Patients were excluded if they had severe ascites or had received chest or abdominal radiotherapy. The study protocol was approved by the Institutional Review Board at each institution. An independent Data and Safety Monitoring Committee evaluated safety throughout the study.

***UGT1A1* genotyping assay.** Genomic DNA was extracted from peripheral blood using a QIAamp blood kit (Qiagen, Hilden, Germany). An Invader UGT1A1 Molecular Assay kit (Third Wave Technologies, Madison, WI, USA) was used to genotype the *UGT1A1*28* and *UGT1A1*6* polymorphisms.

Classification of *UGT1A1* polymorphisms. We classified the *UGT1A1* polymorphisms into three groups: wild-type *(*1/*1)*, heterozygous *(*28/*1, *6/*1)*, and homozygous *(*28/*28, *6/*6, *28/*6)*. The double heterozygous state *(*28/*6)* was included within the homozygous group, taking into account the findings of previous studies.[11,13] A recent study found no evidence of alleles **28* and **6* existing on the same chromosome; patients harboring **28* and **6* on the same chromosome either do not exist or are extremely rare.[16] Therefore, concurrent **28* and **6* homozygosity was considered irrelevant.

Treatment schedule. Irinotecan was given i.v. over the course of 90 min of every 14-day cycle, for only two cycles. The wild-type group received a fixed dose of 150 mg/m^2 as a reference. This is a borderline dose between the low and medium dose levels, as proposed by Hoskins *et al.*,[9] and is the upper limit of the approved dose of irinotecan in Japan. The starting dose was 100 mg/m^2 in the heterozygous group and 75 mg/m^2 in the homozygous group. The dose was escalated in increments of 25 mg/m^2 up to 150 mg/m^2, as described below. The study treatment comprised two cycles, unless unacceptable toxicity developed during the first cycle, or the patient withdrew consent.

Safety was evaluated in the first and second cycles, and DLT was only assessed in the first cycle. Objective clinical evaluations, blood counts, and hepatic and renal function tests were carried out on days 1 and 8 of each cycle. Dose-limiting toxicity was defined as grade 4 neutropenia, grade 4 thrombocytopenia, febrile neutropenia (neutrophil count < 1000/mm^3 and fever ≥ 38.5°C), or grade 3 diarrhea. If DLT occurred in the first cycle, the dose was reduced by one dose level in the second cycle. Toxicity was evaluated according to the Common Terminology Criteria for Adverse Events version 3.0.

Pharmacokinetic assay. Venous blood for pharmacokinetic analysis was collected in sodium-heparinized, evacuated tubes on days 1 and 2 of the first cycle, before infusion of irinotecan, at the end of infusion, and at 1, 2, 4, 7, and 24 h after infusion. The plasma concentrations of intact irinotecan, SN-38, and SN-38G were determined by HPLC, as previously described.[17] The $AUC_{0-24\ h}$ was calculated using WinNonlin software version 5.0.1 (Pharsight, Mountain View, CA, USA).

Dose escalation/reductions: design and statistical considerations. Eligible patients underwent genotyping and were assigned to the wild-type group, heterozygous group, or homozygous group. They were then registered at the data center. All patients who received at least one dose of irinotecan without major protocol violations were included in the safety and pharmacokinetic analyses.

In the wild-type group, we estimated the probability of DLT occurring at a dose of 150 mg/m^2. A sample size of 40 was planned, assuming that the probability of DLT would be 10% (maximum 20%) with 95% confidence limits.

In the heterozygous and homozygous groups, dose escalation and reductions were carried out according to the CRM. We used a logistic regression model to determine the relationship between dose and toxicity. The model was updated based on the patients' responses, using a Bayesian approach. After enrolling the first patient, the doses given to subsequent patients were determined by the CRM. Each subsequent patient was treated at the dose level where the probability of DLT was closest to 30%. Dose-limiting toxicity was assessed in a maximum of three patients at the same time and dose. The dose was increased or decreased by 25 mg/m^2. The MTD was defined as the dose level at which nearest to 30% of patients were expected to have DLT. The recommended dose was determined based on the results obtained during the first two cycles. Simulation studies indicated that 10–20 patients were required to estimate the MTD. The decision to continue or stop the study was made after safety evaluation of the first 10 patients.

Because of a treatment-related death, patient enrolment was temporarily halted and the protocol was revised. The dose of irinotecan in the homozygous group was reduced by two levels in the second cycle if the patient had grade 3–4 neutropenia in the first cycle.

In accordance with the advice of the Data and Safety Monitoring Committee, medical experts and biostatisticians, all eligible patients in the homozygous group were included in the analysis of MTD, DLT, and the toxicity data, irrespective of protocol amendments.

The Cochran–Armitage trend test was used to analyze trends in grade 3–4 adverse events across the different genotypes. Fisher's exact test was used to compare the frequency of toxicity among the wild-type, heterozygous, and homozygous groups. Pearson's correlation coefficient was used to assess the relationships between laboratory test data and pharmacokinetic parameters during the first cycle. The association between pharmacokinetic parameters and genotype was evaluated using the Cochran–Armitage trend test. Levene's test was used to assess the homogeneity of variances in SN-38 among the genotypes. All analyses were carried out using SAS software version 8.2 (SAS Institute, Cary, NC, USA).

Results

Patient characteristics. Between November 2006 and October 2008, 82 patients were enrolled at 12 institutions and assigned to the wild-type (n = 41), heterozygous (n = 20; **28/*1* [n = 8], **6/*1* [n = 12]), or homozygous (n = 21; **28/*28* [n = 3], **6/*6* [n = 12], **28/*6* [n = 6]) groups. Toxicity and pharmacokinetic parameters were evaluated in 81 patients, excluding one patient in the homozygous group who withdrew consent before treatment. After the first dose, two patients were deemed ineligible. Therefore, 79 patients were eligible for DLT analysis (Table 1).

Dose escalation and identification of MTD. The first four patients in each of the heterozygous and homozygous groups showed no DLT, so the dose was increased to 150 mg/m^2 according to the CRM (Fig. 1). At 150 mg/m^2, DLT occurred in one patient in the wild-type group (grade 3 anorexia and fatigue) and in six patients in the homozygous group (**28/*28* [n = 1], **6/*6* [n = 4], **28/*6* [n = 1]) (grade 4 neutropenia, 6; grade 3 diarrhea, 1), but in no patients in the heterozygous group. Based on these data, the probability of DLT at 150 mg/m^2 was 2.5% in the wild-type group (1/40 patients; 95% CI, 0.1–13.2), 5.9% in the heterozygous group (0/16 patients; 80% CI based on the CRM, 2.2–11.2%), and 37.4% in the homozygous group (6/15 patients; 80% CI based on the CRM, 22.8–52.7%). In the homozygous group, the initial dose of irinotecan (150 mg/m^2) was determined to be the MTD, whereas the MTD in the heterozygous group was estimated to be >150 mg/m^2.

Table 1. Disposition and baseline characteristics of patients with gastrointestinal cancer who participated in this study (n = 82)

Characteristics	Wild-type group		Heterozygous group		Homozygous group	
	n	%	*n*	%	*n*	%
Patients enrolled	41	—	20	—	21	—
Consent withdrawn	0	—	0	—	1	—
Patients receiving study drug	41	—	20	—	20	—
Eligible	40	—	20	—	19	—
Not eligible	1	—	0	—	1	—
DLT analysis	40	—	20	—	19	—
Toxicity analysis	41	—	20	—	20	—
Pharmacokinetic analysis	41	—	20	—	20	—
Sex						
Male	22	53.7	10	50.0	13	65.0
Female	19	46.3	10	50.0	7	35.0
Age (years)						
Median	62	—	63	—	66	—
Range	21–88	—	47–80	—	38–78	—
ECOG performance status						
0	27	65.9	12	60.0	11	55.0
1	14	34.1	8	40.0	9	45.0
Adenocarcinoma histology						
Stomach	19	46.3	9	45.0	4	20.0
Colorectal	22	53.7	11	55.0	16	80.0
Total bilirubin (mg/dL)						
Median	0.5	—	0.6	—	0.8	—
Range	0.2–1.3	—	0.2–1.0	—	0.3–2.0	—
Direct bilirubin (mg/dL)						
Median	0.2	—	0.2	—	0.2	—
Range	0.0–0.5	—	0.1–0.3	—	0.1–0.5	—

Patients were divided into groups according to *UGT1A1* genotype: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). DLT, dose-limiting toxicity; ECOG, Eastern Cooperative Oncology Group; —, not applicable.

Toxicity. The major adverse events in patients treated with 150 mg/m^2 irinotecan are listed in Table 2. The most frequently observed grade 3–4 toxicities were leukopenia and neutropenia. During the first cycle, hematologic toxicity was significantly associated with genotype ($P < 0.001$). Grade 3–4 neutropenia occurred in 9.8% of patients in the wild-type group, 18.8% of patients in the heterozygous group, and 62.5% of patients in the homozygous group. The frequency of severe neutropenia was significantly higher in the homozygous group than in the wild-type and heterozygous groups ($P < 0.001$). A similar trend was observed during the first two cycles (wild-type group, 22.0%; heterozygous group, 25.0%; homozygous group, 81.3%). Unlike hematologic toxicity, non-hematologic toxicity was not associated with genotype and was generally mild to moderate in severity (Table 2).

On *UGT1A1* diplotype analysis, grade 3–4 neutropenia and leukopenia occurred frequently in patients in the homozygous group (Table 3). Grade 3–4 diarrhea occurred in 1/9 patients (11.1%) with **6/*6*. The second cycle was delayed in 5/41 patients (12.2%) in the wild-type group, 4/16 patients (25.0%) in the heterozygous group, and 9/16 patients (56.3%) in the homozygous group. The reasons for delaying treatment were neutropenia in seven patients, infection and stomatitis in one, and diarrhea in one. In the homozygous group treated with 150 mg/m^2 irinotecan, the dose for the second cycle was reduced by two dose levels (or to 100 mg/m^2) in three patients and by one dose level (to 125 mg/m^2) in one patient. One patient who received a reduced dose of 100 mg/m^2 irinotecan developed grade 4 neutropenia again in the second cycle. Four of 16 patients (25.0%) in the homozygous group completed two cycles of therapy without needing to delay treatment or reduce the dose.

There was one treatment-related death in the homozygous group, which was caused by septic shock with grade 4 neutropenia after the second cycle of irinotecan at a dose of 150 mg/m^2. This patient, who was homozygous for *UGT1A1*28*, had no DLT in the first cycle, and the second cycle was delayed because of prolonged neutropenia.

Relationships between *UGT1A1* polymorphisms and pharmacokinetic profile and toxicity of irinotecan. The $AUC_{0-24\ h}$ of SN-38 was significantly higher in the homozygous group than in the wild-type or heterozygous groups ($P < 0.001$) and interpatient variability was also higher in the former group (Table 4). The $AUC_{0-24\ h}$ of SN-38G was significantly higher in the wild-type group than in the heterozygous or homozygous groups ($P = 0.001$). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio was highest in the wild-type group, intermediate in the heterozygous group, and lowest in the homozygous group ($P < 0.001$).

The $AUC_{0-24\ h}$ of SN-38 was slightly higher in patients with the **28/*28* or **6/*6* genotypes than in patients with the **28/*6* genotype (Table 4). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio was slightly lower in patients with the **6/*6* genotype than in those with the **28/*28* or **28/*6* genotypes.

The relationship between adverse events and pharmacokinetic parameters was also analyzed. The $AUC_{0-24\ h}$ of SN-38G was not correlated with hematologic toxicity. In contrast, the $AUC_{0-24\ h}$ of SN-38 was correlated with the frequency of grade 3–4 leukopenia and neutropenia ($r = 0.49$, $P < 0.001$). The $AUC_{SN\text{-}38G}/AUC_{SN\text{-}38}$ ratio also correlated with the frequencies of grade 3–4 leukopenia ($r = 0.25$, $P = 0.023$) and neutropenia ($r = 0.308$, $P = 0.005$).

Discussion

Irinotecan is generally given at a dose of 150 mg/m^2 every 2 weeks in Japanese patients with gastrointestinal cancer. This dose was determined based on the results of clinical trials in the 1990s[18,19] and is the upper limit of the approved dose in Japan.

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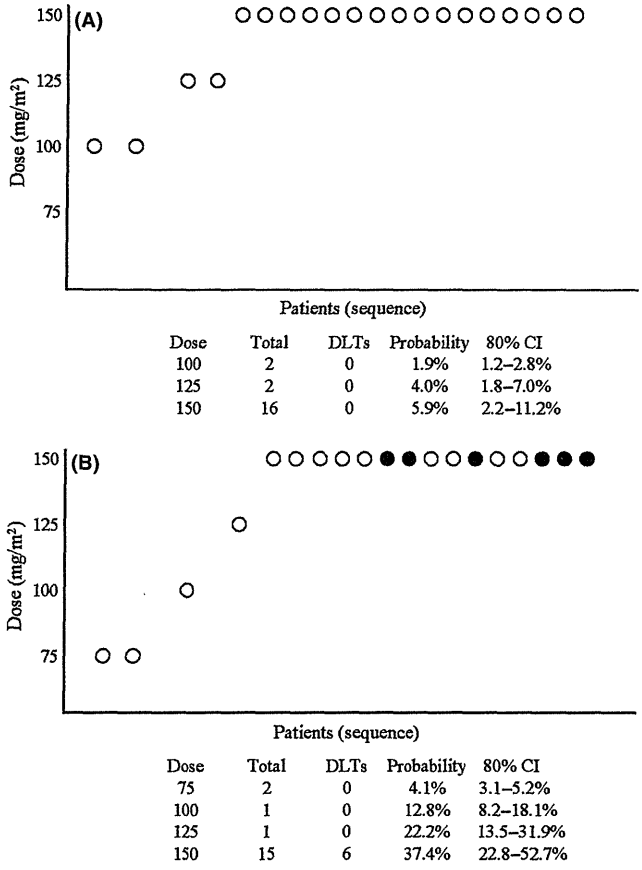

Fig. 1. Probability of dose-limiting toxicity (DLT) of irinotecan in patients with gastrointestinal cancer, determined using the continual reassessment method. Patients were grouped as heterozygous (A) or homozygous (B) according to their *UGT1A1* genotype. (○), Patients registered; (●), occurrence of DLT. CI, confidence interval.

The appropriate dosages of cytotoxic agents have been based on the concept of minimizing the risk of no response within the acceptable toxicity limits. However, genetic information has recently been obtained on the metabolism of CPT-11 and its related toxicities. Hoskins *et al.*[20] showed that the risk of severe hematologic toxicity is higher among patients with the *UGT1A1*28/*28* genotype than among those with the *UGT1A1*28/*1* or *UGT1A1*1/*1* genotypes at medium doses (150–250 mg/m^2) and at higher doses (>250–350 mg/m^2), but not at lower doses (100–125 mg/m^2). However, the results of a recently reported meta-analysis[21] showed that the *UGT1A1*28/*28* genotype was associated with an increased risk of neutropenia not only at medium or high doses of irinotecan, but also at low doses (relative risk [RR], 2.43; 80–145 mg/m^2). To verify these previous findings in a prospective manner, we needed to reset the MTD according to these genetic factors, so we used irinotecan at doses of 75–150 mg/m^2.

Our genotype-directed dose-finding study using the CRM showed that the principal DLT was neutropenia and the MTD of irinotecan was 150 mg/m^2 in Japanese patients carrying the *UGT1A1* variant alleles. In the heterozygous group, the MTD was estimated to be >150 mg/m^2. Our results also showed that the hematologic toxicity of irinotecan at 150 mg/m^2 was significantly more severe, and the $AUC_{0-24\ h}$ of SN-38 was significantly higher and more widely distributed in patients with two variant alleles than in those with one or no variant alleles. These findings are consistent with the results of previous studies.[12,13,22,23] As described above, *UGT1A1* genetic polymorphism is a factor that clearly affects pharmacokinetics, and individual variation in pharmacokinetics was greater in the homozygous group. The recommended dose could not be defined, because it was considered inappropriate to apply the dose obtained by increasing the number of cases to the general population. Moreover, the present study revealed that the $AUC_{0-24\ h}$ of SN-38 in the heterozygous group was similar to that in the wild-type group among patients treated with irinotecan at 150 mg/m^2. The *UGT1A1* polymorphisms were not related to diarrhea in our study, or in previous studies.[10,22,24] Thus, further studies are needed to determine the predictive factors for diarrhea.

When the *UGT1A1*28* and *UGT1A1*6* alleles were evaluated separately, the incidence of neutropenia and the $AUC_{0-24\ h}$ of SN-38 were similar in patients with a homozygous genotype. Although the frequency of patients with *UGT1A1*28/*28* has been reported to be small in Asia,[10–12,22] grade 3–4 neutropenia developed in all of our patients with the *UGT1A1*28/*28* genotype, and one patient died because of treatment-related sepsis. The $AUC_{0-24\ h}$ of SN-38 was also very high in these patients. Therefore, the *UGT1A1*28/*28* genotype is an important determinant of safety, even in Asian patients.

Several studies have addressed the issues of *UGT1A1* polymorphisms and the starting dose of irinotecan,[11,13,22] but most of these studies had limitations, such as small numbers of patients with the rare variant alleles, a retrospective design, or

Table 2. Common adverse events (grades 3–4) associated with 150 mg/m^2 irinotecan in patients with gastrointestinal cancer

Adverse events	First cycle								First and second cycles					
	Wild-type group (n = 41)		Heterozygous group (n = 16)		Homozygous group (n = 16)		P*	P**	Wild-type group (n = 41)		Heterozygous group (n = 16)		Homozygous group (n = 16)	
	G3/G4	%	G3/G4	%	G3/G4	%			G3/G4	%	G3/G4	%	G3/G4	%
Hematologic toxic effects														
Leukopenia	1/0	2.4	0/0	0.0	8/1	56.3	<0.001	<0.001	1/0	2.4	2/0	12.5	10/2	75.0
Neutropenia	4/0	9.8	3/0	18.8	4/6	62.5	<0.001	<0.001	9/0	22.0	3/1	25.0	6/7	81.3
Non-hematologic toxic effects														
Diarrhea	0/0	0.0	0/0	0.0	1/0	6.3	NS	NS	0/0	0.0	0/0	0.0	1/0	6.3
Fatigue	1/0	2.4	0/0	0.0	0/0	0.0	NS	NS	1/0	2.4	0/0	0.0	0/0	0.0
Anorexia	1/0	2.4	0/0	0.0	0/0	0.0	NS	NS	1/0	2.4	1/0	6.3	0/0	0.0

Patients were divided into groups according to *UGT1A1* genotype: wild-type (*1/*1); heterozygous (*28/*1, *6/*1); or homozygous (*28/*28, *6/*6, *28/*6). *Cochrane–Armitage trend test; **Fisher's exact test, wild-type/heterozygous group versus homozygous group. G, grade; NS, not significant.

Table 3. Association between *UGT1A1* genotype in patients with gastrointestinal cancer and the most common grade 3–4 adverse events during the first treatment cycle with 150 mg/m^2 irinotecan

Adverse events	*6/*1 (*n* = 9)		*28/*1 (*n* = 7)		*6/*6 (*n* = 9)		*28/*28 (*n* = 3)		*28/*6 (*n* = 4)	
	G3/G4	%	G3/G4	%	G3/G4	%	G3/G4	%	G3/G4	%
Hematologic toxic effects										
Leukopenia	0/0	0.0	0/0	0.0	5/1	66.7	2/0	66.7	1/0	25.0
Neutropenia	1/0	11.1	2/0	28.6	2/4	66.7	2/1	100	0/1	25.0
Non-hematologic toxic effects										
Diarrhea	0/0	0.0	0/0	0.0	1/0	11.1	0/0	0.0	0/0	0.0
Fatigue	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0
Anorexia	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0	0/0	0.0

G, grade.

Table 4. Relationship between *UGT1A1* genotype and pharmacokinetic parameters for 150 mg/m^2 irinotecan in patients with gastrointestinal cancer

Pharmacokinetic parameter	SN-38$_{0-24\ h}$ (ng × h/mL)	SN-38G$_{0-24\ h}$ (ng × h/mL)	AUC ratio
Wild-type group (*n* = 41)	264 ± 114	1266.8 ± 667.5	5.03 ± 2.25
Heterozygous group (*n* = 16)	279.6 ± 152.0	820.7 ± 378.7	3.25 ± 1.32
*UGT1A1*6/*1* (*n* = 9)	250.2 ± 70.4	723.1 ± 252.8	3.16 ± 1.49
*UGT1A1*28/*1* (*n* = 7)	317.3 ± 219.5	946.1 ± 490.6	3.35 ± 1.16
Homozygous group (*n* = 16)	509.8 ± 261.8	849.0 ± 561.9	1.85 ± 1.13
*UGT1A1*28/*6* (*n* = 4)	251.3 ± 89.5	557.8 ± 148.8	2.34 ± 0.82
*UGT1A1*6/*6* (*n* = 9)	564.9 ± 223.5	673.2 ± 304.1	1.21 ± 0.36
*UGT1A1*28/*28* (*n* = 3)	689.0 ± 327.0	1764.7 ± 631.4	3.10 ± 1.82
Cochrane–Armitage trend test	<0.001	0.001	<0.001
Levene's test	<0.001	0.310	0.013

Analyses were carried out between wild-type versus heterozygous or homozygous groups. AUC, area under the time–concentration curve; AUC ratio, AUC_{SN-38G}/AUC_{SN-38}; SN-38G, SN-38 glucuronide.

the inclusion of patients with various types of cancer. Even prospective studies have included patients who received heterogeneous treatments including irinotecan.[9,10,24] We focused on high-risk patients with relatively rare *UGT1A1* alleles to delineate the important pharmacogenetic determinants of irinotecan-induced neutropenia. To eliminate the potential effects of confounders such as diagnosis and concurrent therapy, we only included patients with gastric or colorectal cancer who received irinotecan monotherapy. We also evaluated the relationship between genotype and the pharmacokinetics of irinotecan and its major metabolites. We believe that these features of our study enhance the validity of our findings. The CRM used in our study offers important advantages over the conventional design with three patient cohorts.[25] We could enroll the patients promptly, and treat approximately 80% of patients at the MTD of irinotecan (150 mg/m^2), as initially estimated.

A previous dose-finding study suggested that the recommended dose of 180 mg/m^2 irinotecan in the FOLFIRI regimen was too low in patients with metastatic colorectal cancer who had the *UGT1A1*1/*1* or *UGT1A1*28/*1* genotype.[19] The incidence of grade 3–4 neutropenia was 24% in that study. In our study, the incidence of grade 3–4 neutropenia was 23% across the wild-type and heterozygous groups, supporting these earlier findings.

In the present study, only 25% of the patients in the homozygous group were able to complete two cycles of treatment at a dose of 150 mg/m^2 without treatment delays and dose reduction. Previous studies[12,20] revealed that low-dose irinotecan (100–125 mg/m^2) carried a low risk of neutropenia, even in patients with a *UGT1A1* homozygous genotype. Although the results of a meta-analysis[21] reported an increased risk of neutropenia not only at medium or high doses of irinotecan, but also at low doses in homozygous group, the results of the present study supported the report of Hoskin *et al.*[20] as, similarly, no DLT occurred at doses of 100 or 125 mg/m^2. This might be because retrospective studies were included and there was heterogeneity (such as cancer type, therapeutic line, regimen) in the meta-analysis,[21] whereas the present study was a prospective study using a single drug in a homogenous population of patients with colorectal cancer and gastric cancer. Moreover, the patients who required dose reduction from 150 to 125 or 100 mg/m^2 were able to receive subsequent treatment safely. Therefore, *UGT1A1* genetic polymorphism testing is useful, because a risk attributable to CPT-11 could be avoided by selecting another therapy, even in homozygous patients with a high risk of side-effects who have a poor performance status or a history of intensive treatment. Irinotecan at doses >150 mg/m^2 has been used in regimens such as cetuximab plus irinotecan (350 mg/m^2)[26] and FOLFIRI (180 mg/m^2) in colorectal cancer.[27] Our results suggest that starting treatment at such high doses of irinotecan would be very risky in patients who have two alleles of *UGT1A1*28* and/or *UGT1A1*6*. The *UGT1A1* genetic polymorphism is a solid factor that affects the pharmacokinetics and a factor for judging the risk of hemotoxicity. Because CPT-11 is used as the second- or third-line rather than the first-line therapy in patients with gastric cancer and colorectal cancer, it would be better to carry out genetic testing before therapy commenced and after a full explanation to all patients. Patients who have two alleles of *UGT1A1*28* and/or *UGT1A1*6* can receive irinotecan at a starting dose of 150 mg/m^2 and must be closely observed by carrying out observations and blood tests weekly, at least during the first cycle.

On the basis of the results obtained in our study, a nationwide close observational study is now ongoing to evaluate the safety and efficacy of irinotecan at a dose of ≤150 mg/m^2 after genetic testing.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Abbreviations

AUC	areas under the time–concentration curves
CI	confidence interval
CRM	continual reassessment method
DLT	dose-limiting toxicity
FOLFIRI	folinic acid/fluorouracil/irinotecan
MTD	maximum tolerated dose
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	SN-38 glucuronide
UGT	uridine diphosphate glucuronosyltransferases

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ORIGINAL ARTICLE

Phase I study of cediranib in combination with cisplatin plus fluoropyrimidine (S-1 or capecitabine) in Japanese patients with previously untreated advanced gastric cancer

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Abstract
Purpose The primary objective of this Phase I study was to assess the safety and tolerability of the vascular endothelial growth factor signalling inhibitor cediranib in combination with cisplatin plus an oral fluoropyrimidine, in Japanese patients with previously untreated advanced gastric cancer.
Methods Patients received continuous, once-daily oral doses of cediranib 20 mg in combination with either cisplatin (60 mg/m^2 iv day 1) plus S-1 (40–60 mg bid, days 1–21) every 5 weeks for a maximum of eight cycles [Arm A]; or cisplatin (80 mg/m^2 iv, day 1) plus capecitabine (1,000 mg/m^2 bid, days 1–14) every 3 weeks for a maximum of six cycles [Arm B]. In both arms, the assessment period for dose-limiting toxicities (DLTs) was the first 21 days of cycle 1.
Results Fourteen patients (Arm A, $n = 6$; Arm B, $n = 8$) were enrolled and received at least one dose of cediranib. One patient in each arm experienced a DLT (Arm A; decreased appetite, grade 3; Arm B, decreased appetite, fatigue and hyponatraemia, all grade 3). Overall, the most common adverse events were decreased appetite, fatigue and nausea (all $n = 13$ [92.9%]). Preliminary efficacy evaluation showed one confirmed (Arm A) and three unconfirmed (Arm A, $n = 1$; Arm B, $n = 2$) partial responses that were ongoing at data cut-off.
Conclusions Cediranib 20 mg/day in combination with cisplatin and S-1 or capecitabine was tolerable, with no new toxicities identified, and showed preliminary evidence of antitumour activity.

Keywords Cediranib · VEGF signalling · Phase I · Gastric cancer · Japanese

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Introduction

Gastric cancer is the most common malignancy in Japan. GLOBOCAN figures revealed that in 2008, there were 102,040 new cases of gastric cancer, and 50,156 deaths were attributed to this disease in Japan [1]. The only curative treatment is surgery, however, over half of patients present with inoperable tumours. For those patients with unresectable tumours and receiving best supportive care, outcomes are extremely poor with median survival times ranging from 3 to 5 months [2–4].

Combination chemotherapy regimens with platinum-based cisplatin plus an oral fluoropyrimidine are commonly used as first-line treatment for advanced gastric cancer in Japan [5]. This treatment regimen is based on early-phase clinical trials that showed cisplatin in combination with 5-fluorouracil (5-FU) or oral fluoropyrimidines yielded overall response rates of approximately 40% and median survival times of 7–13 months [6–10].

Vascular endothelial growth factor (VEGF) plays an essential role in the formation and maintenance of tumour vasculature [11]. The addition of bevacizumab, an anti-VEGF-A antibody, to standard chemotherapy has demonstrated clinical benefit in patients with advanced colorectal cancer [12–14] and non-small-cell lung cancer [15].

Cediranib is an oral, highly potent VEGF signalling inhibitor with activity against all three VEGF receptors [16, 17]. Initial clinical evaluation of cediranib monotherapy demonstrated that it is suitable for once-daily oral dosing in Japanese [18] and Western [19] patients, with biological activity at doses ≥20 mg/day [19]. Subsequent Phase I studies showed that cediranib 30 mg/day was generally well tolerated in combination with various standard anti-cancer treatments, with encouraging preliminary evidence of antitumour activity [20–23]. However, when the protocol for the present study was being developed, emerging data from Phase II and III trials indicated that cediranib 20 mg was the highest tolerable dose suitable for chronic once-daily dosing in combination with chemotherapy, with higher doses not considered to be more effective [24, 25]. Consequently, the dose of cediranib selected for this combination study was 20 mg/day. The primary objective of the current Phase I study (ClinicalTrials.gov, number NCT00960349) was to assess the safety and tolerability of cediranib 20 mg/day in combination with capecitabine/cisplatin or S-1/cisplatin in Japanese patients with previously untreated advanced gastric cancer.

Methods

Patients

Japanese patients ≥20 years of age with histologically or cytologically confirmed previously untreated recurrent or metastatic unresectable gastric adenocarcinoma were eligible for inclusion. Patients were required to have a life expectancy ≥12 weeks and a World Health Organization performance status of 0 or 1. The main exclusion criteria were as follows: significant respiratory, cardiac, hepatic or renal dysfunction; unstable brain metastases; poorly controlled hypertension; significant haemorrhage (>30 ml bleeding/episode in the previous 3 months) or haemoptysis (>5 ml fresh blood in the previous 4 weeks); arterial thromboembolic events in the previous 12 months; history of other malignancies within the previous 5 years; any unresolved toxicity according to Common Terminology Criteria for Adverse Events (CTCAE) from prior radiotherapy; recent (<14 days) major thoracic or abdominal surgery; and incomplete recovery from prior surgery. All patients provided written informed consent. The study was approved by the institutional review board at each participating centre and conducted in accordance with the Declaration of Helsinki, Good Clinical Practice, and the AstraZeneca policy on Bioethics [26].

Study design

This was a multicentre, open-label, non-randomized, Phase I study. Eligible patients received cediranib 20 mg/day orally (starting on day 2 in cycle 1) in combination with either cisplatin (60 mg/m^2 intravenous [iv], day 1) plus S-1 (40–60 mg orally twice daily, days 1–21) [Arm A] or cisplatin (80 mg/m^2 iv, day 1) plus capecitabine (1,000 mg/m^2 orally twice daily, days 1–14) [Arm B] (Fig. 1). One cycle of treatment in Arm A was 5 weeks, and one cycle of treatment in Arm B was 3 weeks. The rest periods in Arms A (2 weeks) and B (1 week) were consistent with standard clinical practice for administration of S-1 and capecitabine, respectively. The chemotherapy treatments in Arms A and B were continued for a maximum of eight and six cycles, respectively. Thereafter, treatment of cediranib plus S-1/capecitabine could be continued until a discontinuation criterion was met. Patients were initially entered into Arm A. Following enrolment of six patients into Arm A, patients were then entered into Arm B.

The primary study objective was to assess the safety and tolerability of cediranib in combination with S-1/cisplatin or capecitabine/cisplatin. After entry of six evaluable patients in each arm, a safety review committee (SRC) discussed whether the regimen was tolerated. The treatment was considered tolerable if ≤1 of the six patients experienced a DLT. If 2–3 of the six patients experienced a DLT, either the SRC recommended the combination was tolerated or the cohort was expanded to include three further evaluable patients. If ≥4 patients experienced a DLT, the treatment was considered intolerable.

In both arms, a DLT was any toxicity considered related to study drug that commenced within the first 21 days of cycle 1 and met any of the following criteria: hypertension or diarrhoea that required cessation of cediranib treatment; an absolute neutrophil count <500/mm^3 for ≥5 days despite growth factor support; a platelet count <50,000/mm^3 for ≥5 days; a dose delay to starting any chemotherapy agent in cycle 2 for longer than 14 days; dose reductions of cediranib due to cediranib-related toxicity; a single increase from baseline in the QT interval corrected for heart rate

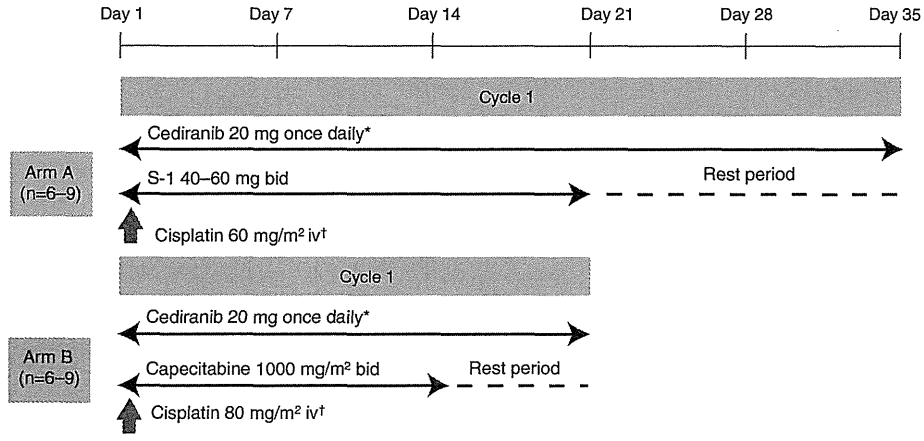

Fig. 1 Study design

*Cediranib initiated on day 2 in cycle 1
†Cisplatin continued for a maximum of eight cycles in Arm A and six cycles in Arm B
Safety assessments were performed on days 1, 8, 21 and 35 (Arm A only)

(QTc) of 60 ms that results in a QTc of at least 460 ms; two QTc measurements >490 ms taken at least 24 h apart; and any other CTCAE grade ≥3 that was, in the opinion of the investigator and the SRC, not clearly related to disease progression, clinically significant and related to the study drug.

Secondary objectives were to determine the steady-state pharmacokinetics (PK) of cediranib alone and in combination with chemotherapy and to investigate the potential effect of cediranib on the PK of the chemotherapy components (cisplatin and S-1/capecitabine [5-FU]). An exploratory objective was to assess the preliminary efficacy of the combination regimens by measurement of tumour response according to the Response Evaluation Criteria In Solid Tumours (RECIST version 1.0) [27].

Assessment of safety and tolerability

After a full physical examination at enrolment, toxicity was monitored throughout the study by the assessment of adverse events (AEs), which were graded according to CTCAE version 3.0. Vital signs (blood pressure [BP], pulse rate and body temperature) were measured, electrocardiograms recorded and samples taken for clinical chemistry, haematology assessment and urinalysis at the screening visit and on days 1, 8 and 21 in both arms; patients in Arm A repeated these assessments on day 35.

Pharmacokinetic assessment

To evaluate steady-state cediranib PK, blood samples were taken immediately before and 1, 2, 4, 6, 8 and 24 h after cediranib treatment on the final day of cycle 1 (cediranib alone) and day 1 of cycle 2 (presence of chemotherapy). To evaluate S-1/capecitabine (5-FU) PK, blood samples were collected immediately before and 0.5, 1, 2, 4, 6 and 8 h after S-1/capecitabine treatment on day 1 of cycle 1 (absence of cediranib) and day 1 of cycle 2 (presence of cediranib). To evaluate cisplatin PK, blood samples were taken pre-dose; 5 min before the end of the 2-h iv infusion; and 2.5, 3, 4, 6, 8 and 24 h post start of infusion on day 1 of cycle 1 (absence of cediranib) and day 1 of cycle 2 (presence of cediranib).

Plasma concentrations of cediranib, capecitabine (5-FU only), S-1 (5-FU only) and cisplatin (total platinum equivalents) were determined using high-performance liquid chromatography with mass spectrometry (LC–MS/MS). PK parameters were calculated using standard non-compartmental analysis.

Assessment of tumour response

Objective tumour assessments determined by RECIST were performed every 12 weeks from the start of treatment until disease progression, death or discontinuation of cediranib due to any other reason.

Results

Patient characteristics

Between August and December 2009, 14 patients were recruited into Arm A ($n = 6$) or Arm B ($n = 8$). Patient demographic and baseline characteristics are summarized in Table 1. At data cut-off (4 January 2010), three patients in Arm A and five patients in Arm B were still receiving cediranib, and one patient in Arm B continued to receive capecitabine and cisplatin. The reasons for discontinuation of cediranib treatment were clinical disease progression

Table 1 Patient demographics and baseline characteristics

Characteristics	Cediranib + S-1 + cisplatin ($n = 6$)	Cediranib + capecitabine + cisplatin ($n = 8$)	Total ($n = 14$)
Age, years			
Median	59.5	60.5	60.5
Range	53–71	27–72	27–72
Sex, *n* (%)			
Male	4 (66.7)	5 (62.5)	9 (64.3)
Female	2 (33.3)	3 (37.5)	5 (35.7)
WHO performance status, *n* (%)			
0	3 (50.0)	4 (50.0)	7 (50.0)
1	3 (50.0)	4 (50.0)	7 (50.0)
Number of metastatic sites (%)			
1	1 (16.7)	0	1 (7.1)
>1	5 (83.3)	8 (100.0)	13 (92.9)
Recurrence, *n* (%)	0	1 (12.5)	1 (7.1)
Stage IV, *n* (%)	6 (100)	7 (87.5)	13 (92.9)
Measurable target lesion, *n* (%)	5 (83.3)	6 (75.0)	11 (78.6)
Histology, *n* (%)			
Adenocarcinoma (intestinal)	1 (16.7)	3 (37.5)	4 (28.6)
Adenocarcinoma (diffuse)	1 (16.7)	0	1 (7.1)
Tubular adenocarcinoma	3 (50.0)	2 (25.0)	5 (35.7)
Signet ring carcinoma	1 (16.7)	3 (37.5)	4 (28.6)

WHO World Health Organization

(Arms A and B, $n = 1$), AEs (Arms A and B, $n = 1$) and withdrawal of consent (Arm A, $n = 1$). One patient in Arm B was revealed ineligible at cycle 2 due to a pulmonary embolism at baseline; this patient discontinued study treatment but was included in safety analyses.

Safety and tolerability

All patients received at least one dose of cediranib and were therefore evaluable for safety. The median (range) daily cediranib dose was 16.0 (12.9–20.0) mg in Arm A and 15.9 (13.7–20.0) mg in Arm B, and median (range) duration of actual exposure to cediranib was 72.5 days (13–127) for Arm A and 38.5 days (13–62) for Arm B. The median (range) number of chemotherapy cycles received was 2.5 (1–4) for both arms.

Overall, 12 (86%) [Arm A, $n = 5$; Arm B, $n = 7$] patients experienced one or more cediranib dose interruptions, with one patient from each arm having a dose reduction to 15 mg/day. All six patients in Arm A experienced a dose reduction or interruption of S-1 and seven patients (87.5%) in Arm B experienced a dose reduction or interruption of capecitabine. Five patients in each arm (Arm A, 83.3%; Arm B, 62.5%) had a dose reduction or dose delay of cisplatin. Two patients in Arm A (alopecia, $n = 1$; diarrhoea, stomatitis, fatigue, decreased appetite and hyponatraemia, $n = 1$) and one patient in Arm B (diarrhoea, fatigue, decreased appetite and hypomagnesaemia) experienced AEs that led to permanent discontinuation of cediranib treatment.

Table 2 Most common adverse events (incidence > 30% in total population)

AE, preferred term	All grades, *n* (%)		
	Cediranib + S-1 + cisplatin ($n = 6$)	Cediranib + capecitabine + cisplatin ($n = 8$)	Total ($n = 14$)
Decreased appetite	5	8	13 (92.9)
Fatigue	5	8	13 (92.9)
Nausea	5	8	13 (92.9)
Constipation	3	7	10 (71.4)
Diarrhoea	5	5	10 (71.4)
Stomatitis	4	6	10 (71.4)
Hypertension	3	6	9 (64.3)
Weight decreased	5	4	9 (64.3)
Neutropenia	5	3	8 (57.1)
Vomiting	3	5	8 (57.1)
Alopecia	2	4	6 (42.9)
Dysphonia	2	4	6 (42.9)
Hiccups	1	4	5 (35.7)
Leukopenia	3	2	5 (35.7)
Proteinuria	3	2	5 (35.7)

AE adverse event

DLTs were reported in one patient in Arm A (decreased appetite, grade 3) and one patient in Arm B (decreased appetite, fatigue and hyponatraemia; all grade 3). In Arm A, the investigator assessed that decreased appetite was related to S-1 and/or cisplatin. In Arm B, the investigator judged decreased appetite and hyponatraemia related to cediranib, S-1 and cisplatin, and stomatitis related to cediranib and S-1. The SRC decided neither DLT warranted cohort expansion for further evaluation of safety.

The most commonly reported AEs were decreased appetite, fatigue and nausea (all $n = 13$ [92.9%]) [Table 2]. Five (83%) patients in Arm A and six (75%) patients in Arm B experienced AEs grade ≥3 (Table 3). Hypertension was reported as an AE in nine patients (Arm A, $n = 3$; Arm B, $n = 6$), only one (Arm B) of which was

Table 3 Any CTCAE grade ≥3 adverse events

	Grade	*Cediranib* + S-1 + cisplatin ($n = 6$)	*Cediranib* + capecitabine + cisplatin ($n = 8$)	*Total* ($n = 14$)
Neutropenia	3	3	2	5 (35.7)
Hypokalaemia	3	0	3	3 (21.4)
Hyponatraemia	3	1	2	3 (21.4)
Decreased appetite	3	1	1	2 (14.3)
Fatigue	3	0	2	2 (14.3)
Anaemia	3	0	1	1 (7.1)
Diarrhoea	3	1	0	1 (7.1)
Haemoglobin decreased	3	1	0	1 (7.1)
Hyperbilirubinaemia	3	0	1	1 (7.1)
Hyperglycaemia	3	0	1	1 (7.1)
Hypertension	3	0	1	1 (7.1)
Hypomagnesaemia	3	0	1	1 (7.1)
Platelet count decreased	3	1	0	1 (7.1)
Pulmonary embolism	4	0	1	1 (7.1)
Stomatitis	3	1	0	1 (7.1)
Syncope	4	1	0	1 (7.1)
White blood cell count decreased	3	1	0	1 (7.1)
Wound infection	3	1	0	1 (7.1)

grade 3; no action was taken regarding dose adjustment. One patient in Arm A experienced grade 4 transient syncope on day 6, cycle 2. A head computed tomography (CT) scan showed no cerebral haemorrhage and the syncope resolved on the same day it appeared. The investigator considered this event to be related to cediranib, S-1 and cisplatin. One patient from Arm B experienced a grade 4 pulmonary embolism that was identified on day 18, cycle 2 after the patient complained of chest pain. After careful review of the baseline CT scan, the pulmonary embolism was found to be pre-existing at study entry. The investigator judged the event as worsening of the pulmonary embolism related to cediranib, capecitabine and cisplatin. Increases in thyroid stimulating hormone were observed in both arms, but free T4 and T3 remained within normal limits for the majority of these patients. Increases were observed in alanine aminotransferase and aspartate aminotransferase in both arms, but most values were generally within the normal ranges. There were no clinically relevant results related to electrocardiogram, physical findings or other safety observations.

Five serious AEs (SAEs) were reported in three patients in Arm A (decreased appetite, $n = 2$; hyponatraemia, $n = 1$; stomatitis, $n = 1$; syncope, $n = 1$), and in addition to the pulmonary embolism in one patient, three other SAEs were reported in a separate patient in Arm B (decreased appetite, hyponatraemia and fatigue). All SAEs, except for the pulmonary embolism, had resolved by data cut-off. There were no deaths in the period to data cut-off in either arm.

Pharmacokinetics

A summary of PK parameters for cediranib, cisplatin and S-1/capecitabine is shown in Table 4. Only six patients (Arm A, $n = 2$; Arm B, $n = 4$) were evaluable for PK analysis, having completed the planned sampling schedule; therefore, limited data were available for within-patient comparison. In Arm A ($n = 2$), the PK parameters for S-1 in combination with both cediranib and cisplatin were similar to those for S-1 when administered with cisplatin alone, and the PK parameters for cediranib were similar in the presence and absence of chemotherapy; however, there were insufficient data to draw meaningful conclusions on the PK in Arm A. Based on limited data from Arm B ($n = 4$), the cediranib PK parameters were similar in the absence and presence of capecitabine/cisplatin. The PK profile of capecitabine was generally similar in the absence and presence of cediranib; one patient (patient 4 in Table 4) had a higher exposure in the presence of cediranib, but the reason for this is not clear as no interaction would be expected. In all patients (Arms A and B), slight increases in exposure to cisplatin (total platinum equivalents; maximum plasma concentration [C_{max}] and area under plasma concentration–time curve from time zero to 8 h [AUC_{0-8h}]) were observed when cediranib was administered with chemotherapy compared with chemotherapy alone; however, samples collected in the absence of cediranib were obtained following single-dose cisplatin, whereas those collected in the presence of cediranib were obtained following multiple-dose cisplatin.

Efficacy

Seven patients (Arm A, $n = 4$; Arm B, $n = 3$) had a post-baseline scan and were therefore evaluable for efficacy. Tumour shrinkage was observed in five of these patients (Fig. 2); the mean largest change from baseline was −41.8% in Arm A ($n = 4$) and −26.3% in Arm B ($n = 3$). One patient in Arm A had a partial response that was ongoing at data cut-off (duration >79 days). Among the four patients with stable disease ($n = 2$ in each arm), three had unconfirmed partial responses at data cut-off. One patient in each arm had a best response of progressive disease.