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Tumor *KRAS* Status Predicts Responsiveness to Panitumumab in Japanese Patients with Metastatic Colorectal Cancer

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Objective: Mutation status of the *KRAS* gene in tumors has been shown to be a predictive biomarker of response to anti-epidermal growth factor receptor antibody therapy in patients with metastatic colorectal cancer. This retrospective analysis examined the association between efficacy and safety of the fully human anti-epidermal growth factor receptor antibody panitumumab and *KRAS* mutation status in Japanese metastatic colorectal cancer patients using data from two clinical trials with adherence to good clinical practices.

Methods: An exploratory, integrated analysis of data from *KRAS* evaluable patients enrolled in a Phase 1 study (Study 20040192) and a Phase 2 study (Study 20050216) was performed. Paraffin-embedded tumor samples were analyzed for *KRAS* status. Primary efficacy endpoint of this analysis was objective tumor response per modified response evaluation criteria in solid tumors; a key secondary endpoint was progression-free survival. Safety endpoints included incidence of adverse events.

Results: Tumor samples with known *KRAS* status were available from 8 of 13 (62%) metastatic colorectal cancer patients in the Phase 1 study and 16 of 53 patients (30%) in the Phase 2 study. Overall, 14 (58%) patients had wild-type *KRAS* tumors and 10 (42%) patients had mutated *KRAS* tumors. Four (17%) patients had a partial response; all responders had tumors with wild-type *KRAS*. Results of all secondary efficacy endpoints also favored patients with wild-type *KRAS*. Treatment-related adverse events were predominantly mild to moderate and skin related, and were similar between patients with tumors with wild-type and mutated *KRAS* in this small patient population.

Conclusions: Mutated *KRAS* status in tumors of Japanese patients with metastatic colorectal cancer is associated with lack of response to panitumumab therapy.

Key words: panitumumab – epidermal growth factor receptor – colorectal cancer – KRAS

INTRODUCTION

Expression of the epidermal growth factor receptor (EGFR) is frequently associated with malignant transformation in human cancers (1). This observation led to the development of anti-EGFR therapies for the treatment of EGFR-expressing tumors (2). While anti-EGFR antibody therapies have demonstrated efficacy in patients with metastatic colorectal cancer (mCRC) (3), the level of EGFR expression has not been shown to be associated with response to anti-EGFR antibodies (4,5). Biomarkers that can identify patients who are likely to respond to anti-EGFR therapy are needed.

Downstream signaling pathways are activated when EGFR binds to its ligand. The *KRAS* gene codes for a protein that is a member of the ras family of small G-proteins involved in intracellular signaling. When EGFR binds to ligand, its tyrosine kinase function is activated, ultimately resulting in the activation of the Ras-Raf-MAP kinase signaling cascade (6). Ras is activated by binding to GTP and deactivation of

ras in normal cells is accomplished by hydrolysis of GTP. Mutations in the *KRAS* gene that abolish the intrinsic GTPase activity result in constitutively active ras proteins that are oncogenic (7). It is possible that downstream signaling pathways are therefore constitutively activated and can become EGFR independent. In support of this hypothesis, mutations in *KRAS* have been shown to predict non-responsiveness to anti-EGFR antibody therapies in patients with mCRC (8–11).

KRAS mutations occur in ~30–50% of all patients with colorectal cancers (8–10,12,13). *KRAS* mutations have been reported in ~30% of Japanese patients with colorectal cancers (14,15). Anti-EGFR antibody therapies are therefore likely to be ineffective in at least one-third of Japanese patients with mCRC, highlighting the importance of screening for these mutations in tumors.

Panitumumab is a fully human monoclonal antibody against EGFR that is indicated as monotherapy for treatment of EGFR-expressing mCRC in the USA and EGFR-expressing plus wild-type *KRAS*-expressing mCRC in the European Union (EU) (16,17). In retrospective analyses of data from panitumumab clinical trials, including a Phase 3 trial comparing panitumumab monotherapy with best supportive care, the presence of a mutated *KRAS* gene in tumors was associated with lack of response (18,19). This retrospective integrated analysis of data from two clinical trials (20,21) is the first study to examine the efficacy and safety of panitumumab monotherapy according to tumor *KRAS* status in Japanese patients with mCRC.

PATIENTS AND METHODS

Study Design

This was a retrospective, exploratory integrated analysis of data from two clinical trials (20,21) in Japanese patients with mCRC to examine the efficacy and safety of panitumumab monotherapy according to tumor *KRAS* status.

Study 20040192 was a Phase 1 clinical trial of panitumumab monotherapy in Japanese patients with advanced solid tumors (20). Key objectives of this study were to evaluate the safety, pharmacokinetics, immunogenicity and clinical efficacy of panitumumab at various dose/dosing schedules in Japanese patients with advanced solid tumors. Patients with documented, advanced solid tumors that were refractory to standard chemotherapy or for which no standard therapy was available were eligible. Patients were sequentially enrolled into one of three panitumumab dosing cohorts: 2.5 mg/kg once weekly (QW), 6.0 mg/kg once every 2 weeks (Q2W) and 9.0 mg/kg once every 3 weeks (Q3W). These doses are all considered to reach clinically active panitumumab exposures. Objective responses were determined by the investigators using modified response evaluation criteria in solid tumors (RECIST) (22). Eighteen patients (six per cohort) were enrolled in the study. Only patients with mCRC were included in this analysis.

Study 20050216 was an open-label, single-arm, Phase 2 clinical trial of panitumumab monotherapy in Japanese patients with EGFR-expressing mCRC, who had developed disease progression while on or after prior fluoropyrimidine, irinotecan and oxaliplatin therapy (21), which were the same eligibility criteria as those used in the global Phase 3 trial (23). Key objectives of this study were to assess the effect of treatment with panitumumab on best overall objective response rate, progression-free survival, overall survival, safety and pharmacokinetics of Japanese patients with mCRC. Patients received panitumumab 6.0 mg/kg Q2W until disease progression or intolerability. Objective responses were determined by central radiographic review and by the investigators using modified RECIST, as defined in the pivotal trial (23). Fifty-three patients enrolled in the study and received at least one dose of panitumumab.

Participation in the biomarker analysis was optional in the two studies and additional informed consent was required to participate. Therefore, only the subset of patients with the additional informed consent enrolled in the studies were included in the analysis reported here. Overall, tumor samples from 28 of the total 66 patients with mCRC enrolled in the studies were available for biomarker analyses.

Study Endpoints

The primary efficacy endpoint was the objective tumor response rate. A key secondary efficacy endpoint was progression-free survival time. An *ad hoc* analysis of change in target lesions (sum of target lesion diameters) from baseline was also performed. Safety endpoints included the incidence of treatment-emergent adverse events. Pharmacokinetics of panitumumab were characterized for selected patients.

Tumor *KRAS* Assessments

A retrospective analysis of *KRAS* mutation status (wild-type or mutated) was conducted using existing paraffin-embedded tumor tissues. Most specimens were from the primary tumor; three specimens were from metastatic sites. Samples were tested using the K-RAS Mutation kit (RUO KR-02) from DxS (Manchester, UK), which was the convenient, commercially available method used to detect *KRAS* mutations in the pivotal panitumumab trial. DNA was extracted from paraffin-embedded tumor samples using the QIAamp® DNeasy kit (QIAGEN, Inc., Valencia, CA, USA). All testing was performed by a central laboratory (HistoGeneX, Antwerp, Belgium); personnel performing the assays were blinded to the clinical outcomes.

The K-RAS Kit utilizes the amplification refractory mutation system (ARMS®) (24) for mutation-specific amplification and Scorpions® (25,26) technology to detect the mutations. ARMS technology is based on the observation that *Taq* DNA polymerase is ineffective at amplifying oligonucleotides with a mismatched 3′ residue. Primers for seven specific mutations in the *KRAS* gene (with the mutations appearing at the 3′ end of the primers) are used to amplify

mutated *KRAS* sequences in PCR reactions: Gly12Ala, Gly12Asp, Gly12Arg, Gly12Cys, Gly12Ser, Gly12Val and Gly13Asp. Scorpions, bifunctional molecules comprising a PCR primer covalently linked to a probe, are included in the PCR reaction. The Scorpion probe consists of a fluorophore and a quencher that are separated by the specific probe sequence. Complementary stem sequences flanking the specific probe sequence cause the Scorpion probe to form a hairpin structure in which the fluorophore and quencher are brought together, resulting in loss of fluorescence from the fluorophore. When the Scorpion probe is heat-denatured and then allowed to cool and bind to its target amplicon (a mutated *KRAS* sequence that has been amplified by an ARMS probe), the fluorophore and quencher are separated and fluorescence is increased. The fluorescence is measured in a LightCycler® 480 Instrument (Roche Applied Science, Indianapolis, IN, USA) using software version LCS480 1.2.9.11. This kit has the ability to detect ~1% mutated DNA in a background of wild-type genomic DNA. The failure rate of the *KRAS* assay was 4% in a large data set of patients with mCRC in a prior panitumumab clinical study (18).

Statistical Analyses

All analyses were descriptive evaluations to assess the relationship between clinical outcome and tumor *KRAS* mutation status in Japanese patients with mCRC who had measurable disease at baseline. Because of the small number of patients with samples available for *KRAS* testing, data from the two studies were pooled for these analyses, ignoring the potential heterogeneity in the analysis sets between the studies. All efficacy and safety analyses were performed on the *KRAS* analysis sets (enrolled patients who had: given consent for biomarker analysis; measurable disease at baseline; evaluable *KRAS* status and received at least one dose of panitumumab). Analyses were stratified by *KRAS* status; no other covariates were considered. No hypothesis testing was performed to compare endpoints between wild-type *KRAS* and mutated *KRAS* strata.

For continuous endpoints, the mean and standard deviation (SD) values are provided. The frequency and percentage distributions are provided for discrete data. The objective response rate and its two-sided 95% confidence interval (CI) were calculated; the 95% CI was based on the *F* distribution method (27). Kaplan–Meier estimates for progression-free survival time and 95% CIs were calculated; the 95% CI was based on a sign test (28). No imputation for missing or incomplete data was performed. All analyses were performed using SAS version 8.2 or higher (SAS Institute Inc., Cary, NC, USA) on the Sun/UNIX platform (Sun Microsystems, Inc; Santa Clara, CA, USA).

RESULTS

Patients

Participation in the biomarker analyses was optional in these studies and required additional written consent. Of the 66 patients with mCRC enrolled in the two studies, consent to participate in the study was obtained from 28 patients. Of these, 24 had known *KRAS* status. Patient demographics and disease characteristics at baseline are shown in Table 1.

At the time of data cut-off (12 April 2007), all patients had ended treatment because of disease progression ($n = 28$). All patients completed the protocol-specified safety follow-up. No patient included in this analysis withdrew from the studies. All patients had baseline measurable disease.

KRAS Status

KRAS status was determined in 24 (34%) patients, including 8 patients from Study 20040192 and 16 patients from Study 20050216. Of these, 14 (58%) had tumors with wild-type *KRAS* and 10 (42%) had tumors with mutated *KRAS*. The *KRAS* test failed for four patients: there was insufficient DNA in the tumor samples for three patients, and the tissue failed pathology review (i.e. no tumor sample) for one patient.

Table 1. Patient demographics, disease characteristics and dose assignments at baseline

	Panitumumab		
	Wild-type *KRAS* ($n = 14$)	Mutated *KRAS* ($n = 10$)	All patients ($n = 24$)
Sex, *n* (%)			
Men	9 (64)	4 (40)	13 (54)
Women	5 (36)	6 (60)	11 (46)
Age, years			
Mean (SD)	55.4 (12.3)	63.6 (10.1)	58.8 (11.9)
Range	32–71	45–77	32–77
Primary diagnosis, *n* (%)			
Colon cancer	6 (43)	5 (50)	11 (46)
Rectal cancer	2 (14)	3 (30)	5 (21)
Colorectal cancer[a]	6 (43)	2 (20)	8 (33)
ECOG performance status, *n* (%)			
0	11 (79)	8 (80)	19 (79)
1	3 (21)	2 (20)	5 (21)
Assigned dose cohort in Study 20040192, *n*			
N[b]	6	2	8
2.5 mg/kg QW	3	0	3
6.0 mg/kg Q2W	1	2	3
9.0 mg/kg Q3W	2	0	2

ECOG, Eastern Cooperative Oncology Group; SD, standard deviation; QW, once weekly; Q2W, every 2 weeks; Q3W, every 3 weeks.
[a]Specification of cancer site (colon vs. rectum) was not required in Study 20040192.
[b]Number of patients in Study 20040192 only.

Efficacy Outcomes

In the *KRAS* analysis set, four patients had a partial response to panitumumab therapy; all four responders had tumors expressing wild-type *KRAS* (Table 2). Six patients with tumors with wild-type *KRAS* had stable disease (median duration of 13.2 weeks; 95% CI: 11.1, 15.1). Of patients with tumors expressing mutated *KRAS*, none had a partial response and one had stable disease for 11.4 weeks. Patients with wild-type *KRAS*-expressing tumors had longer progression-free survival (median 13.2 weeks) than patients with mutated *KRAS* (median 7.3 weeks) (Fig. 1).

In an *ad hoc* analysis, the maximum decrease in the sum of target lesion diameters was determined for all patients. Of the 14 patients with tumors expressing wild-type *KRAS*, the sum of target lesion diameters was decreased in 10 patients; 4 of these patients had a partial response and 6 patients had stable disease (Fig. 2). The remaining four patients with wild-type *KRAS* in their tumors had an increase in the sum of target lesion diameters and had progressive disease. All patients with mutated *KRAS* in their tumors had an increase in the sum of their target lesion diameters.

Table 2. Best objective response and progression-free survival

	Panitumumab		
	Wild-type *KRAS* ($n = 14$)	Mutated *KRAS* ($n = 10$)	All patients ($n = 24$)
Best objective response, *n* (%)			
Partial response	4 (29)	0	4 (17)
Stable disease	6 (43)	1 (10)	7 (29)
Progressive disease	4 (29)	9 (90)	13 (54)
Objective response rate			
Patients with partial response, *n*	4	0	4
Rate, %	28.6	0	16.7
95% CI	8.4–58.1	0–30.9	4.7–37.4
Progression-free survival			
Median weeks	13.2	7.3	7.8
95% CI	8.0–23.1	7.1–7.6	7.4–11.4

CI, confidence interval.

Safety Outcomes

All 24 patients in the *KRAS* analysis set experienced an adverse event (related or unrelated to panitumumab therapy) during the study. Six (25%) patients had an adverse event with the worst grade of 3; 3 (13%) patients had an event with the worst grade of 4 and 7 (29%) patients had a serious adverse event. Treatment-related adverse events also occurred in all patients, including two (8%) patients with an adverse event with a worst grade of 3 and one (4%) patient with a serious adverse event. All skin-related events were Grade 1 or 2 in severity. Hypomagnesemia was reported in five (36%) patients with tumors expressing wild-type *KRAS* and in four (40%) patients with tumors expressing mutated *KRAS*. Adverse events occurring in 20% or more of the patients are shown in Table 3. No patient had an investigator-reported adverse event with a preferred term indicative of an infusion reaction or a reaction to panitumumab. No deaths or withdrawals due to adverse events were reported in the *KRAS* analysis set. No marked difference in adverse events was observed based on *KRAS* mutation status.

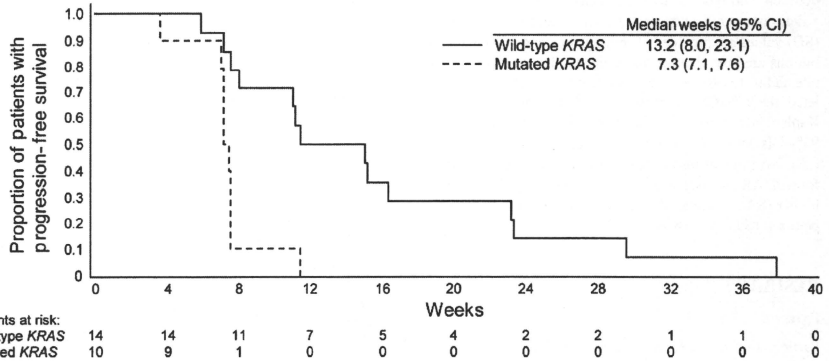

Figure 1. Progression-free survival. The proportion of patients with progression-free survival over time (weeks) is shown.

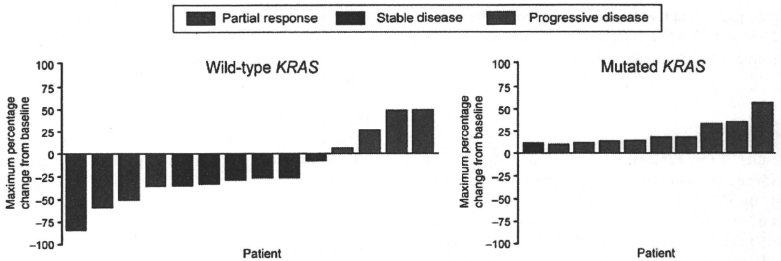

Figure 2. Changes in target lesions by *KRAS* status. The maximum percentage changes in the sum of target lesions for individual patients are shown for patients with tumors expressing wild-type *KRAS* (left panel) and mutated *KRAS* (right panel). The best overall response is designated by green bars for a partial response, blue bars for stable disease and red bars for progressive disease.

Table 3. Summary of adverse events and adverse events occurring in 20% or more of the patients[a]

Adverse event, *n* (%)	Panitumumab					
	Wild-type *KRAS* ($n = 14$)		Mutated *KRAS* ($n = 10$)		All patients ($n = 24$)	
	Any grade	Grade ≥3	Any grade	Grade ≥3	Any grade	Grade ≥3
Patients with any adverse event	14 (100)	6 (43)	10 (100)	3 (30)	24 (100)	9 (38)
Anorexia	7 (50)	3 (21)	8 (80)	0	15 (63)	3 (13)
Dry skin	8 (57)	0	7 (70)	0	15 (63)	0
Fatigue	9 (64)	3 (21)	6 (60)	0	15 (63)	3 (13)
Rash	7 (50)	0	7 (70)	0	14 (58)	0
Paronychia	9 (64)	0	4 (40)	0	13 (54)	0
Acne	6 (43)	0	3 (30)	0	9 (38)	0
Hypomagnesemia	5 (36)	0	4 (40)	0	9 (38)	0
Diarrhea	5 (36)	0	3 (30)	0	8 (33)	0
Pruritus	6 (43)	0	2 (20)	0	8 (33)	0
Back pain	3 (21)	0	3 (30)	0	6 (25)	0
Constipation	6 (43)	0	0	0	6 (25)	0
Acneiform dermatitis	6 (43)	0	0	0	6 (25)	0
Nausea	4 (29)	0	2 (20)	0	6 (25)	0
Abdominal pain	4 (29)	0	1 (10)	0	5 (21)	0
Pyrexia	3 (21)	0	2 (20)	1 (10)	5 (21)	1 (4)

[a]Includes events that were related or unrelated to treatment with panitumumab; adverse events were coded using the Medical Dictionary for Regulatory Activities (MedDRA) version 9.0; severity was graded according to the National Cancer Institute Common Toxicity Criteria (NCI CTC) version 2.0 with the exception of skin toxicities, which were graded using the modified Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Pharmacokinetic Evaluation

This pharmacokinetic analysis only included patients who received panitumumab at 6 mg/kg Q2W. Panitumumab pharmacokinetic data were available for 10 patients with tumors expressing mutated *KRAS* (from 2 patients in Study 20040192 and 8 patients in Study 20050216) and 9 patients with tumors expressing wild-type *KRAS* (from 1 patient in Study 20040192 and 8 patients in Study 20050216). The pharmacokinetic profiles of panitumumab were similar between patients with wild-type and mutated *KRAS* status. On the basis of the population pharmacokinetic model (29), the mean (SD) areas under the curves at steady-state were 1110 (385) and 863 (240) μg·day/ml, the mean maximum concentrations were 168 (38.8) and

137 (27.0) μg/ml and the mean minimum concentrations were 38.4 (21.8) and 27.9 (13.2) μg/ml for patients with wild-type and mutated *KRAS* status, respectively.

DISCUSSION

The results of this exploratory integrated analysis of safety and efficacy of panitumumab monotherapy in Japanese patients by *KRAS* status are consistent with those of other studies of anti-EGFR antibodies in patients with mCRC (9–11,18,19). The distribution of *KRAS* status (wild-type vs. mutated) was also similar to those seen in these other studies. Results of efficacy endpoints were favorable in patients with tumors expressing wild-type *KRAS*, and mutated tumor status was associated with lack of response to anti-EGFR therapy. In this limited analysis, response to panitumumab therapy was seen only in patients with tumors expressing wild-type *KRAS*. Patients with tumors with wild-type *KRAS* showed a trend toward longer progression-free survival.

Technical issues in sample processing resulted in the loss of four samples for testing, including lack of sufficient material for testing ($n = 3$) and *KRAS* assay failure ($n = 1$). Assay failure can be caused by inappropriate tissue fixation at the time of tissue collection. It is important, therefore, for investigators enrolling patients in clinical trials to ensure availability of proper materials and procedures for tissue collection. These precautions should enhance the quality and quantity of data obtained in clinical trials for these types of analyses.

The safety profile of panitumumab in this study was also consistent with prior studies of panitumumab monotherapy in patients with mCRC (23,30,31). Skin toxicities and hypomagnesemia are known effects of EGFR inhibition but are generally manageable. Because of the small sample size and the varying doses of panitumumab received by patients in the 20040192 study, it is not possible to draw meaningful conclusions regarding potential differences in the incidence or severity of adverse events between patients with tumors expressing wild-type or mutated *KRAS*.

Panitumumab exhibits pharmacokinetics that are consistent with target-mediated drug disposition, involving saturable binding to EGFR and subsequent internalization and degradation inside the cells (32). In addition, panitumumab is cleared by the reticuloendothelial system, similar to other endogenous immunoglobulins. As it is unlikely that *KRAS* is involved in the clearance of panitumumab, it was not unexpected that pharmacokinetic profiles of panitumumab were similar between patients with tumors expressing mutated *KRAS* and patients with tumors expressing wild-type *KRAS*.

The results of this study also support the need for *KRAS* testing to assist in identification of patients who are unlikely to respond to panitumumab therapy. Expression of EGFR on tumors is a requirement of both the USA (16) and EU (17) labels, although EGFR expression has now been shown to have no predictive value with respect to response to anti-EGFR therapy (4,5). Data from our analysis support the suggestion that panitumumab therapy should be restricted to patients whose tumors express wild-type *KRAS*.

In conclusion, the efficacy of panitumumab in the treatment of mCRC is similar in Japanese patients and Western patients. Additionally, panitumumab efficacy according to *KRAS* status is similar in Japanese and non-Japanese patients.

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Conflict of interest statement

Toshihiko Doi, Makoto Tahara, Takayuki Yoshino and Tomohide Tamura have no disclosures. Kentaro Yamazaki has been granted a contribution or research support from Taiho, Pfizer, Takeda and Chugai. Yasuhide Yamada has been a lecturer and received fees at lectures sponsored by Taiho, Chugai, Novartis, Bayer, Pfizer Japan and Yakult Honsha. Bing-Bing Yang and Kelly Smith Oliner are compensated employees and shareholders of Amgen Inc. Satoru Otani and Daisuke Asahi are compensated employees of Takeda Bio Development Center Limited.

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PHASE I STUDIES

Management of axitinib (AG-013736)-induced fatigue and thyroid dysfunction, and predictive biomarkers of axitinib exposure: results from phase I studies in Japanese patients

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Summary *Background* Axitinib is an oral, potent and selective inhibitor of vascular endothelial growth factor receptors (VEGFRs) 1, 2 and 3. We report on data obtained from 18 Japanese patients with advanced solid tumors in two phase I trials that evaluated the safety, pharmacokinetics and antitumor activity of axitinib and also examined potential biomarkers. *Methods* Six patients received a single 5-mg dose of axitinib followed by 5 mg twice daily (BID), and an additional six patients received axitinib 5 mg BID only. Another six patients received axitinib at 5-mg, 7-mg and 10-mg single doses followed by 5 mg BID. *Results* Plasma pharmacokinetics following single doses of axitinib was generally linear. Common treatment-related adverse events were fatigue (83%), anorexia (72%), diarrhea (67%), hand–foot syndrome (67%) and hypertension (61%). Sixteen patients (89%) experienced thyroid-stimulating hormone (TSH) elevation. Grade 3/4 toxicities included hypertension (33%) and fatigue (28%). No grade 3/4 fatigue occurred in patients who started thyroid hormone replacement therapy when TSH was elevated. Thyroglobulin elevation was observed in all patients who continued treatment with axitinib for ≥3 months. Abnormal TSH correlated with exposure to axitinib (r=0.72). Decrease in soluble (s) VEGFR-2 levels significantly correlated with exposure to axitinib (r=–0.94). Axitinib showed antitumor activity across multiple tumor types. *Conclusions* Axitinib-related thyroid dysfunction could be due to a direct effect on the thyroid gland. Grade 3/4 fatigue and hypothyroidism appear to be controllable with use of thyroid hormone replacement therapy. sVEGFR-2 and TSH may act as biomarkers of axitinib plasma exposure.

Keywords Axitinib · Fatigue · Thyroid dysfunction · Biomarker · Pharmacokinetics · Vascular endothelial growth factor receptor

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Introduction

Axitinib (AG-013736) is an oral, potent and selective inhibitor of vascular endothelial growth factor (VEGF) receptors (VEGFRs) 1, 2 and 3. Inhibition of the associated receptor tyrosine kinases is seen at picomolar concentrations [1]. Inhibition of the VEGF/VEGFR pathway by axitinib reduces VEGF-stimulated receptor autophosphorylation, blocking the

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growth and survival of endothelial cells, reducing tumor vascularization and inducing cell death [2]. Antitumor effects have been documented in a range of preclinical models, using single-agent axitinib or in combination with chemotherapy [3, 4]. Clinical activity with axitinib monotherapy, at a recommended starting dose of 5 mg twice daily (BID), has been observed in phase II studies in patients with metastatic renal cell carcinoma (mRCC) refractory to treatment with cytokines [5] or sorafenib [6]. Two multicenter phase III studies of axitinib in advanced RCC are ongoing. Antitumor activity also has been noted in trials of axitinib in patients with advanced non-small cell lung cancer (NSCLC) [7], thyroid cancer [8], melanoma [9] and breast cancer [10].

A phase I study (Study 1) was conducted in 12 Japanese patients with solid tumors to determine the recommended starting dose for this patient population. This study also investigated the safety, pharmacokinetics following axitinib 5-mg single dosing and continuous dosing, the antitumor activity and potential biomarkers associated with single-agent axitinib [11]. Subsequently, a second phase I study (Study 2) was conducted in an additional six Japanese patients with solid tumors to evaluate pharmacokinetics following axitinib 5-mg, 7-mg, 10-mg single dosing and continuous dosing. In Study 1, fatigue and abnormal thyroid-stimulating hormone (TSH) levels were frequently observed in 12 patients. In Study 2, therefore, free T3, free T4 and thyroglobulin were prospectively measured to further evaluate the effects of axitinib on the thyroid. Study 2 also explored biomarkers and antitumor activity. In this report, we newly reported the axitinib pharmacokinetic profile following 5-mg, 7-mg and 10-mg single dosing, thyroid function tests for free T3, free T4 and thyroglobulin, and management of fatigue with thyroid hormone replacement therapy. In addition, we updated safety data, antitumor activity, and the relationship between TSH level or soluble (s) VEGFR-2 versus axitinib exposure in 18 patients compared with the 12 patients evaluated in the initial phase I study (Study 1) [11].

The objectives of this analysis were to (1) evaluate the safety of axitinib in Japanese patients, including effects on fatigue and thyroid function; (2) evaluate plasma pharmacokinetics following axitinib 5-mg, 7-mg and 10-mg single and continuous dosing; (3) investigate plasma levels of VEGF, sVEGFR-2, sVEGFR-3 and soluble stem cell factor receptor (sKIT) and their potential as biomarkers; and (4) assess the preliminary antitumor activity of axitinib in this patient population.

Methods

Patient population

Patients were eligible for these studies if they had histologically or cytologically diagnosed advanced malignant solid tumors and were either ineligible for, or refractory to, standard therapies; aged 20–75 years in Study 1 and ≥20 years in Study 2; expected survival ≥3 months; adequate bone marrow, renal and hepatic function; Eastern Cooperative Oncology Group performance status (ECOG PS) 0, 1 or 2; and no proteinuria (dipstick value <1+ or <500 mg per 24-h urine collection if ≥1+ in Study 1 and <1+ or <2 g per 24-h urine collection if ≥1+ in Study 2).

Patients with uncontrolled hypertension at screening (defined as blood pressure [BP] >130/80 mm Hg in Study 1 and >140/90 mm Hg in Study 2) were excluded, although use of antihypertensive agents was permitted. No anticancer therapy in the previous 4 weeks was allowed and no hormonal therapy, radiotherapy, oral fluorinated pyrimidine drug or a molecularly targeted agent was permitted for ≥2 weeks prior to initiation of axitinib.

Other key exclusion criteria included the presence of central lung lesions involving major blood vessels; active gastrointestinal (GI) bleeding or GI disorders that could affect axitinib ingestion or absorption; active seizure disorder or brain metastases with symptoms or requiring treatment; cardiovascular disease in the previous 12 months, including myocardial infarction, severe or unstable angina, coronary artery/peripheral artery bypass graft or symptomatic congestive heart failure; cerebrovascular disorders, such as transient cerebral ischemic attack, deep vein thrombosis or pulmonary embolus, in the previous 12 months; and grade ≥3 hemorrhage within 4 weeks or hemoptysis (>2.5 mL bright-red blood per day) within the week prior to enrollment. Patients were also excluded if they were receiving or likely to receive drugs or food that could act as potent cytochrome P450 (CYP) 3A4 inhibitors or CYP3A4 or CYP1A2 inducers, as were patients requiring anticoagulant therapy.

Study protocols were approved by the Institutional Review Board of the National Cancer Center, Tokyo, Japan (Study 1) and Kobe University Hospital, Kobe, Japan (Study 2). All patients provided written informed consent. The studies were conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization guidelines on Good Clinical Practice, as well as all applicable local regulatory requirements and laws.

The trials reported here are registered on the clinical trials site of the US National Cancer Institute website (Study 1, http://www.clinicaltrials.gov/ct/show/NCT00447005; Study 2, http://www.clinicaltrials.gov/ct/show/NCT00726752).

Drug administration

In Study 1 (N=12), the first six patients received a single 5-mg dose of axitinib. If well tolerated over the initial 48 h, they received continuous axitinib 5 mg BID for the first 28-day cycle (Fig. 1). Axitinib single 5-mg dosing was administered to the first 6 patients only in order to evaluate

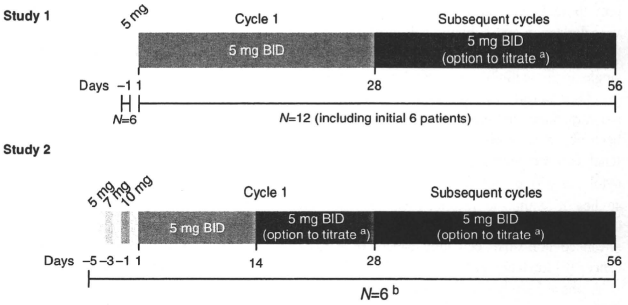

Fig. 1 Study schemas. *BID*, twice daily. [a] 5 mg BID could be titrated up to 7 mg BID, then 10 mg BID or interrupted/ reduced to 3 mg BID, then 2 mg BID. [b] All patients in Study 2 received axitinib at 5-mg, 7-mg and 10-mg single doses on days −5, −3 and −1

axitinib terminal-phase plasma half-life. The observation period (48 h) was set to evaluate the pharmacokinetics of axitinib up to 32 h after axitinib single 5-mg dosing. This observation period was also used in the first-in-human (FIH) study previously conducted in Caucasian patients. The same observation period was used to maintain consistency between both phase I studies conducted in Western and Japan. If axitinib was well tolerated with no treatment-related grade >2 adverse events occurring over a consecutive 2-week period in subsequent cycles, the dose of axitinib could be increased to 7 mg BID and then to a maximum 10 mg BID continuous dosing unless systolic BP (sBP) was >150 mm Hg, diastolic BP (dBP) >90 mm Hg and the patient was receiving antihypertension medication. The axitinib dose could be reduced to 3 mg BID and then to 2 mg BID in the event of treatment-related toxicity or patient intolerance. An additional six patients in this study were started on continuous axitinib dosing at 5 mg BID without receiving an initial single dose.

In Study 2 (N=6), patients received a single dose of 5 mg axitinib followed by a single dose of 7 mg and then 10 mg (Fig. 1). If these doses were safely administered, patients could receive continuous axitinib 5-mg BID dosing for the first 14 days. Subsequently, these patients also had the option of dose titration (increase or decrease) during the continuous dosing period.

In both studies, axitinib was administered orally in a fed state. A food effect study conducted in Western healthy volunteers demonstrated that lower plasma exposure of axitinib was obtained in the fed state versus overnight fasting state [12]. However, there was no difference in exposure of axitinib between the fed state and shorter fasting durations (i.e. 2 h before and 2 h after dosing, and 1 h before and 1 h after dosing, respectively). Since it was considered impractical for patients to be "overnight fasted" around each of the two daily doses of axitinib, and in order to avoid fluctuations in plasma exposures between morning and evening doses of the drug, axitinib administration in the fed state was recommended.

The axitinib dose was reduced or interrupted in both trials if drug-related toxicity occurred according to predefined dose-modification criteria. Axitinib treatment was interrupted or the dose was reduced if patients developed hemoptysis, hypertension (sBP >150 mm Hg or dBP >100 mm Hg) despite institution of maximal antihypertensive therapy or proteinuria (≥2 g/24 h). Patients continued axitinib therapy until disease progression, intolerable toxicity or withdrawal of consent.

Study assessments

Safety

Adverse events were assessed throughout the studies using Common Toxicity Criteria for Adverse Events, version 3.0. In Study 1, TSH measurements were performed every week in cycle 1, then every 2 weeks. In Study 2, antithyroperoxidase (anti-TPO) and antithyroglobulin antibody titers were determined at screening; TSH, free triiodothyronine (free T3), free thyroxine (free T4) and thyroglobulin were measured every week in cycle 1 and every 2 weeks thereafter. In addition, total cholesterol and creatine phosphokinase (CPK), which were potentially relevant to thyroid function, were also measured. Previous studies have found evidence of hypothyroidism in patients treated with other antiangiogenic agents, such as sunitinib, and related symptoms (including fatigue) improved with administration of thyroid hormone replacement therapy [6, 13]. In Study 2, thyroid function–test values were prospectively monitored at every visit, and thyroid hormone replacement therapy was proactively given to patients, as appropriate according to TSH levels, to maintain a euthyroid state.

Pharmacokinetics

To assess axitinib pharmacokinetics, blood samples were collected from patients who received 5-mg, 7-mg and 10-mg single doses (pre-dose and 0.5, 1, 2, 4, 6, 8, 10, 24 and 32 h

post dose) in Study 2. The blood samples were also collected during continuous dosing (pre-dose and 0.5, 1, 2, 4, 8 and 12 h post dose) at cycle 1 day 1 and cycle 1 day 15 in Study 1 and at cycle 1 day 15 in Study 2. Pharmacokinetic parameters evaluated for single doses included maximum plasma concentration (C_{max}), area under the plasma concentration–time curve (AUC) from time zero to infinity (AUC_{inf}), time to first occurrence of C_{max} (T_{max}) and terminal-phase plasma half-life ($t_{1/2}$). AUC over the dosing interval (AUC from time zero to 12 h [AUC_{12}]) and accumulation ratio (R_{ac}) were also measured following continuous dosing.

Blood samples were centrifuged at 1,000 ×g for 15 min and plasma was stored frozen at –20°C. Plasma samples were analyzed for axitinib concentration at Charles River Laboratories, Inc. (Worcester, MA, USA) using a validated high-performance liquid chromatography–tandem mass spectrometric method; the lower limit of quantification was 0.1 ng/mL.

Soluble biomarkers

Plasma samples were collected to assess changes in soluble protein biomarkers that might be associated with axitinib-induced VEGF/VEGFR inhibition. Plasma concentrations of VEGF, sVEGFR-2, sVEGFR-3 and sKIT were measured in patient blood samples at baseline and during treatment on day 1 of each cycle up to 12 cycles and at the end of treatment. Each biomarker was measured using an enzyme-linked immunosorbent assay (ELISA) kit at Alta Analysis (San Diego, CA, USA). Exploratory analyses were conducted to investigate the relationship between these endpoints and pharmacokinetics.

Preliminary antitumor activity

Antitumor activity was assessed at screening, every 8 weeks during continuous dosing and at the end of study, according to Response Evaluation Criteria in Solid Tumors (RECIST, version 1.0) [14].

Results

Patient demographics

Patient demographics and baseline characteristics for the two studies are shown in Table 1. Of the 18 patients enrolled in the two studies, the majority had an ECOG PS of 0 or 1. The most common tumor type was colorectal cancer. Most patients were heavily pretreated; 14/18 patients (78%) had received ≥3 prior systemic treatment regimens. Six patients were receiving concurrent antihypertension medication at the start of axitinib therapy.

Table 1 Patient demographics and baseline characteristics (pooled data from Study 1 and Study 2)

	N=18
Median age (range), y	63 (25–75)
Gender, *n* (%)	
Male	10 (56)
Female	8 (44)
ECOG PS, *n* (%)	
0	7 (39)
1	10 (56)
2	1 (6)
Tumor type, *n* (%)	
Colorectal cancer	6 (33)
Renal cell cancer	2 (11)
Liposarcoma	2 (11)
Other [a]	8 (44)
No. of prior systemic treatment regimens, *n* (%)	
1	3 (17)
2	1 (6)
3	5 (28)
4	4 (22)
≥5	5 (28)
Concurrent antihypertensive agent, *n* (%)	
Yes	6 (33)
No	12 (67)

[a] Ovarian cancer; non-small cell lung cancer; thymic cancer; synovial sarcoma; esophageal malignant melanoma; hypopharynx cancer; gastric cancer; pancreatic cancer

ECOG PS Eastern Cooperative Oncology Group performance status

Safety

Common treatment-related adverse events are shown in Table 2 and treatment-related laboratory abnormalities for the two studies are listed in Table 3. The most common adverse events (all grades) were fatigue, anorexia, diarrhea, hand–foot syndrome and hypertension. Grade 3/4 adverse events were infrequent and mainly limited to fatigue and hypertension. The most frequent treatment-related laboratory abnormality was increased TSH, but all cases were low-grade in severity. Proteinuria occurred in nine patients, with grade 3/4 proteinuria seen in two patients.

Pharmacokinetics

Plasma pharmacokinetic parameters following single and continuous dosing of axitinib in Study 2 (*N*=6) are shown in Table 4. The plasma pharmacokinetics following single dosing of 5 mg, 7 mg and 10 mg axitinib seemed to be generally linear. The plasma axitinib concentration profiles

Table 2 Common treatment-related adverse events (pooled data from Study 1 and Study 2; $N=18$)

Adverse event	All grades, *n* (%)	Grade 3/4, *n* (%)
Fatigue	15 (83)	5 (28)
Anorexia	13 (72)	0
Diarrhea	12 (67)	0
Hand–foot syndrome	12 (67)	1 (6)
Hypertension	11 (61)	6 (33)
Stomatitis	10 (56)	0
Hoarseness	9 (50)	0
Constipation	7 (39)	0
Rash	6 (33)	0
Epistaxis	5 (28)	0
Nausea	4 (22)	0
Headache	4 (22)	0

after single-dose administration are shown in Fig. 2. Following a single dose of axitinib, the plasma concentration reached a maximum (C_{max}) at ~4 h after administration and was eliminated with a half-life of 4.8–5.9 h. The mean (% coefficient of variation) R_{ac} after continuous dosing was 1.37 (28%), consistent with the value predicted from the mean half-life of axitinib.

Thyroid function

In the two studies, 16 patients (89%) experienced elevation of TSH above the upper limit of normal range. Changes in TSH, free T3, free T4 and thyroglobulin levels over time for patients in Study 2 are shown in Fig. 3a–e. While the kinetics of response varied, all three patients who continued treatment with axitinib for ≥3 months exhibited an increase in thyroglobulin levels between day 100 and day 200 following initiation of axitinib therapy, which subsequently returned to baseline (Fig. 3e). Normalization of thyroglobulin levels was associated with the elevation of TSH levels in these patients. One of six patients had positive antithyroglobulin antibody titers at baseline and another one patient tested positive for anti-TPO antibody in Study 2. Changes in thyroid function tests were observed in patients regardless of baseline antithyroglobulin/anti-TPO antibody status.

Thyroid hormone replacement therapy was proactively given to patients enrolled in Study 2, according to TSH levels measured at each visit, to maintain a euthyroid state. As shown in Table 5, while grade 3/4 fatigue occurred in 42% of patients in Study 1, no cases of grade 3/4 fatigue were noted in Study 2. Proactive administration of thyroid hormone replacement therapy appeared to prevent the development of axitinib-related grade 3/4 fatigue.

In Study 2, a slight TSH decrease below the lower limit of normal range was observed in two patients between day 120 and day 170, and between day 320 and day 340 (Fig. 3b). This observation occurred during thyroid hormone replacement therapy and TSH decrease normalized with dose reduction or a temporary interruption of thyroid hormone replacement therapy.

In the three patients in Study 2 who received at least three cycles of axitinib treatment, changes in TSH levels mirrored the kinetics of change in total cholesterol levels. Meanwhile, no clear correlation was observed between TSH levels and CPK.

The relationship between changes in levels of TSH and axitinib plasma AUC was also investigated. When data for patients in both studies were analyzed, TSH change (baseline to cycle 2 day 1) was found to increase linearly with AUC_{12}, with a correlation coefficient r of 0.723 (95% confidence interval [CI] 0.361 to 1.000; $P=0.0180$) (Fig. 4).

Soluble plasma biomarkers

Table 6 shows changes in plasma concentrations of VEGF, sVEGFR-2, sVEGFR-3 and sKIT from baseline to cycle 2 day 1 in both studies. Significant increases in median VEGF levels (+242%) and decreases in median plasma sVEGFR-2 and sVEGFR-3 levels (–38% and –53%, respectively) were observed. An inverse relationship was seen between percent change in sVEGFR-2 levels (baseline to cycle 2 day 1) and axitinib AUC_{12} with a correlation coefficient r of –0.940 (95% CI –1.000 to –0.841; $P<0.0001$) (Fig. 5).

Table 3 Common treatment-related laboratory abnormalities (pooled data from Study 1 and Study 2; $N=18$)

Adverse event	All grades, *n* (%)	Grade 3/4, *n* (%)
Non-hematologic		
TSH increased	13 (72)	0
Proteinuria	9 (50)	2 (11)
Hematuria	7 (39)	0
Lipase increased	6 (33)	0
Blood glucose increased	5 (28)	0
ALP increased	4 (22)	1 (6)
AST increased	4 (22)	0
TSH decreased	4 (22)	0
Hematologic		
Platelet count decreased	6 (33)	0
Neutrophil count decreased	3 (17)	1 (6)
Lymphocyte count decreased	1 (6)	0

ALP alkaline phosphatase; *AST* aspartate aminotransferase; *TSH* thyroid-stimulating hormone

Table 4 Pharmacokinetic parameters following single and continuous dosing of axitinib (Study 2; *N*=6)

Single dosing				
Dose	Mean [a] (%CV) C_{max}, ng/mL	Mean [a] (%CV) AUC_{Inf}, ng·hr/mL	Mean [a] (%CV) $t_{1/2}$, h	Median (min, max) T_{max}, h
5 mg	17.0 (70)	142 (86)	4.8 (59)	4.10 (3.95, 6.02)
7 mg	23.3 (88)	181 (80)	5.1 (51)	4.00 (0.983, 9.88)
10 mg	34.9 (115)	288 (91)	5.9 (59)	4.02 (2.05, 6.00)
Continuous dosing (cycle 1 day 15)				
Dose	Mean [a] (%CV) C_{max}, ng/mL	Mean [a] (%CV) AUC_{12}, ng·hr/mL	Median (min, max) T_{max}, h	Mean [a] (%CV) R_{ac}
5 mg BID	21.4 (84)	138 (78)	4.04 (3.93, 7.70)	1.37 (28)

[a] Values are measured in geometric mean for C_{max}, AUC_{inf}, AUC_{12}, and R_{ac} and arithmetic mean for $t_{1/2}$

%CV percent coefficient of variation; AUC_{12} area under the plasma concentration–time curve from time zero to 12 h; AUC_{Inf} area under the plasma concentration–time curve from time zero to infinity; *BID* twice daily; C_{max} maximum plasma concentration; R_{ac} accumulation ratio; $t_{1/2}$ terminal-phase plasma half-life; T_{max} time to first occurrence of C_{max}

Preliminary antitumor activity

The majority of patients (n=16; 89%) had disease progression before enrollment. Following treatment with axitinib, a reduction in tumor size was observed in 11 of 17 (65%) evaluable patients who had a target lesion. Tumor size decreased by≥30% in two patients with colorectal cancer and by≥20% in two patients (one each with NSCLC and thymic cancer). Additionally, stable disease (≥24 weeks) was noted in six patients (one each with colorectal cancer, NSCLC, thymic cancer, synovial sarcoma, esophageal malignant melanoma and pancreatic cancer).

Discussion

Preliminary population pharmacokinetic analyses of cytokine-refractory mRCC patients indicated that patients who achieved a higher exposure to axitinib (AUC) while receiving 5 mg BID had higher response rate and longer overall survival. Additionally, retrospective analyses of two clinical trials of axitinib in mRCC demonstrated that patients who developed at least one dBP measurement ≥90 mm Hg during therapy had a longer overall survival, higher probability of a partial response and greater decreases in tumor size [15]. These findings suggested that increasing the axitinib dose above a starting dose of 5 mg BID in a select group of patients who tolerate the drug without experiencing hypertension may result in improved efficacy. Thus, in axitinib clinical trials, dose titration from a starting dose of 5 mg BID to 7 mg BID and then to a maximum 10 mg BID is recommended if no treatment-related adverse events grade >2 are observed and BP is ≤150/90 mm Hg for 2 consecutive weeks with axitinib 5 mg BID. However, the pharmacokinetic profile following 5-mg, 7-mg and 10-mg single dosing of axitinib has not been evaluated in either Western or Japanese subjects. In Study 2, axitinib pharmacokinetics following 5-mg, 7-mg and 10-mg single dosing was further evaluated.

Single and continuous dosing of axitinib was generally well tolerated in the Japanese patients studied here. The adverse event profile was similar to that seen in other studies of axitinib in Caucasian populations [5–9, 16]. Toxicities associated with axitinib therapy were generally manageable in the current studies. Major grade 3/4 adverse events included fatigue and hypertension. Fatigue (all grades) occurred in 83% of patients in the two studies, with grade 3/4 fatigue seen in 42% of patients in Study 1. Comparable rates of fatigue were reported by Rini et al [6] in a study of

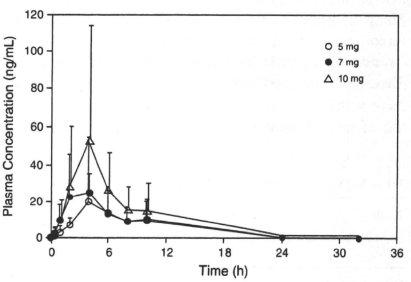

Fig. 2 Axitinib plasma concentration over time following single dosing in Study 2; pharmacokinetic parameters were measured following a single dose of axitinib (*N*=6)

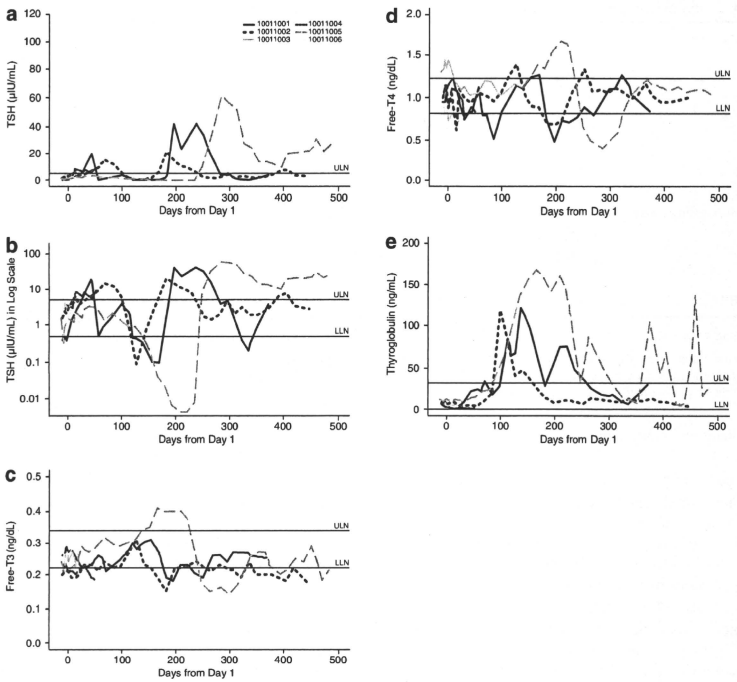

Fig. 3 Changes over time in Study 2 patient laboratory levels: **a** thyroid-stimulating hormone (absolute values); **b** thyroid-stimulating hormone (log scale); **c** free triiodothyronine (free T3); **d** free thyroxine (free T4); **e** thyroglobulin. *LLN* lower limit of normal range; *ULN* upper limit of normal range

axitinib in sorafenib-refractory RCC. For the six patients in Study 2, proactive thyroid hormone replacement therapy (according to TSH levels) seemed useful to prevent the development of grade 3/4 fatigue related to axitinib treatment. These results suggest that axitinib-related grade 3/4 fatigue may be preventable through careful monitoring of TSH levels and use of thyroid hormone replacement therapy as needed to maintain a euthyroid state. It is known that anemia is frequently associated with hypothyroidism [17, 18]. In the present two phase I studies, anemia was not reported as an adverse event. Elevation of hemoglobin was reported in patients treated with VEGFR inhibitors such as axitinib, sunitinib and sorafenib [11, 19–21]. This could obscure the development of anemia-associated hypothyroidism, and it thus may be difficult to investigate the relationship between anemia and hypothyroidism with the treatment of VEGFR inhibitors.

Table 5 Treatment-related fatigue in Study 1 and Study 2

Study	Fatigue	
	All grades, *n* (%)	Grade 3/4, *n* (%)
Study 1 (*N*=12)	10 (83)	5 (42)
Study 2 (*N*=6) [a]	5 (83)	0

[a] Four patients received thyroid hormone replacement therapy

Elevation of thyroglobulin was observed in all three patients who continued treatment with axitinib for ≥3 months in Study 2. Thyroglobulin is synthesized by the thyroid gland to produce the thyroid hormones T4 and T3. Increased serum levels of thyroglobulin can suggest Graves' disease or some forms of thyroiditis such as subacute thyroiditis or destructive thyroiditis. The observed changes in thyroglobulin suggest that transient subacute thyroiditis and subsequent hypothyroidism

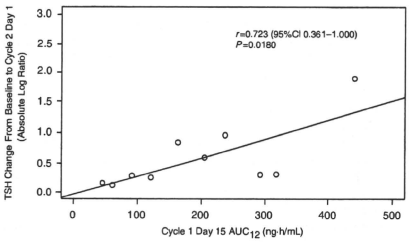

Fig. 4 Relationship between change in thyroid-stimulating hormone (TSH) levels from baseline to cycle 2 day 1 (absolute log ratio) and AUC_{12} in Studies 1 and 2 (pooled data): $n=10$—eight patients were excluded (two with abnormal baseline TSH; three received thyroid hormone replacement therapy or had axitinib dose titrated before cycle 2 day 1; two discontinued treatment before cycle 2 day 1; and one did not receive cycle 1 day 14 AM dose). AUC_{12} area under the plasma concentration–time curve from time zero to 12 h; *CI* confidence interval

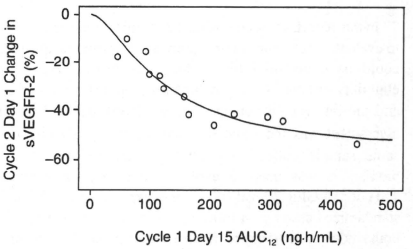

Fig. 5 Relationship between percentage change in soluble VEGFR-2 level from baseline to cycle 2 day 1 and AUC_{12} in Studies 1 and 2 (pooled data): $n=13$—five patients were excluded (two discontinued treatment before cycle 2 day 1; two had axitinib dose titrated before cycle 2 day 1; and one did not receive cycle 1 day 14 AM dose). AUC_{12} area under the plasma concentration–time curve from time zero to 12 h; *VEGFR-2* vascular endothelial growth factor receptor-2

observed following axitinib treatment could be due to a direct effect of this agent on the thyroid gland.

The mechanism for axitinib-induced hypothyroidism is currently unclear; however, *in vitro* and *in vivo* studies have demonstrated that VEGF and VEGFR mRNA and protein are expressed in normal thyroid follicular cells, which is mediated in part by thyroid-stimulating hormone [22–24]. These data suggest it is possible that axitinib, a VEGFR inhibitor, decreases thyroid function by interfering with VEGF function and/or impairing thyroid blood flow, resulting in hypothyroidism. It has been postulated that hypothyroidism associated with sorafenib may be caused by altered thyroid hormone metabolism, increasing type 3 deiodination [25]. Hypothyroidism and thyroid dysfunction are commonly observed with sunitinib (36–85% of patients) and sorafenib (~20%) [13, 26–29]. A study of 16 patients with mRCC treated with sunitinib, sorafenib or axitinib for ≥12 months found that thyroid dysfunction was a common toxicity, occurring in ~40% of patients [30]. Development of severe hypothyroidism associated with use of these agents in mRCC is infrequent, however, and typically can be corrected by use of thyroid hormone replacement therapy. Regular monitoring of thyroid-function abnormalities and use of thyroid hormone replacement therapy in patients receiving axitinib and related tyrosine kinase inhibitors is therefore indicated.

Table 6 Change in plasma concentration of biomarkers from baseline to cycle 2 day 1 (pooled data from Study 1 and Study 2; $N=16$ [a])

Biomarker	% Change in concentration from baseline to cycle 2 day 1, median (range)
VEGF	+242 (15 to 1446)
sVEGFR-2	–38 (–58 to –10)
sVEGFR-3	–53 (–94 to –16)
sKIT	–2.9 (–24 to 34)

[a] Two patients who discontinued treatment before cycle 2 day 1 were excluded

sKIT soluble stem cell factor receptor; *VEGF* vascular endothelial growth factor; *sVEGFR-2/3* soluble VEGF receptor-2/3

In Study 2, it was possible to maintain a euthyroid state in hypothyroid patients with careful monitoring of TSH and thyroid hormone dose adjustments. The decreases in sensitive TSH below the lower limit of normal range during thyroid hormone replacement therapy were not associated with any symptoms of hyperthyroidism and were successfully managed with temporary interruption or dose reduction of thyroid hormone.

The time-course of change in TSH levels and that of total cholesterol appeared to be correlated in some patients. This finding suggests that the observed changes in total cholesterol values were due to axitinib-related hypothyroidism.

A correlation between occurrence of hypothyroidism and progression-free survival was seen in some studies of sunitinib in mRCC [31, 32]. Tyrosine kinase inhibitor–induced thyroid dysfunction, therefore, has been proposed as a possible surrogate marker of efficacy with antiangiogenic tyrosine kinase inhibitors, rather than being viewed as an unwanted toxicity [31, 33]. In the current phase I studies, increased TSH levels were highly correlated with exposure to axitinib ($r=0.72$). Additionally, a significant correlation was noted between change in sVEGFR-2 level and exposure to axitinib ($r=-0.94$). Other clinical trials of axitinib have also observed preferential decreases in sVEGFR levels in patients with thyroid cancer [8] or poor-prognosis acute myeloid leukemia/myelodysplastic syndrome [16]. These data suggest that both sVEGFR-2 and TSH levels may serve as biomarkers of axitinib exposure.

In this report, we used pooled data from two phase I studies to evaluate safety, antitumor activity and biomarkers, and thus could have potential biases for these results. However, eligibility criteria in these two phase I studies were similar, and the interval for patient visit, laboratory test and tumor assessment was the same between the two protocols. The same sample collection point for pharmacokinetics and biomarkers was used to explore the correlation between TSH or sVEGFR-2 level and axitinib exposure. In addition, a standardized case report form was used for data collection in both studies, which may reduce measurement and/or reporting biases. Therefore, biases introduced by using pooled data to evaluate these data could not be substantial.

In conclusion, these results demonstrate that single and continuous dosing of axitinib were generally well tolerated in Japanese cancer patients. While axitinib caused thyroid dysfunction (hypo- and hyperthyroidism), grade 3/4 fatigue and hypothyroidism appear to be controllable by the use of thyroid hormone replacement therapy. Since changes in sVEGFR2 and TSH levels correlated with axitinib plasma levels, both sVEGFR2 and TSH may potentially act as biomarkers of axitinib exposure. Axitinib showed antitumor activity across multiple tumor types in Japanese patients with solid tumors. Further clinical investigation is necessary to clarify the clinical usefulness of these potential biomarkers.

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N-myc downstream-regulated gene 1/Cap43 expression promotes cell differentiation of human osteosarcoma cells

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Abstract. The N-myc downstream regulated gene 1 (NDRG1)/Cap43 is closely associated with cell differentiation, and its expression is induced by hypoxia and increasing intracellular calcium levels. Whether the NDRG1/Cap43 expression in cancer cells is a predictive marker of good or poor prognosis in patients, depends upon tumor types and differentiation status. In this study, we examined whether the NDRG1/Cap43 expression was involved in the differentiation of osteosarcoma cells, using three osteosarcoma cell lines, MG63, U2OS and SaOS2. The NDRG1/Cap43 expression in MG63 and U2OS was significantly enhanced by vitamin D_3, which also induced the production of osteocalcin, a differentiation marker of osteoblasts. The knockdown of NDRG1/Cap43 using small interfering RNA also suppressed the production of osteocalcin and enhanced cell proliferation, accompanied by the suppression of p21 expression. Furthermore, the acquired invasiveness of osteosarcoma cells during the invasion in Matrigel resulted in the decreased expression of NDRG1/Cap43. On the basis of these results, our proposed role for NDRG1/Cap43 would be in the capacity of differentiation and invasion in osteosarcoma cells.

Introduction

The N-myc downstream regulated gene 1 (NDRG1)/Cap43 gene, also known as Ndrg-1 (1), Drg-1 (2), RTP (3), and RIT42 (4), was isolated using the differential display technique, as was done previously with the gene induced by nickel compounds in human bronchoalveolar epithelial cells (5). This gene expresses a 3.0-kb mRNA encoding a 43-kDa protein. The NDRG1/Cap43 protein possesses three unique 10-amino-acid tandem repeats at the COOH-terminal end. Analysis of the amino acid sequence predicted that the protein contains seven or more phosphorylation sites and that it is phosphorylated by protein kinase A (6). Many factors and chemical agents control the expression of NDRG1/Cap43. The expression of NDRG1/Cap43 is repressed by c-myc and N-myc/Max complex *in vitro* (1). p53 induces the expression and nuclear translocation of NDRG1/Cap43 in response to DNA-damaging agents (4). In addition, NDRG1/Cap43 is up-regulated by androgens (7) and various chemical agents including homocysteine and nickel compounds. The NDRG1/Cap43 expression is mostly observed in epithelial cells (8). NDRG1/Cap43 is also an essential factor for the maintenance of myelin sheaths in peripheral nerves (9). In particular, NDRG1/Cap43 is associated with the terminal differentiation of Schwann cells during nerve regeneration (10), and also with the differentiation of the kidney and brain (11). NDRG1/Cap43 is expressed at lower levels in tumor cells including prostate, breast and colorectal cancers than in normal tissue (2,4,12), and can suppress metastasis by inducing cell differentiation and reversing the metastatic phenotype (13,14). However, NDRG1/Cap43 is highly expressed in certain tumors, such as hepatocellular and colorectal carcinoma (15,16). The NDRG1/Cap43 expression is positively associated with tumor angiogenesis in cervical adenocarcinoma (17). The positive or negative association between NDRG1/Cap43 and tumor angiogenesis depends upon tumor types and differentiation status (18). Although there is certain evidence that NDRG1/Cap43 is involved in cell differentiation and in the metastasis of cancer cells, little is known about the relationship between the NDRG1/Cap43 expression and the differentiation of mesenchymal cells.

Vitamin D_3 can induce normal and leukemic hematopoietic cells to differentiate into cells with mature monocyte/macrophage phenotypes, and can also induce a block in cell proliferation (19,20). Vitamin D_3 also affects cell differentiation in other cell types, including poorly differentiated

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Key words: N-myc downstream regulated gene 1, Cap43, osteosarcoma, small interfering RNA

keratinocytes (21), prostate cancer (22), and several breast cancer cells (23). Vitamin D_3 is essential for calcium homeostasis and bone metabolism, and plays an important role in osteoblast phenotypes and in the differentiation of osteosarcoma cells. We have previously demonstrated that vitamin D_3 induces cell differentiation by the transcriptional activation of p21 in p53-deficient osteosarcoma cells (24).

In the present study, we first examined whether NDRG1/Cap43 could play a critical role in cell differentiation in osteosarcoma cells treated with vitamin D_3. On the basis of our results, using small interfering RNA (siRNA) against human NDRG1/Cap43, we showed how NDRG1/Cap43 plays a role in cell differentiation and invasion by osteosarcoma cells.

Materials and methods

Cell culture and treatment. The human osteosarcoma cells, MG63, SaOS2 and U2OS, were used in this study. The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) (Nissui, Japan) supplemented with 10% (v/v) fetal calf serum (FCS) (Bioserum, Victoria, Australia), 100 units/ml penicillin, and 10 mg/ml streptomycin (Gibco/Life Technologies, Gettysburg, MD, USA).

In vitro cell proliferation assay. The cells were seeded into a 96-well culture plate ($1x10^4$ cells/well) and incubated for one day in a medium containing 0.1 % FCS and indicated concentrations of vitamin D_3. Cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. In order to estimate the influence of the NDRG1/Cap43 knockdown on cell proliferation in the U2OS cells, the cells ($5x10^3$/well) were plated in 6-well plates in a medium supplemented with 10% serum. The cells were trypsinized and counted at 0, 1, 3 and 5 days with a coulter counter (Beckman Coulter, Miami, FL).

Production of osteocalcin by osteosarcoma cells. For the measurement of osteocalcin, the cells were seeded in a 6-well culture plate and cultured to 70% confluence. The cells were washed three times with phosphate buffered saline (PBS) and incubated in serum-free medium for 24 h before the experiment. The cells were incubated for 36 h in a medium containing 0.1% FCS and indicated concentrations of vitamin D_3. After the treatment, the medium was collected for the measurement of osteocalcin. This was performed using an Osteocalcin EIA kit (Takara, Japan).

NDRG1/Cap43 siRNA expression vector construction and establishment of NDRG1/Cap43 siRNA expressing cells. The pcDNA3_GFP_hU6 siRNA expression plasmid was kindly provided by Dr H. Izumi (University of Occupational and Environmental Health, Kitakyushu, Japan). In order to obtain the pcDNA3_GFP_hU6/Cap43 siRNA expression plasmid, the following double-stranded oligonucleotides were inserted into the pcDNA3_GFP_hU6 siRNA expression plasmid (Invitrogen, San Diego, CA) designed for *Bam*HI and *Hin*dIII. The NDRG1/Cap43 siRNA oligonucleotides were as follows: sense, 5'-GATCCGCGTGAACCCTTGTGCGGAA TTCAAGAGATTCCGCACAAGGGTTCACGTTTT TTGGAAA-3'; and antisense, 5'-AGCTTTTCCAAAAAAC GTGAACCCTTGTGCGGAATCTCTTGAATTCCGCA CAAGGGTTCACGCG-3' (Invitrogen). The Cells were transfected with the pcDNA3_GFP_hU6/Cap43 siRNA expression plasmid (NDRG1/Cap43 siRNA) or the pcDNA3_GFP_hU6 siRNA expression plasmid (mock) using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

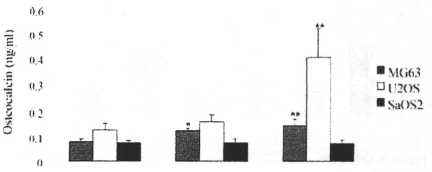

Figure 1. Effects of vitamin D3 on the production of osteocalcin in MG63, U2OS and SaOS2 cells. The cells were incubated in a medium containing 0.1% FCS with or without various concentrations of vitamin D_3 ($5x10^{-8}$ and $1x10^{-7}$ mol/l) for 36 h, and the conditioned medium was then collected. The measurement of osteocalcin was performed using an Osteocalcin EIA kit.

Western blot analysis. The cells were washed with ice-cold PBS, scraped from the plates, lysed, and harvested on ice in RIPA buffer supplemented with PMSF, protease inhibitor cock-tail and sodium orthovanadate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The homogenates were clarified by centrifugation at 15,000 x g for 15 min at 4°C, and the supernatant was collected. The proteins were separated using SDS-PAGE, loading 50 μg aliquots of protein into each well, and transferred to Immobilon membranes (Millipore, Bedford, MA). After the transfer, the blots were incubated with blocking solution and probed with antibodies. The antibodies used were the following: Rabbit polyclonal antibody directed against NDRG1/Cap43, mouse monoclonal anti-p21 Waf1/Cip1 (DCS60) antibody (Cell Signaling Technology, Beverly, MA), and mouse monoclonal anti-GAPDH antibody (Sigma-Aldrich, St. Louis, MO).

Statistical analysis. The significance of the differences in cell proliferation and the production of osteocalcin was estimated using the unpaired Student's t-test. The results were recognized as statistically significant at a probability value of $p<0.05$.

Results

Effects of vitamin D_3 on cell differentiation, cell proliferation and NDRG1/Cap43 expression. We first examined whether vitamin D_3 could induce cell differentiation in three osteosarcoma cell lines, MG63, U2OS and SaOS2. The secretion of osteocalcin is a representative biomarker of cell differentiation in osteoblasts. Osteocalcin was increased in a dose-dependent manner by vitamin D_3 in MG63 and U2OS, but not in SaOS2 (Fig. 1). There was an ~2-fold and 4-fold increase in the production of osteocalcin by vitamin D_3 in MG63 and U2OS, respectively. In all three cell lines, cell proliferation was not affected after exposure to vitamin D_3 (Fig. 2).

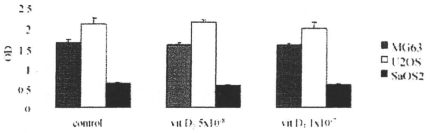

Figure 2. Effects of vitamin D_3 on the cell proliferation in the MG63, U2OS and SaOS2 cell lines. The cells were incubated in a medium containing 0.1% FCS and various concentrations of vitamin D_3 (none, $5x10^{-8}$ and $1x10^{-7}$ mol/l) for 48 h, and the effects were analyzed using an MTT assay. $^{*}p<0.05$, $^{**}p<0.01$.

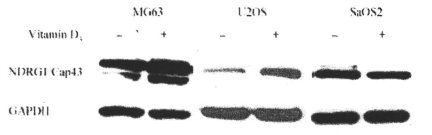

Figure 3. Effects of vitamin D_3 on the expression of NDRG1/Cap43 in the MG63, U2OS and SaOS2 cell lines. Cells ($1x10^5$) were plated in 6-well plate and cultured in DMEM containing 0.1% FCS with or without vitamin D_3 ($1x10^{-7}$ mol/l) treatment. The expression of NDRG1/Cap43 was analyzed by Western blot analysis.

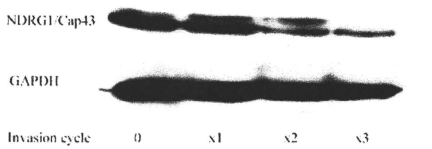

Figure 4. The decreased expression of NDRG1/Cap43 in highly invasive SaOS2 cells. The cells ($2x10^5$) were plated onto a coated filter in a Matrigel Invasion chamber. Migrated cells were collected after 24-h incubation, and were cultured under normal conditions. Subsequently, invasive cells were also incubated in the chamber for 24 h. This procedure was performed three times. A fraction of the cells was collected and examined for NDRG1/Cap43 expression via Western blot analysis.

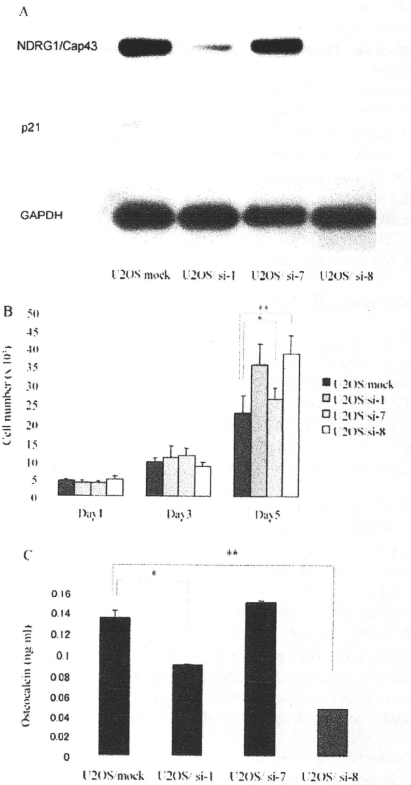

Figure 5. The effects of siRNA transfection on the expression of NDRG1/Cap43 and p21 and the production of osteocalcin. Three human osteosarcoma cell lines constitutively expressing NDRG1/Cap43 siRNA, U2OS/si-1, U2OS/si-7 and U2OS/si-8, derived from human osteosarcoma U2OS cells, were established. The expression of the NDRG1/Cap43 protein was decreased in both the U2OS/si-1 and U2OS/si-8 cells compared to the U2OS/si-7 and the parental cells. The p21 protein was also suppressed in these cell lines (A), resulting in an increase in cell proliferation in the U2OS/si-1 and U2OS/si-8 cells (B). The osteocalcin protein level was determined using EIA kits. The production of osteocalcin was significantly suppressed by the decreased expression of NDRG1/Cap43 in U2OS/si-1 and U2OS/si-8 cells (C). Each value represents the mean concentration of osteocalcin ± SD. $^{*}p<0.05$, $^{**}p<0.01$.

Western blot analysis showed the expression of NDRG1/Cap43 in MG63, SaOS2, and U2OS under exponentially growing conditions. The NDRG1/Cap43 expression was enhanced by vitamin D_3 in MG63 and U2OS, but not in SaOS2 (Fig. 3).

NDRG1/Cap43 expression and invasiveness of osteosarcoma cells. The *in vitro* invasion assay was designed to test whether the invasive potential of the tumor cells had any correlation with the NDRG1/Cap43 expression and cell differentiation in a Matrigel-coated transwell chamber. SaOS2 was found to have the greatest invasive potential among the three cell lines (data not shown), and therefore we used SaOS2 for the invasion assay in this study. We isolated SaOS2x1, SaOS2x2 and SaOS2x3 at each cycle of the invasion assay, and compared the expression levels of NDRG1/Cap43 in those cell lines. There was a markedly decreasing expression of NDRG1/Cap43 during the acquirement of the invasive potential by the SaOS2 cells. NDRG1/ Cap43 often showed two major bands: The upper band was phosphorylated and the lower band was relatively phosphorylated, as the treatment with phosphatase resulted in the almost complete disappearance of the upper band of NDRG1/ Cap43 (unpublished data). There was a much lower