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厚生労働科学研究費補助金

難病・がん等の疾患分野の医療の実用化研究事業
（がん関係研究分野）

進行非小細胞肺癌を対象としたエルロチニブとYM155の分子標的治療薬併用第Ⅰ相試験

平成24年度　総括研究報告書

研究代表者　中　川　和　彦

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厚生労働科学研究費補助金研究報告書表紙

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厚生労働科学研究費補助金

難病・がん等の疾患分野の医療の実用化研究事業

進行非小細胞肺癌を対象としたエルロチニブとYM155の分子標的治療薬併用第I相試験

平成２４年度　総括研究報告書

研究代表者　中川　和彦

平成２５（２０１３）年　３月

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別紙３

厚生労働科学研究費補助金（難病・がん等の疾患分野の医療の実用化研究事業）
総括研究報告書

進行非小細胞肺癌を対象としたエルロチニブとYM155の分子標的治療薬併用第I相試験

研究代表者　中川　和彦
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研究要旨　進行非小細胞肺癌患者を対象に、EGFRチロシンキナーゼ阻害剤(EGFR-TKI)エルロチニブに併用するYM155の推奨投与量の設定、及び用量制限毒性（DLT）を明らかにし、推奨投与量における安全性と抗腫瘍効果、及び効果に関わるバイオマーカーを探索する

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Ａ．研究目的

EGFR陽性進行非小細胞肺癌患者を対象に、EGFRチロシンキナーゼ阻害剤(EGFR-TKI)エルロチニブに併用するYM155の推奨投与量の設定、及び用量制限毒性(DLT)を明らかにし、推奨投与量における安全性と抗腫瘍効果、及び効果に関わるバイオマーカーを探索する。

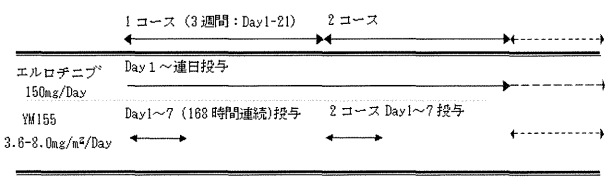
Ｂ．研究方法

[研究計画・方法]
分子標的治療薬併用第Ｉ相臨床試験(医師主導治験)として、EGFR陽性進行非小細胞肺癌に対する化学療法を受ける患者を対象にエルロチニブとYM155併用投与の両薬剤推奨投与量の設定、用量制限毒性（DLT）および最大耐用量（MTD）を明らかにし、両分子標的治療薬の推奨投与量における安全性と抗腫瘍効果について検討する。

[対象症例]
EGFR陽性進行非小細胞肺癌に対する化学療法を受ける患者、20歳以上、ECOG Performance Status（PS）0-2、主要臓器機能が保持された症例。患者本人の自由意思による文書同意を必須とする。

【Primary endpoint】
エルロチニブとYM155併用投与の安全性プロファイル（有害事象）、用量制限毒性（DLT：dose limiting toxicity）、最大耐用量（MTD：maximum tolerated dose）および推奨投与量の決定。

【Secondary endpoint】
推奨投与量における安全性と抗腫瘍効果、及び抗腫瘍効果に関わるバイオマーカーの探索。



エルロチニブは１日１錠（150mg）の連日投与とし、YM155（アステラス製薬より供給予定）は（シリンジポンプを用いた）168時間（7日間）の持続点滴静脈内投与とする。併用治療開始時点を1コースday１とする。エルロチニブは連日投与、YM155は1週間(168時間)投与2週間休薬をもって1コース（21日間隔）とする。以後、腫瘍の増悪、新病変の出現または投与継続が困難な有害事象の発現を認めるまで、1コースを21日間隔として治療を継続する。パート1(dose escalation cohort)の症例では、プロトコール本文に記載のスケジュールにてエルロチニブ及びYM155の薬物動態測定を行う。また同意が得られた患者に対し、抗腫瘍効果に関わるバイオマーカーの探索として1)YM155投与前後における腫瘍組織中のサバイビン蛋白質量の測定とアポトーシス誘導の有無を確認、2)肺癌組織の体細胞変異解析にあたり、LungCarta、Bio-plex（Ligand panel)等のマススクリーニングパネルを用いた半網羅的体細胞変異解析を行う

[予定症例数及び研究期間]
医師主導治験による第Ｉ相臨床試験として、12-24例。試験期間は2012年12月1日より2015年11月31日（準備期間：1年、登録期間:１年、追跡期間：1年）とする。

［研究体制］
研究代表者（医師主導治験実施責任者）は研究の統括・計画を実施する。研究分担者は近畿大学医学部腫瘍内科において研究の計画・測定・解析を実施、症例登録を行う。バイオマーカーの測定は近畿大学医学部ゲノム生物学教室で測定する。近畿大学医学部・医学部附属病院臨床研究センターおよび外部CROであるクインタイルズ・ジャパン・データマネジメント部および日本臨床研究オペレーションズ（JCRO）は近畿大学医学部腫瘍内科と共同して本医師主導治験運用に必須であるセンターデータマネージメント、モニタリング業務、治験薬管理（薬剤供与企業との連携）、CRC業務およびローカルデータマネージメント業務を遂行する。統計解析は近畿大学医学部臨床研究管理センター腫瘍統計学部門および外部CROであるクインタイルズ・ジャパン・データマネジメント部が行う。研究実施環境については研究施設・研究資料・研究フィールド・現在の研究環境の状況等インフラ整備されており問題はない。

（倫理面への配慮）
試験に関係するすべての研究者は、ヘルシンキ宣言および臨床研究に関する倫理指針にしたがって本試験を実施し，以下の事項を厳守する．
1．登録に先立って、すべてに患者に施設の倫理審査委員会（IRB）承認が得られた説明文書を用いて十分な説明を行い，考慮の時間を設けた後に患者自身の自由意志による同意を文書にて取得るする。
2．個人情報および診療情報などのプライバシーに関する情報は個人の人格尊重の理念の下、厳重に保護され慎重に取り扱われるべきものと認識し、万全な管理対策を講じ、プライバシー保護に努める。データの取り扱いに関しては直接個人を識別できる情報を用いず，データベースのセキュリティーを確保し，個人情報の保護を厳守する。
本研究に組み込まれるバイオマーカー研究は蛋白発現、体細胞DNAを対象に解析するものであり、「ヒトゲノム・遺伝子解析研究に関する倫理指針」の対象ではないが、その趣旨を踏まえた対応を行う。

C．研究結果
研究計画に関する現在までの研究成果・取組進行状況は以下に示す通りである。平成24年6月27日本研究計画に関する採択通知受領後、直ちに同年 7月より同医師主導治験実施体制準備開始に至り10月23日施設内治験審査委員会（IRB）承認を得たのちに11月22日医薬品医療機器総合機構（PMDA当局）へ治験届を提出、12月10日PMDA当局より審査承認確認を得た。平成24年 12月12日第1回目サイトトレーニング（CRC・薬剤師等を対象）施行、12月20日治験キックオフミーティングを兼ねた第2回目サイトトレーニング（医師・CRC・看護師・薬剤師等対象）施行、12月21日最終CRFフォーム固定（クインタイルズ・ジャパン・データマネジメント部）、12月21日アステラス製薬より 近畿大学医学部附属病院薬剤部へ治験薬（YM155）搬入完了。12月25日クインタイルズ・トランスナショナル・ジャパンと同治験委受託契約完了を行った。平成25年1月4日PK（薬物動態測定解析用）用検査キット米国より輸入通関完了（Advion/Quintiles and PPD, USA)。平成25年1月23日日本臨床研究オペレーションズ（JCRO)と同治験業務委受託契約完了。平成25年1月25日第1コホート第1症例の治験登録平成25年1月29日第1症例の第 1サイクル投与開始。平成25年2月6日第2症例の第 1サイクル投与開始。平成25年2月19日第3症例の第 1サイクル投与開始。第1コホートレベル（治験薬YM155 3.6mg/㎡/day）では用量制限毒性（DLT）発現及び臨床上有意な毒性を全3症例において認めず、平成25年3月25日に効果安全性委員会を開催、同委員会の外部委員による審査にて次コホート（第2コホートレベル）への用量増加が承認され、平成25年4月より第2コホート症例の治験登録が開始予定とされた。同治験薬剤のCIOMSフォームを用いた海外における有害事象（SAE）報告に関しても近畿大学医学部腫瘍内科、クインタイルズ・ジャパンおよび日本臨床研究オペレーションズ（JCRO）による海外SAE報告プロセスのSOPに従いPMDAへの定期報告を行っている。

D．考察
第1コホートレベル（治験薬YM155 3.6mg/㎡/day）では用量制限毒性（DLT）発現及び臨床上有意な毒性を全3症例において認めず、既に1回目の外部委員による効果安全性委員会承認を経て次年度以降（平成25年4月以降）は治験プロトコールに準じて予定通り次コホート（第2コホートレベル）への用量増加による治験登録が開始予定とされている。また、治験薬投与前後の腫瘍組織採取（気管支鏡生検等）も既に採取施行可能例には被験者の同意取得のもとに実施されており、抗腫瘍効果に関わるバイオマーカーの探索として1)YM155投与前後における腫瘍組織中のサバイビン蛋白質量の測定とアポトーシス誘導の有無を確認、2)肺癌組織の体細胞変異解析にあたり、LungCarta、Bio-plex（Ligand panel)等のマススクリーニングパネルを用いた半網羅的体細胞変異解析を行うための病理組織サンプルを病院病理部にて保管中である。

E．結論
未承認薬を用いた分子標的治療薬併用医師主導治験（第Ⅰ相試験）の実施運用に関する施設内インフラ体制は概ね整備が整っている状況となっている。
近畿大学医学部附属病院腫瘍内科単施設にて治験開始後初めの1ヶ月以内で第1レベルの合計3症例全ての治験症例登録完了および治験薬投与開始が実施出来ており、症例集積に関しても何ら問題の無い状況である。第1コホートレベル（治験薬YM155 3.6mg

/㎡/day）では用量制限毒性（DLT）発現及び臨床上有意な毒性を3症例全てにおいて認めず安全性・忍容性を確認済みであり、引き続き平成25年度においても当初の研究計画書・治験実施計画書に基づいた第2コホートレベル以降の第Ⅰ相試験実施を継続する予定である。

Ｆ．研究発表

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Ｇ．知的財産権の出願・登録状況

1. 特許取得　なし
2. 実用新案登録　なし
3. その他　　なし

別紙４

研究成果の刊行に関する一覧表レイアウト

雑誌

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A note on bounds for the causal infectiousness effect in vaccine trials

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Principal stratification

ABSTRACT

In vaccine trials, the vaccination of one person might prevent the infection of another. This dependency makes it difficult to estimate the effect of a vaccine on infection. To deal with this issue, causal inference along with a principal stratification framework has been discussed. Unfortunately, however, no standard method has been established for estimating the causal infectiousness effect (CIE). Recently, in a setting of two persons per household, it has been reported that the crude estimator becomes the upper bound of the CIE under two plausible assumptions. Here, we present the lower bound for the CIE by strengthening one of these two assumptions.

1. Introduction

The vaccine status of one person may affect whether another person becomes infected. This phenomenon is sometimes referred to as interference in the statistics literature (Hong and Raudenbush, 2006; Rosenbaum, 2007; Hudgens and Halloran, 2008; VanderWeele, 2010) and as an indirect effect in the infectious disease context (Halloran and Struchiner, 1991, 1995), and it makes it difficult to estimate the causal effect of a vaccine on infection, *i.e.*, the causal infectiousness effect (CIE). In this paper, we consider households of two individuals in which one of the two individuals (individual 1) is randomized to receive a vaccine or control, and the other (individual 2) receives nothing. In this setting, using the counterfactual notation (Little and Rubin, 2000) and principal stratification framework (Frangakis and Rubin, 2002; Rubin, 2004), the CIE is defined as the causal effect for individual 2 in the principal stratum of households for whom individual 1 would have been infected irrespective of whether individual 1 was vaccinated (Hudgens and Halloran, 2006; Tchetgen Tchetgen and VanderWeele, 2012).

Recently, VanderWeele and Tchetgen Tchetgen (2011) have demonstrated that the crude estimator becomes the upper bound of the CIE under two assumptions: (i) there is no one who would be infected if vaccinated but uninfected if unvaccinated, and (ii) the probability of infection for individual 2 if both individuals 1 and 2 were unvaccinated would be lower in the subgroup of households for which individual 1 was unvaccinated and infected than in that for which individual 1 was vaccinated and infected. Here, we present the lower bound by strengthening the second assumption.

The paper is organized as follows. In Section 2, we present the notation and definitions used throughout this paper. In Section 3, we present two assumptions and bounds for the CIE derived under them. Section 4 concludes with a discussion.

Note that some readers may think that this paper is similar to my other paper in this journal (Chiba, in this issue), which discussed bounds for the complier average causal effect (CACE) (Angrist et al., 1996; Cheng and Small, 2006) in the context of randomized trials with noncompliance, but there are differences between these two papers. The differences can clearly be explained by using directed acyclic graphs (Pearl, 2009). Figs. 1 and 2 show a setting of this paper and that of the CACE paper, respectively. The first difference is the relationships among variables; a direct arc from A_1 to Y_2 exists in Fig. 1, while a direct arc from Z to Y does not exist in Fig. 2. The second difference is causal effects of interest; the CIE focuses on the causal

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ORIGINAL ARTICLE

Pharmacokinetics of sepantronium bromide (YM155), a small-molecule suppressor of survivin, in Japanese patients with advanced solid tumors: dose proportionality and influence of renal impairment

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Abstract
Purpose The purpose of this analysis was to investigate the pharmacokinetics (PK) of sepantronium (YM155), a small-molecule suppressor of the expression of the antiapoptosis protein survivin, in Japanese patients with advanced solid tumors and to evaluate the effect of renal impairment on the PK profile of sepantronium.
Methods Sepantronium was administered as a continuous intravenous infusion of 1.8–10.6 mg/m^2/day for 168 h (7 days) to 33 patients. PK parameters were estimated via non-compartmental method. Renal function was categorized for the analysis based on the chronic kidney disease guidance using eGFR values at pre-dose.
Results The PK of sepantronium was dose proportional in the dose range of 1.8–10.6 mg/m^2/day. Age and sex did not significantly affect the PK of sepantronium. Results suggested that total clearance and renal clearance in patients with moderate renal impairment were 0.7-fold lower than those in patients with normal renal function, resulting in 1.3-fold higher steady-state concentration and area under the curve values. The PK parameters of sepantronium in patients with mild renal impairment were comparable to those in the patients with normal renal function.
Conclusions While age and sex did not significantly affect the PK of sepantronium, moderate renal impairment increased exposure of sepantronium by about 30 %. The results suggest that no dose adjustment is required for patients with mild renal impairment.

Keywords Sepantronium bromide · YM155 · Pharmacokinetics · Renal impairment · Advanced solid tumor

Introduction

Sepantronium bromide (sepantronium, YM155, 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)-4,9-dihydro-1*H*-naphtho[2,3-*d*]imidazolium bromide), a small-molecule survivin suppressant, was identified by cell-based, high-throughput screening and lead optimization. Sepantronium bromide selectively suppresses survivin expression, resulting in activation of caspases and apoptosis induction in hormone refractory prostate cancer cells. Sepantronium bromide showed broad spectrum antitumor activity and induced tumor regressions in various xenograft models rather than uncertainness of mode of action. Continuous infusion of sepantronium bromide has also been found to induce tumor regression and intratumoral survivin suppression in established human hormone refractory prostate cancer (HRPC), non-Hodgkin lymphoma (NHL), and non-small cell lung cancer (NSCLC) tumor xenografts [1–4].

Phase 2 studies to evaluate the safety, efficacy, and pharmacokinetics (PK) of sepantronium were conducted with a continuous intravenous infusion (CIVI) for 7 days and showed modest single-agent clinical activity in patients with NSCLC, HRPC, or unresectable stage III or IV melanoma, respectively [5–7]. Sepantronium is currently being

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investigated in phase 1 and phase 2 studies in combination therapy, enrolling patients with diffuse large B-cell lymphoma and other solid tumors [8–10].

The results of a monotherapy phase 1 study to evaluate tolerability, safety, efficacy, and PK of sepantronium in Japanese patients with advanced solid tumors have been reported previously [11]. Sepantronium was administered by CIVI for 7 days (168 h) every 21 days at 1.8, 3.6, 4.8, 6.0, 8.0, and 10.6 mg/m^2/day. Sepantronium was generally well tolerated, and the maximum tolerated dose was estimated to be 8.0 mg/m^2/day when administered as the CIVI for 7 days in Japanese patients. Dose-limiting toxicities (DLT) observed in the study were increased blood creatinine, grade 3 increased serum aspartate aminotransferase (AST), and grade 4 anemia. Steady-state conditions were achieved 24 h after start of infusion for all doses administered. The concentrations of unchanged drug declined rapidly following a biphasic manner after termination of infusion. It appeared that systemic exposure increased with increasing doses and no accumulation was noted with repeated doses. Mean values for elimination half-life ($t_{1/2}$) and total body clearance (CL) of sepantronium seemed constant across the dose ranges. The urinary excretion ratio of unchanged drug ranged from 25 to 42 % and showed no relationship with the dose administered. Non-clinical studies in rats showed that three metabolites were identified in bile and urine after a single intravenous dose of sepantronium; however, sepantronium was minimally metabolized when incubated with human cryopreserved hepatocytes [12].

Given these characteristics, it is anticipated that a substantial reduction in renal function may affect the renal clearance of sepantronium, and consequently its PK, as well as possibly, its safety, and tolerability.

The present analysis was performed to evaluate the effect of renal impairment on PK of sepantronium using a linear mixed effect model with data obtained from a previously reported phase 1 study in Japan. In addition, dose proportionality of sepantronium was evaluated, and an exploratory analysis to investigate the effect of demographics on the PK of sepantronium was performed.

Methods

A retrospective analysis of data obtained from the previously reported phase 1 study in Japanese subjects [11] was performed.

Study design

The study was an open-label, single center, phase 1, dose-escalation study with administration of CIVI dose of sepantronium as monotherapy over 7 days (168 h) every 21 days. The safety, tolerability, efficacy, and PK of sepantronium were evaluated in male and female Japanese patients with advanced solid tumors. Sepantronium was prepared for administration by dilution of the appropriate volume of concentrated stock solution in 5 % dextrose in a light- and temperature-controlled environment.

The study consisted of 6 dose cohorts of 3–6 patients each treated with 1.8, 3.6, 4.8, 6.0, 8.0, or 10.6 mg/m^2/day. Doses were expressed as those of the cationic moiety of sepantronium bromide. Each 21-day cycle included a 7-day (168-h) administration period and a 14-day observation period (1 cycle). This study was conducted at the Department of Medical Oncology, Kinki University Hospital, Osaka, Japan. The protocol was approved by an independent ethics committee for the study site, and the study was conducted in accordance with the principles of the Declaration of Helsinki. Results regarding the tolerability, safety, and basic PK profile of sepantronium have been reported previously [11].

Population

The study enrolled Japanese male and female patients with advanced solid tumors. Eligibility criteria for patients enrolled in the study included refractory advanced solid tumors for which no standard therapy was available; histologic or cytologic diagnosis of cancer; age at least 20 years; life expectancy of at least 12 weeks; Eastern Cooperative Oncology Group performance status of <3; and adequate hematopoietic, hepatic, and renal functions (absolute neutrophil count of $\geq 1.5 \times 10^9$/L, platelets of $\geq 100 \times 10^9$/L, hemoglobin of ≥ 9 g/dL, bilirubin within 1.5 × upper limit of normal, transaminases of $\leq 2.5 \times$ upper limit of normal, and creatinine of $\leq$ upper limit of normal) [11].

Blood and urine sampling

Venous blood samples were collected in tubes containing heparin sodium from a site other than the infusion site before and at 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, 48, 72, 96, 120, and 144 h after start of infusion, as well as at the end of infusion (168 h), and at the following time points thereafter: 168.25, 168.5, 169, 170, 171, 172, 174, 180, 192, and 216 h after the start of infusion. Blood samples were centrifuged immediately, and the plasma samples obtained were stored at −20 °C before analysis. To determine the urinary concentration of unchanged sepantronium, urine samples were collected over the 216-h period after start of CIVI and stored at −20 °C before analysis. Blood and urine samples for PK evaluation were collected during cycle 1 and cycle 2 [11].

Table 1 Patient characteristics

Descriptive statistics for patient demographics		Number of PK data sets and frequency of renal function			
	No. of patients		Cycle 1	Cycle 2	Total
Total patients	33	Total	31	15	46
Male/female	23/10	Renal function			
Age (years)		Normal	12	7	19
Median (range)	59 (26–81)	Mild	14	7	21
Body weight (kg)		Moderate	5	1	6
Median (range)	54 (40–88)				
BSA					
Median (range)	1.6 (1.3–2.0)				

Thirty-two of 33 patients who received sepantronium administration had concentration data and were PK evaluable patients. Fourteen of 32 had both cycle 1 and cycle 2 data, 17 patients had cycle 1 data, and remaining 1 patient had cycle 2 data

Bioanalytical procedures

Measurement of sepantronium concentration in plasma and urine samples was performed by Astellas Europe B.V. EDD using liquid chromatography tandem mass spectrometry (LC–MS/MS). The lower limit of quantitation for sepantronium was 0.05 ng/mL in plasma and 1.0 ng/mL in urine. Concentrations were expressed as those of the cationic moiety of sepantronium bromide [11, 13]. The precision and the accuracy of inter- and intra-assay for the LC–MS/MS methods were within ±20 % (unpublished data).

Pharmacokinetic analysis

PK parameters of sepantronium in plasma and urine were calculated using WinNonlin Professional® version 5.0.1 (Pharsight Corporation, Mountain View, CA, USA) and the SAS® system (SAS Institute Inc., Cary, NC, USA). The area under the curve (AUC) was calculated according to the linear trapezoidal rule from zero to time t of the last measurable concentration above the lower limit of quantitation. Steady-state concentration (C_{SS}) was the mean value of daily concentrations taken through 7-day CIVI (the mean value of concentration at 24, 48, 72, 96, 120, 144, and 168 h after start of infusion). Terminal elimination half-life ($t_{1/2}$) of sepantronium was calculated as follows: $t_{1/2}$ = ln 2/terminal elimination rate constant. CL is the total systemic clearance, estimated by: CL = total amount of dose/AUC from time zero to infinity. CL_R is renal clearance, estimated by: CL_R = cumulative amount excreted in urine/AUC. V_d is apparent volume of distribution, estimated by: V_d = CL/terminal elimination rate constant. Ae is amount excreted in urine. Fe is fraction excreted in urine estimated by: Fe = Ae/Dose.

Statistical analysis

Statistical analysis was performed using the SAS® system. Dose proportionality in PK parameters of sepantronium was evaluated via power model regression using a mixed effect model. The effect of cycle, dose, and demographics (age and sex) upon PK parameters as a fixed effect was investigated.

Renal function was categorized into normal renal function with eGFR of ≥90 mL/min/1.73 m^2 (normal group), mild decrease in eGFR (60–89 mL/min/1.73 m^2) (mild renal impairment group), or moderate decrease in eGFR (30–59 mL/min/1.73 m^2) (moderate renal impairment group) based on the chronic kidney disease guidance [14]. The eGFR for a Japanese population was calculated using the following equation [15]:

$$\mathrm{eGFR}\left(\mathrm{mL/min/1.73\,m^2}\right) = 194 \times (\text{serum creatinine})^{-1.094} \times (\text{age})^{-0.287} (\times 0.739 \text{ if female})$$

PK parameters among renal function groups were compared using geometric mean ratios (GMRs) and their 90 % confidence intervals (CIs) with renal function and cycle as fixed effects and subject as a random effect in the mixed effect model. Results of patients with normal eGFR (normal group) were considered as reference data. Analysis was performed using natural-log transformed PK parameters. The point estimates and their 90 % CIs were exponentiated, and the results were presented as a natural scale. Analysis was performed using dose-normalized AUC (AUC/Dose), dose-normalized C_{SS} (C_{SS}/Dose), CL, and CL_R.

Results

Study populations

Patient characteristics are summarized in Table 1. A total of 33 male (n = 23) and female (n = 10) patients with advanced solid tumors received sepantronium as a CIVI for at least 1 cycle. The PK was evaluated based on data from cycle 1 and cycle 2. After exclusion of 1 patient who had

Table 2 Descriptive Statistics for PK Parameters

	Cohort 1 1.8 mg/m^2/day (n = 6)	Cohort 2 3.6 mg/m^2/day (n = 10)	Cohort 3 4.8 mg/m^2/day (n = 9)	Cohort 4 6.0 mg/m^2/day (n = 8)	Cohort 5 8.0 mg/m^2/day (n = 8)	Cohort 6 10.6 mg/m^2/day (n = 5)
AUC (ng h/mL)	538 ± 119	1,186 ± 385	1,738 ± 685	2,239 ± 952	2,233 ± 489	3,235 ± 526
$t_{1/2}$ (h)	7 ± 3	21 ± 9	15 ± 10	16 ± 6	21 ± 9	29 ± 14
CL (L/h)	42 ± 7	39 ± 13	35 ± 11	34 ± 11	41 ± 14	34 ± 7
C_{SS} (ng/mL)	3 ± 1	7 ± 2	10 ± 4	14 ± 6	13 ± 3	19 ± 3
V_d (L)	436 ± 175	1,197 ± 568	759 ± 541	795 ± 347	1,169 ± 484	1,544 ± 955
	(n = 6)	(n = 6)	(n = 9)	(n = 7)	(n = 4)	(n = 4)
Ae (mg)	7 ± 1	16 ± 5	16 ± 5	19 ± 6	25 ± 2	46 ± 8
Fe (%)	31 ± 4	35 ± 8	30 ± 9	28 ± 6	29 ± 4	42 ± 8

Values are mean ± standard deviation. *AUC*, area under the curve from zero to time t of the last measurable concentration above the limit of quantitation: $t_{1/2}$, terminal elimination half-life, *CL* total systemic clearance, C_{SS} steady-state concentration, V_d apparent volume of distribution, *Ae* amount excreted in urine, *Fe* fraction excreted in urine

no concentration data, 32 patients were included in the analysis.

Fourteen of 32 patients had both cycle 1 and cycle 2 data, while the 17 patients had cycle 1 data and remaining 1 had cycle 2 data, yielding a total of 46 data sets for use in the analysis. The baseline of serum creatinine for enrolled patients ranged from 0.4 mg/dL to 1.2 mg/dL, and were under the upper limit of normal level (1.3 mg/dL). The number of data sets in moderate renal impairment group (30–59 mL/min/1.73 m^2) was 6. The number of data sets in normal group (≥90 mL/min/1.73 m^2) (n = 19) and mild renal impairment group (60–89 mL/min/1.73 m^2) (n = 21) was comparable. All dose cohorts included normal group and mild renal impairment group. Moderate renal impairment group were in the dose cohorts of 3.6 (n = 3), 4.8 (n = 1), or 6.0 mg/m^2/day (n = 2). There were no patients who had severe decrease in eGFR or who required dialysis. Renal function in 2 patients changed from cycle 1 to cycle 2 (from mild to normal in one patient and vice versa in the other).

Pharmacokinetics

Descriptive statistics for PK parameters of sepantronium by dose cohort were presented in Table 2. The relationship between dose and AUC was presented in Fig. 1.

There was no difference in PK parameters between cycle 1 and cycle 2 [11], and the analysis was performed using combined data from cycle 1 and cycle 2. Inter-individual variability of sepantronium PK was moderate as shown in Table 2. Slopes [90 % CIs] by power model regression for AUC and C_{SS} versus dose were 0.981 [0.868–1.094] and 0.998 [0.885–1.110], respectively. The results suggested that AUC and C_{SS} increased in a dose proportional manner.

Significance as a fixed effect of age and sex was evaluated by adding to the power model; however, for these

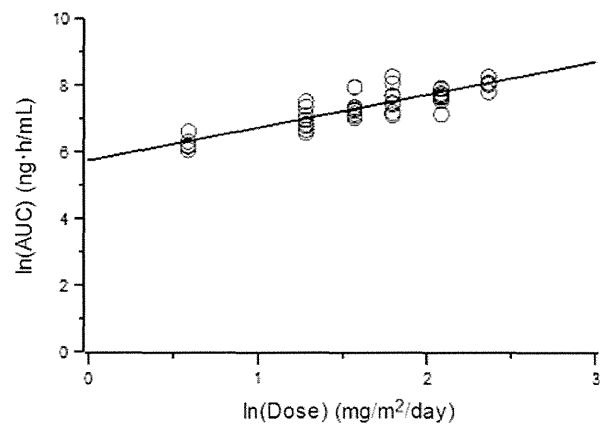

Fig. 1 Relationship between dose and AUC of sepantronium. *Line*: power model regression, *empty circle*: individual value. Slopes and their 90 % confidence intervals obtained from power model regression between the dose and AUC at a dose range of 1.8–10.6 mg/m^2/day was 0.981 (0.868–1.094)

models, either fit statistics were not improved by adding age and sex as fixed effects, or the effect was not significant (age, $p > 0.1$; sex, $p > 0.04$). No demographics were therefore added to the model as a fixed effect.

Summary statistics of PK parameters by renal function are presented in Table 3. Mean plasma concentration versus time profile of sepantronium is presented in Fig. 2. The relationship between PK parameter and renal function is presented in Fig. 3.

Mean plasma concentrations in the moderate renal impairment group were slightly higher than the concentrations in other groups after termination of the sepantronium infusion. Mean PK parameters in the mild impairment group were comparable to those in the normal group. The GMR for the mild renal impairment group versus normal group was nearly equal to 1, and the 90 %

Table 3 Summary of pharmacokinetic parameters of sepantronium after continuous intravenous infusion of 1.8, 3.6, 4.8, 6.0, 8.0, or 10.6 mg/m^2/day for 168 h

PK parameters (mean ± SD)	Normal (n = 19)	Mild (n = 21)	Moderate (n = 6)
AUC/Dose (ng h/mL/mg)	29 ± 14	27 ± 7	37 ± 10
C_{SS}/Dose (ng/mL/mg)	0.17 ± 0.08	0.16 ± 0.04	0.22 ± 0.07
CL (L/h)	39 ± 11	39 ± 11	28 ± 9
	(n = 14)	(n = 14)	(n = 4)
CL_R (L/h)	13 ± 5	13 ± 2	9 ± 4

PK Parameter comparison, GMR (90 % CI)	Mild/normal	Moderate/normal
AUC/Dose (ng h/mL/mg)	0.976 (0.819–1.162)	1.340 (1.033–1.738)
C_{SS}/Dose (ng/mL/mg)	0.989 (0.824–1.187)	1.273 (0.969–1.672)
CL (L/h)	1.021 (0.857–1.217)	0.740 (0.570–0.960)
CL_R (L/h)	1.107 (0.887–1.383)	0.695 (0.499–0.968)

SD standard deviation, *AUC/Dose* dose-normalized area under the curve from zero to time t of the last measurable concentration above the limit of quantitation, *C_{SS}/Dose* dose-normalized steady-state concentration, *CL* total systemic clearance, *CL_R* renal clearance, *GMR* geometric mean ratio, *CI* confidence interval

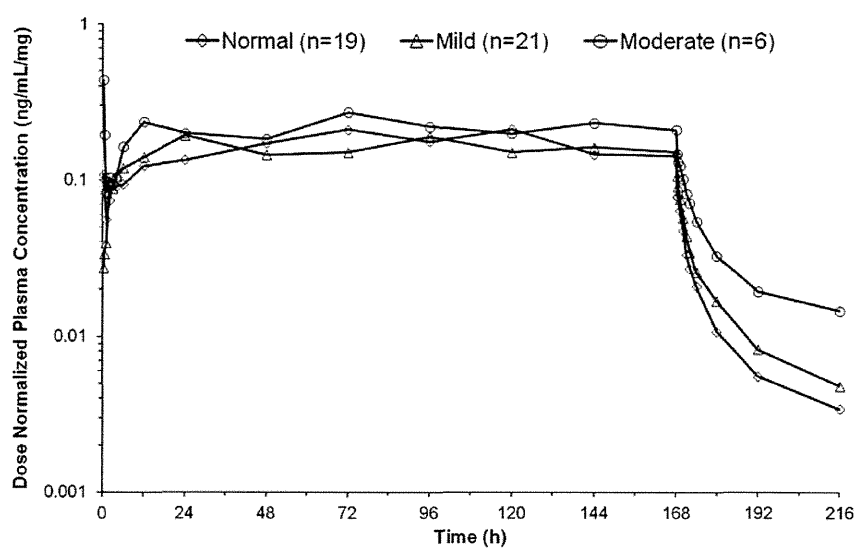

Fig. 2 Mean dose-normalized plasma concentration versus time profile of sepantronium after continuous intravenous infusion of 1.8–10.6 mg/m^2/day for 168 h in Japanese patients with advanced solid tumors

CIs were almost in the range of 0.8–1.25. Renal function in 2 patients changed from cycle 1 to cycle 2 (from mild to normal in one and vice versa in the other), and PK parameters in these patients were similar for both cycles. Results for the moderate renal impairment group showed lower mean CL and CL_R compared to the normal group, and AUC/Dose and C_{SS}/Dose in the moderate renal impairment group were 1.3-fold higher than those in the normal group.

Two of the five patients in the highest dose cohort (10.6 mg/m^2/day) had DLT of increased blood creatinine. Of note is the fact that these two patients with the DLT had mild renal impairment. The AUC values of these two patients were 3975 and 3098 ng h/mL, respectively, and were equal to or greater than the values in the other patients in the same dose cohort (2510–3351 ng h/mL).

Discussion

We evaluated the effect of renal impairment on PK of sepantronium in patients with advanced solid tumors using the data obtained from an open-label, phase 1 study. Sepantronium was administered as CIVI at a dose and rate of 1.8–10.6 mg/m^2/day over 7 days. Overall, PK parameters of sepantronium were similar in patients with mild impairment and patients with normal renal function; however, patients with moderate impairment had a slightly

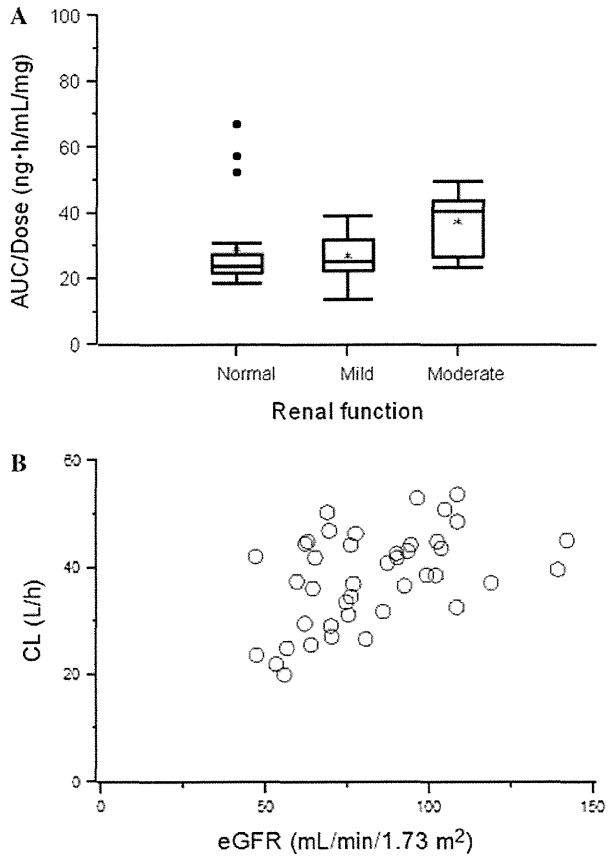

Fig. 3 Relationship between renal function and PK parameters of sepantronium. *Star* represents a mean value: *box* represents a range of 50 % interval: a *bar* in each *box* represents a median value: *bar* under the *box* represents a 25 percentile: *bar* over the *box* represents a 75 percentile: *fixed circle* represents outlier in **a**. Pearson's correlation coefficient between CL and eGFR = 0.46, $p = 0.0021$ in **b**

lower clearance of sepantronium. The GMR for the mild renal impairment group compared to the normal group was nearly equal to 1, and the 90 % CIs were in the range of 0.8–1.25 which is commonly accepted as an equivalence range. The results indicated that there was no clinically significant difference in PK between patients with normal renal function and patients with mild renal impairment.

Two phase 1 studies have consistently reported excretion ratios of sepantronium as unchanged drug into urine of approximately 30 %, results which indicate that urinary excretion is an important elimination routes of sepantronium [11, 13]. The results that moderate renal impairment reduced the CL_R of sepantronium and mild renal impairment had no effect on the PK are in agreement with the above assumption.

Impaired renal function often alters a drug's PK profile, when the drug is eliminated primarily by renal excretion. In vitro and animal studies have suggested that renal impairment may affect or down-regulate various CYP enzymes and transporters that may lead to clinically relevant changes in non-renal clearance [16, 17]. The CL and CL_R of sepantronium decreased in parallel in the moderate renal impairment group in the present analysis. Although the elimination mechanism of sepantronium is yet not fully known, it is assumed that a decrease in CL reflects a reduction in CL_R linearly.

The majority of the PK data sets were comprised of normal group ($n = 19$) and mild renal impairment group ($n = 21$). Only 6 were in the moderate renal impairment group. None of the patients had severe renal impairment. Inter-subject variability of sepantronium PK parameters was moderate as shown in Table 3, and there were three outliers in the normal renal function group which were presented as fixed circle in Fig. 3a. Figure 3b shows a relationship between the eGFR and CL of sepantronium. A weak correlation was observed between eGFR and CL of sepantronium when outliers were excluded (Pearson's correlation coefficient between CL and eGFR = 0.46, $p = 0.0021$). There is a possibility that a strong relationship will be observed between eGFR and CL of sepantronium if further investigation is conducted using comparable number of patients with moderate and severe renal impairment compared to patients with normal renal function.

Most patients enrolled in the study had normal serum alanine aminotransferase and AST values, and an analysis to evaluate the effect of hepatic impairment on PK of sepantronium was not performed. Of interest was a weak correlation observed between the CL_R and baseline value of alkaline phosphatase (ALP), which was above upper normal range in 15 of 46 PK data sets at the baseline (Pearson's correlation coefficient = 0.40, $p = 0.0229$). However, no similar relationship was observed between CL and ALP (Pearson's correlation coefficient = 0.15, $p = 0.3106$). The reason for this finding is unclear.

It was reported that 3 metabolites were identified in bile and urine samples obtained after a single intravenous dose of sepantronium to rats. The proposed metabolic pathways of sepantronium in rats involve *N*-dealkylation, *o*-demethylation, and the oxidation of a methyl group to a carboxylic acid. Sepantronium is minimally metabolized when incubated with human cryopreserved hepatocyte [12]. It was suggested that human organic cation transporter 1 (OCT1) was the predominant transporter for the hepatic uptake of sepantronium, and that excretion into bile was an important elimination pathway of sepantronium in humans. It has also been reported that the transporter-mediated uptake clearance observed in vitro may account for the in vivo intrinsic hepatic clearance [18]. The contribution ratio of hepatic metabolism to metabolic clearance in humans is unclear presently; however, given these results from in vitro and non-clinical studies, the

contribution ratio of hepatic metabolism in humans is likely small. Further investigation for metabolites in human will help to clarify the effect of hepatic impairment on PK of sepantronium.

Frequently, the effects of renal impairment are clarified by conducting a clinical study enrolling patients with renal impairment and comparing them with those in matched healthy subjects, or by performing a model analysis using a non-linear mixed effect model [17]. The present analysis is another approach to investigate the effect of renal or hepatic impairment on the PK of compounds early in the clinical trials.

Dose proportionality of the PK of sepantronium was evaluated via the power model regression. Slopes and their 90 % CIs for AUC and C_{SS} versus dose were within the range of 0.8–1.25, indicating that exposure of sepantronium increased in a linear dose proportional manner. Other PK parameters were similar among dose cohorts. These results suggested that the PK of sepantronium was linear at a dose range from 1.8 to 10.6 mg/m^2/day. In agreement with the present findings, an earlier phase 1 study for sepantronium conducted in the United States found that the values of C_{SS} and AUC increased in a dose proportional manner, and CL was independent of dose over the range of 1.8 to 4.8 mg/m^2/day [13]. The effect of cycle on sepantronium PK was evaluated by adding cycle numbers to the model as a fixed effect; however, the effect was not significant ($p > 0.1$). These results indicate that there was no difference in PK parameters between cycle 1 and cycle 2 and accumulation with repeated dosing was not observed. Demographics such as age and sex did not significantly affect the PK of sepantronium.

A previous non-clinical toxicology study found that short-term exposure at high plasma concentrations caused nephrotoxicity [11]. Two of five patients in the highest dose cohort (10.6 mg/m^2/day) had DLT of increased blood creatinine [11]. Of note is the fact that these two patients with the DLT had mild renal impairment. The AUC values of these 2 patients were equal or greater than the values in the other patients in the same dose cohort. The PK of sepantronium was linear, and individual AUC and C_{SS} values in lower-dose cohorts were not in excess of those patients receiving 10.6 mg/m^2/day, although inter- and intra-patient variability was moderate.

In conclusion, while age and sex did not significantly affect the PK of sepantronium; moderate renal impairment increased exposure of sepantronium by about 30 %. The CL and CL_R of sepantronium were lower in patients with moderate renal impairment relative to the patients with normal renal function. The PK in patients with mild renal impairment was comparable to those for patients with normal renal function. The results suggest that no dose adjustment is required for patients with mild renal impairment. It will be necessary to monitor the safety in patients with moderate renal impairment.

Conflict of interest Yumiko Aoyama, Tetsuya Nishimura, Taiji Sawamoto, and Masataka Katashima are employees of Astellas Pharma Inc.

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FGF3/FGF4 Amplification and Multiple Lung Metastases in Responders to Sorafenib in Hepatocellular Carcinoma

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The response rate to sorafenib in hepatocellular carcinoma (HCC) is relatively low (0.7%-3%), however, rapid and drastic tumor regression is occasionally observed. The molecular backgrounds and clinico-pathological features of these responders remain largely unclear. We analyzed the clinical and molecular backgrounds of 13 responders to sorafenib with significant tumor shrinkage in a retrospective study. A comparative genomic hybridization analysis using one frozen HCC sample from a responder demonstrated that the 11q13 region, a rare amplicon in HCC including the loci for *FGF3* and *FGF4*, was highly amplified. A real-time polymerase chain reaction–based copy number assay revealed that *FGF3/FGF4* amplification was observed in three of the 10 HCC samples from responders in which DNA was evaluable, whereas amplification was not observed in 38 patients with stable or progressive disease ($P = 0.006$). Fluorescence *in situ* hybridization analysis confirmed *FGF3* amplification. In addition, the clinico-pathological features showed that multiple lung metastases (5/13, $P = 0.006$) and a poorly differentiated histological type (5/13, $P = 0.13$) were frequently observed in responders. A growth inhibitory assay showed that only one *FGF3/FGF4*-amplified and three *FGFR2*-amplified cancer cell lines exhibited hypersensitivity to sorafenib *in vitro*. Finally, an *in vivo* study revealed that treatment with a low dose of sorafenib was partially effective for stably and exogenously expressed *FGF4* tumors, while being less effective in tumors expressing *EGFP* or *FGF3*. *Conclusion:* *FGF3/FGF4* amplification was observed in around 2% of HCCs. Although the sample size was relatively small, *FGF3/FGF4* amplification, a poorly differentiated histological type, and multiple lung metastases were frequently observed in responders to sorafenib. Our findings may provide a novel insight into the molecular background of HCC and sorafenib responders, warranting further prospective biomarker studies. (HEPATOLOGY 2013;57:1407-1415)

Abbreviations: 5FU, 5-fluorouracil; CGH, comparative genomic hybridization; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; FFPE, formalin-fixed, paraffin-embedded; FISH, fluorescence in situ *hybridization; HCC, hepatocellular carcinoma; IC_{50}, 50% inhibitory concentration; mRNA, messenger RNA; PCR, polymerase chain reaction; PIVKA-II, protein induced by vitamin K absence or antagonist-II; RPMI-1640, Roswell Park Memorial Institute 1640; RT-PCR, reverse-transcription PCR.*

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Hepatocellular carcinoma (HCC) is the sixth most common cancer-related cause of death in the world annually, and the development of new primary tumors, recurrences, and metastasis are the most common causes of mortality among patients with HCC.[1,2] Sorafenib (Nexavar; Bayer Healthcare Pharmaceuticals Inc.) is a small molecule kinase inhibitor that is classified as an anti-angiogenic inhibitor.[3] Sorafenib inhibits the kinase activities of Raf-1 and B-Raf in addition to vascular endothelial growth factor receptors, platelet-derived growth factor receptor β, Flt-3, and c-KIT. Two large randomized controlled trials reported a significant clinical benefit of single-agent sorafenib in extending overall survival in both Western and Asian patients with advanced unresectable HCC.[4,5] Consequently, sorafenib is now used as a standard therapy for HCC. The mechanisms of action that lead to these remarkably prolonged overall survival periods are thought to result from the anti-angiogenic effects of sorafenib and its characteristic inhibitory effect on Raf-1 and B-Raf signaling. In these trials, a partial response was observed in 0.7% (2/299) and 3.3% (5/150) of the patients treated with sorafenib.[4-5]

Recently, emerging evidence has demonstrated that some responders exhibit rapid tumor regression as a result of sorafenib treatment for HCC. Complete responses were observed in two patients with advanced HCC and multiple lung metastases, with rapid tumor regression observed even after short-term treatment with sorafenib.[6,7] The drastic tumor response to sorafenib seems to be similar to the tumor response obtained using other tyrosine kinase inhibitors to target a deregulated signal in cancer cells. For example, constitutively active mutations of epidermal growth factor receptor (EGFR) tyrosine kinase in non–small cell lung cancer are associated with a striking treatment response to gefitinib, a selective EGFR tyrosine kinase inhibitor.[8,9] We hypothesized that these HCC cells may harbor a genetic background conducive to a drastic response to sorafenib, rather than the typical anti-angiogenic effect. In this study, we retrospectively searched for genetic changes using mainly formalin-fixed, paraffin-embedded (FFPE) samples from patients with HCC who had undergone sorafenib treatment.

Patients and Methods

Reagent and Cell Culture. Sorafenib was provided by Bayer Healthcare Pharmaceuticals Inc. (Montville, NJ). All cell lines used in this study were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma, St. Louis, MO) except for IM95, OUMS23, Colo320, WiDr, HLF, HLE, Huh7, and HepG2 (Dulbecco's modified Eagle's medium [DMEM]; Nissui Pharmaceutical, Tokyo, Japan); LoVo (F12; Nissui Pharmaceutical, Tokyo, Japan); KYSE180, KYSE220, and KYSE270 (RPMI-1640:F12, 1:1); KYSE150 (F12); and KYSE70 (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) or 2% FBS for the KYSE series plus penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. These cell lines were obtained from the American Type Culture Collection (Manassas, VA) and the Japanese Collection of Research Bioresources Collection (Sennan-shi, Osaka, Japan).

Patients and Samples. The inclusion criteria for the study were as follows: patients with histologically confirmed HCC who had been treated with sorafenib, from whom pretreatment tumor samples were available. Finally, the clinical characteristics of a total of 55 cases of HCC from 12 medical centers were evaluated retrospectively. In the gene copy number analysis, four samples were excluded because of an insufficient quantity of DNA, two samples were excluded because of the poor quality of the DNA and two samples were response not evaluable. One not evaluable sample was poor DNA quality. Thus, the copy number assay was performed using the remaining 48 samples. Meanwhile, a series of 82 HCC samples were obtained from frozen specimens of surgical specimens at the Kinki University Faculty of Medicine. The tumor response was evaluated using computerized tomography according to the Response Evaluation Criteria in Solid Tumors; the response was then classified as a complete response, a partial response, stable disease, progressive disease, or not evaluable. The clinico-pathological features evaluated included age, sex, viral infection, alpha-fetoprotein level, protein induced by vitamin K absence or antagonist-II (PIVKA-II), clinical stage, primary tumor size, metastatic lesion, histological type, treatment response, and duration of sorafenib

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treatment. The present study was approved by the institutional review boards of all the centers involved in the study, and informed consent was obtained from the patients.

Isolation of Genomic DNA. Genomic DNA samples were extracted from deparaffinized tissue sections preserved as FFPE tissue using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA samples were extracted from surgical frozen sections using a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instructions. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA).

Comparative Genomic Hybridization Analysis. The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) was used to perform array comparative genomic hybridization (CGH) on genomic DNA from HCC and paired liver samples according to the manufacturer's instructions. A total of 250 ng of genomic DNA was digested with both Nsp I and Sty I in independent parallel reactions, subjected to restriction enzymes, ligated to the adaptor, and amplified using polymerase chain reaction (PCR) with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and GeneChip Operating Software version 1.4. In the array CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek Inc., St. Louis, MO).

Copy Number Assay. The copy numbers for *FGF3* and *FGF4* were determined using commercially available and predesigned TaqMan Copy Number Assays according to the manufacturer's instructions (Applied Biosystems, Foster City, CA) as described.[10] The primer IDs used for the *FGFs* were as follows: *FGF3*, Hs06336027_cn; *FGF4*, HS01235235_cn. The *TERT* locus was used for the internal reference copy number. Human Genomic DNA (Clontech) and DNA from noncancerous FFPE tissue were used as a normal control.

Real-Time Reverse-Transcription PCR. Real-time reverse-transcription PCR (RT-PCR) was performed as described.[11] In brief, complementary DNA was prepared from the total RNA obtained from each surgical frozen section using a GeneAmp RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed using a Thermal Cycler Dice (TaKaRa, Otsu, Japan) in accordance with the manufacturer's instructions under the following conditions: 95°C for 5 minutes, followed by 50 cycles of 95°C for 10 seconds and 60°C for 30 seconds. The primers used for the real-time RT-PCR were as follows: *FGF3*, 5′-TTT GGA GAT AAC GGC AGT GGA-3′ (forward) and 5′-CGT ATT ATA GCC CAG CTC GTG GA-3′ (reverse); *FGF4*, 5′-GAG CAG CAA GGG CAA GCT CTA-3′ (forward) and 5′-ACC TTC ATG GTG GGC GAC A-3′ (reverse); *GAPD*, 5′-GCA CCG TCA AGG CTG AGA AC-3′ (forward) and 5′-ATG GTG GTG AAG ACG CCA GT-3′ (reverse). *GAPD* was used to normalize expression levels in the subsequent quantitative analyses.

Fluorescence* In Situ *Hybridization Analysis. Fluorescence *in situ* hybridization (FISH) was performed as described.[10] Probes designed to detect the *FGF3* gene and *CEN11p* on chromosome 11 were labeled with fluorescein isothiocyanate or Texas red and were designed to hybridize to the adjacent genomic sequence spanning approximately 0.32 Mb and 0.63 Mb, respectively. The probes were generated from appropriate clones from a library of human genomic clones (GSP Laboratory, Kawasaki, Japan).

Immunoblotting. Western blot analysis was performed as described.[11] The following antibodies were used: monoclonal FGF3 (R&D Systems, Minneapolis, MN), FGF4 and FGFR2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), and phosphorylated FGFR and horseradish peroxidase–conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA). NIH-3T3 cells were exposed to the indicated concentrations of sorafenib for 2 hours and were then stimulated with FGF4-conditioned medium for 20 minutes.

Cell Growth Inhibitory Assay. To evaluate growth inhibition in the presence of various concentrations of sorafenib, we used an MTT assay as described.[12]

Plasmid Construction, Viral Production, and Stable Transfectants. The methods used in this section have been described.[12] The complementary DNA fragment encoding human full-length *FGF3* or *FGF4* was isolated using PCR and Prime STAR HS DNA polymerase (TaKaRa, Otsu, Japan) with following primers: *FGF3*, 5′-GG GAA TTC GCC GCC ATG GGC CTA ATC TGG CTG CTA-3′ (forward) and 5′-CC CTC GAG GCC CAG CTA GTG CGC ACT GGC CTC-3′ (reverse); *FGF4*, 5′-GG GAA TTC GCC GCC ATG TCG GGG CCC GGG ACG GCC GCG GTA GCG C-3′ (forward) and 5′-CC CTC GAG