IV. 研究成果の刊行物・別刷

第2章　膀胱がん

病因・病理

1. 病　因

要旨

明確な遺伝性がんのカテゴリーを持つ腎がんと比較すると，膀胱がんでは喫煙や職業性化学発がんなどの環境要因のがん化への関与が特徴に挙げられ，がん化の解明は後塵を拝した感は拭えない．しかし，1980 年代にがん遺伝子が世界で最初に単離されたのは膀胱がん細胞株であり，最近になりそのシグナルが再注目されてきている．本稿では 膀胱がんの病因について，① がん化危険因子としての環境要因と ② がん化や進展に関与する遺伝子との 2 項目に分けて記す．

●キーワード

膀胱がん
がん遺伝子
がん抑制遺伝子
職業性化学発がん
喫煙

はじめに

1982 年にマサチューセッツ工科大学の Weinberg らによって，膀胱がん細胞株 EJ / T24 からヒトのがん遺伝子として *H-Ras* が世界で最初に単離され，*H-Ras* がん遺伝子の恒常的な活性化がたった 1 アミノ酸置換によって起るという実に衝撃的な研究成果が報告された[1]．しかし，家族発生がまれであるということが膀胱がんの特徴として挙げられ[2~4]，がん抑制遺伝子 *VHL* の変異による von Hippel-Lindau 病など家族性のカテゴリーを持つ腎がんと比較して，原因遺伝子の解明に後塵を拝した感は拭えない．一方，膀胱がんは喫煙や職業性化学発がんなどの環境要因ががん化に関与していることが特徴的である．本稿では，① 危険因子としての環境要因と ② がん化や進展に関与する遺伝子との 2 項目に分けて記す．

がん化危険因子としての環境要因

膀胱がんは早くから実験的発がんについて研究されてきたがんの 1 つである．現在でもマウスやラットの膀胱化学発がんモデルとして，経口摂取により高率に膀胱がんを生じるブチルブタノールニトロソア

ミン（BBN）はよく用いられている．実際に，臨床においても，化学物質への曝露による膀胱発がんは古くより認識されており，代表的なものとしては，職業性化学発がんと喫煙がある．そのほかにも食事，尿路感染症や薬剤などに加えて，これらの環境因子に対する代謝や解毒といった宿主側の因子が挙げられる．

1．職業性化学発がん

職業性化学発がんは 1775 年に Pott らによって煙突掃除夫に生じた陰嚢がんとして初めて報告されたが，膀胱がんにおいても 1895 年に Rehn によりゴム工場での職業性膀胱発がんを報告され[5]，その後の研究により 2-naphthylamine や benzidine などに代表される芳香族アミンに発がんが起因していることが判明した．1972 年の Cole らは，20％ もの膀胱がんが職業性に長期間化学物質に曝露されたことに由来すると報告している[6]．我が国においても，2-naphthylamine や benzidine などの生産は 1972 年の労働衛生法によって製造が完全に禁止されたが，すでに曝露された該当者の膀胱発がんは現在も重大な問題である．このほかにも職業性膀胱がんとして，アルミニウム製造業，塗装業，皮革業，トラックなどの運転手や石炭ガス化業が報告されている．

2．喫　煙

喫煙は肺がんなどさまざまながんの危険因子とされているが，膀胱がんでも顕著な危険因子である．膀胱がんの罹患危険率について，喫煙者は非喫煙者に比較すると 4 倍となり，喫煙量と期間に応じて高くなるとされている[7]．膀胱がん患者の 1／3 は喫煙と関連性を認めるという報告もあり，喫煙は膀胱発がんの大きな危険因子である[8]．また，禁煙すると 30～60％ の危険率減少が認められるとされるが，ベースラインに戻るには 20 年という長期間が必要であるとも言われている[9]．タバコ中のがん危険因子となる化学成分は特定されていないが，nitrosamines，2-naphthylamine，4-aminobiphenyl や triptophan 代謝物の増加が候補物質として挙げられている[10]．

3．食　事

動物実験では，人工甘味料のサッカリンは膀胱発がん作用があると報告されている[11]．しかし，大規模な case-control study では，サッカリンの摂取量と膀胱がんの発生率に有意な関係は認めず，現時点で

はその関連性は明らかではない[12]．膀胱がんと食物に関する 38 の研究結果をまとめた meta-analysis では，野菜や果物をたくさん摂取するグループではそれぞれ危険率が 0.7 倍，0.8 倍と減少し，反対に高脂肪食グループでは 1.4 倍の危険率があるが，肉食やレチノイン酸，カロチンと有意差は認められなかったとされている[13]．

4．尿路感染症

胃がんにおけるピロリ菌感染症のように，膀胱がんにおいても炎症や感染症と発がんが関連するとされており，典型的なものとして Schistosomiasis 住血吸虫症と膀胱発がんの関係はよく知られている．住血吸虫が膀胱の粘膜下に産卵し，反復する細菌感染と慢性炎症を誘発することで産生された，nitrosamines に膀胱粘膜が曝露されることによるとされている[14]．エジプトや北アフリカなどの Schistosomiasis 住血吸虫症の好発地域では膀胱がんの発生が多く，組織型では扁平上皮がんが約 70％みられることも特徴である．

5．薬　剤

動物実験ではシクロオキシゲナーゼ（COX）-2 阻害薬などの非ステロイド性抗炎症薬は膀胱発がんの抑制作用があると報告されており[15]，これは増殖シグナルの 1 つであるホスフォチジルイノシトール 3 キナーゼ（PI3K）伝達シグナルを抑制されるためと説明されている．Case-control study においても非ステロイド性抗炎症薬の使用者は，コントロールと比較して 0.81 倍と危険率を減少させるが，反対に別の非ステロイド性抗炎症薬であるフェナセチンの長期連用者では 1.52 倍と危険率を高めると報告されている[16]．

6．環境因子に対する宿主側因子

環境中の発がん物質に対する宿主側の解毒・分解，代謝は，さまざまな酵素の活性によって発がん感受性が規定され，同じ環境因子に曝露されても発症リスクには個人差がある．現在まで膀胱発がんの感受性を規定する遺伝子的因子として N-acetyltransferase-2（*NAT2*），Glutathione S-transferase M1（*GSTM1*）などの遺伝子多型と喫煙との関連が報告されており，膀胱がんに対するゲノムワイド関連解析（GWAS）でもこれらの遺伝子と喫煙との関連が示唆されている[17][18]．

NAT2 タンパクは芳香族アミンの解毒，代謝であるアセチル化を触媒する．*NAT2* は 6 つの遺伝子多型があるのが知られており，活

性の高い速効型と活性の低い遅延型に分けられる．遅延型のものでは速効型と比較して膀胱発がんの発生率が1～2倍高いが，特に発がん化学物質に曝露される職業の場合は，10倍以上と顕著である[19]．アジア人では遅延型は5～25％と比較的少ないのに対して，白人では40～60％，アラブ人では90％と，人種間での差がみられる．

GSTM1はPAHなどの発がん物質とグルタチオンとの包合を触媒する酵素であるが，この遺伝子は50％の人で完全欠損型を認める．喫煙者においては，この遺伝子の完全欠損型は，少なくとも片方が野生型と比較して1.8倍の膀胱がんの発生率を認めたが，非喫煙者では差を認めなかったとの報告もある[20]．

がん化や進展に関与する遺伝子

環境因子やあるいは宿主側の感受性によって，正常の尿路上皮細胞の遺伝子に変異やメチル化，脱メチル化などの異常が生じる．膀胱がんでは低異型度で表在性の乳頭状がん，高異型度の上皮内がん（CIS），そして浸潤がんに大別され，それぞれ変異あるいは異常発現する遺伝子，活性化あるいは不活化されるシグナルや転写因子など原因は異なると考えられている．膀胱発がん，進展に関して，現在まですべてが明らかにはされていないが，代表される遺伝子異常について記述する．

表在性乳頭状がんに主としてみられる遺伝子変化

表在性乳頭状がんでは，線維芽細胞増殖因子受容体3（FGFR3）や上皮増殖因子受容体（EGFR）などのreceptor型チロシンリン酸化酵素の変異や発現異常から，Ras-MAPKおよびPI3K-Aktの亢進がみられる（図1A）とされている．

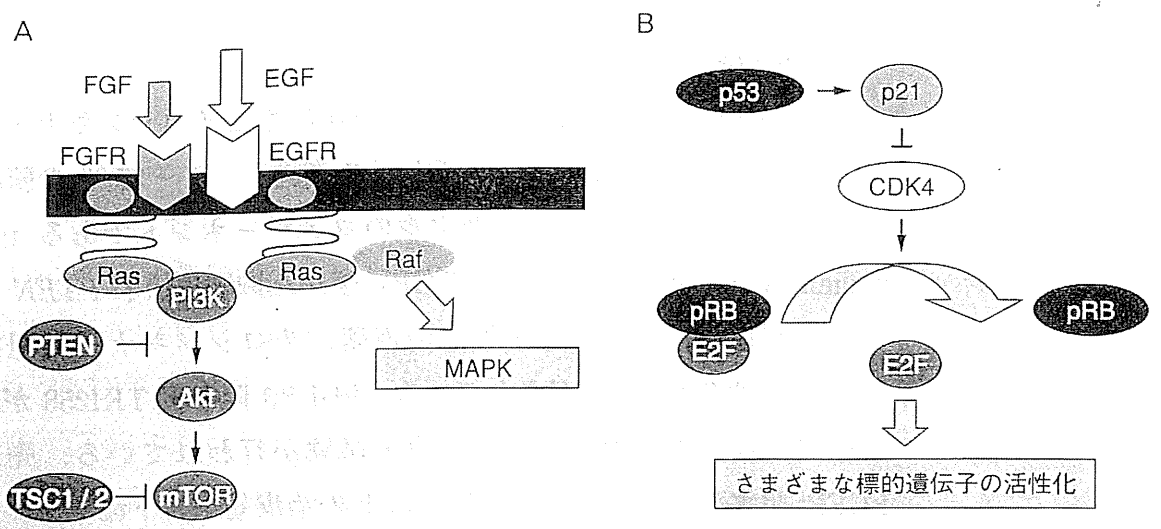
1．FGFR3

多くの乳頭状がんでは*FGFR3*遺伝子の点突然変異がみられる[21]．特に，low grade，low stageのがんに多くみられ，pTaでは68～88％，pT1では21％，pT2-4では16％，そして興味深いことには，CISでは全く検出されなかったと報告されている[22]．

2．EGFR

EGFRはFGFRと同様にチロシンリン酸化酵素で，EGFなどの成長因子の刺激を受けてRAS-MAPKなどの増殖シグナルを活性化す

図1　表在性乳頭状がんおよび上皮内がん（CIS）のシグナル変化



A：EGFR, FGFR3 などのレセプター型チロシンリン酸化酵素から Ras-MAPK, PI3K-Akt の恒常的活性化は，表在性乳頭状がんの発がんや，CIS の進行に関連するようである．
B：一方，p53-RB 経路の欠失は，CIS の発がんや，浸潤がんへの進行に関連するようである．

る．マウスの実験では EGFR 過剰発現が低異型度の表在性乳頭状がんでみられ，さらに RB タンパクと P53 経路を抑制すると腫瘍増殖が活性化されると報告されている[23]．これらから EGFR 阻害薬エルロチニブが表在性がんの再発予防効果や浸潤がんに対する補助療法として期待されているほか，転移性尿路上皮がんに対しては標準抗がん化学療法であるゲムシタビン，シスプラチン（GC）療法へのゲフィチニブ併用効果が試されるなど，分子標的薬の臨床試験も進んでいる．

3．Ras-MAPK シグナル

Ras はヒトの発がんにかかわる最初に発見されたがん遺伝子であり，*H-Ras*, *K-Ras*, *N-Ras* の 3 種類のファミリーが見いだされ，それぞれヒトの第 11 染色体，第 12 染色体，第 1 染色体に存在しているが，膀胱がんでは *H-Ras*, *K-Ras* は同程度に変異が観察されるが，*N-Ras* の変異の頻度は高くないようだ．Ras は FGFR や EGFR などのチロシンリン酸化酵素から細胞膜上でシグナルを受け取り，マイトジェン活性化タンパクキナーゼ（MAPK）や PI3K などさまざまなシグナルを活性化し，細胞の増殖や分化に関与している．コドン 12, 13 や 61 の 1 塩基置換により生じた Ras タンパクの変異体は常

時活性化され，増殖シグナルを伝達することでさまざまながんと関連している．

4．PI3K-Akt シグナル

PI3K-Akt シグナルも，EGFR や FGFR などチロシンキナーゼの下流シグナルで，*PIK3CA* がん遺伝子の変異が 13～27％ の膀胱がんに同定されている[24)25)]．シグナルのコンポーネントである mammalian target of rapamycin（mTOR）やがん抑制遺伝子 *PTEN* などの研究が進み，さらに分子標的治療薬，チロシンキナーゼ阻害薬（TKI）の開発が現在注目されている．FGFR3 阻害薬 TKI258 が進行期尿路上皮がんに対して第Ⅱ相臨床試験が行われている．先述の EGFR 阻害薬にもあてはまるが，TKI の治療効果と下流シグナルの遺伝子変異との相関は大腸がんや肺がんなど，ほかのがん種でも明らかであり，今後ますます研究が進むと考えられる．

5．chromosome 9

ほとんどの膀胱がんで第 9 染色体の欠失がみられ，早くから発がん早期のイベントとされ，がん抑制遺伝子の存在が示唆されている[26)]．しかし，幾つかのがん抑制遺伝子が報告されているものの，発がんとの関連について明確な説明はなされていない．

現在までに 9p21，9q32-33，9q34 など幾つかのがん抑制遺伝子の候補領域が報告されている．9p21 では $p15^{INK4b}$ をコードしている *CDKN2B* 遺伝子 や $p16^{INK4A}$ と $p14^{ARF}$（ARF は alternative reading flame の略で 1 つの遺伝子から alternative splicing によって 2 つのがん抑制遺伝子が翻訳される．）をコードしている *CDKN2A* 遺伝子が候補とされ，完全欠損がしばしばみられている[27)]．9q32-33 では deleted in bladder cancer chromosome region 1（*DBCCR1*）遺伝子の過剰メチル化が高頻度に報告され[28)]，9q34 では tuberous sclerosis complex-1（*TSC1*）が候補遺伝子として報告されている[29)]．

上皮内がん（CIS）や浸潤性がんに主としてみられる遺伝子変化

CIS では乳頭状がんとは別の経路の遺伝子異常が示唆されており，特に p53-RB タンパクによる抑制機構が障害され，DNA 障害が修復されないまま増殖し，アポトーシスが誘導されず変異が蓄積していく（図１Ｂ）．このため，CIS がしばしば悪性度の高い浸潤がんに移行しやすいという説明がなされている．

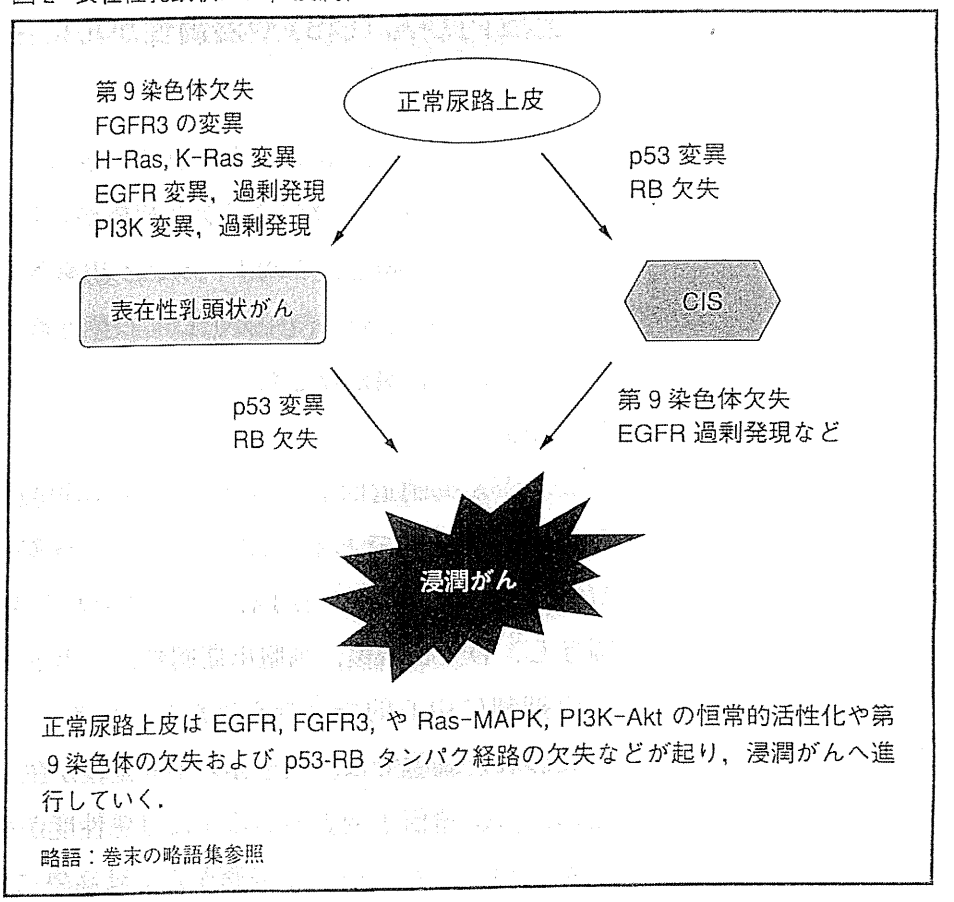
１．p53

p53 がん抑制遺伝子は第 17 番染色体短腕上（17p13.1）に存在し，その遺伝子産物である p53 タンパクは転写因子として，*GADD45*, *MDM2*, $p21^{CIP1/WAF1}$, *BAX*, *14-3-3δ* など多くの遺伝子群の発現に関与し，DNA 修復，細胞周期制御，アポトーシスを誘導など，発がんの抑制に中心的な役割を果たしている．したがって，正常な p53 が失われた細胞では，染色体の不安定性が生じて連鎖的に変異を起すため，*p53* 遺伝子変異のあるがんは悪性度が高いとされている．膀胱がんにおいても，CIS や浸潤がんでは高率に変異がみられ[30]，喫煙と p53 変異との相関も示唆されている．transgenic mouse を用いた実験でも，SV40 遺伝子によって，p53 や RB の経路を阻害すると，高異型度，浸潤性の膀胱がんがみられる[23]．*p53* 遺伝子の多彩な機能を利用して，がんの治療に応用しようとする試みがなされているが，p53 は４量体として活性するため，１つの変異アレルの影響がみられてしまう，いわゆる dominant negative 効果がみられるため，単純にはいかないようである．

２．RB

網膜芽細胞腫の原因遺伝子である *RB* 遺伝子は，13q14 染色体上に位置するがん抑制遺伝子としても知られ，産物である RB タンパクは転写因子 E2F を不活化し，細胞増殖や分化の抑制，アポトーシス誘導などさまざまな機能を担っている．RB タンパクが不活化すると E2F が解離し，さまざまな増殖シグナルが活性化されることになる．膀胱がんにおいても，*RB* 遺伝子の発現が欠失したものや，反対に高発現のものなどの発現異常は予後不良との報告や[31]，RB 遺伝子の LOH が，表在性膀胱がんでは 19％ にみられたのに対して，浸潤が

図2　表在性乳頭状がん，浸潤性がんにおける遺伝子異常やタンパク発現変化



正常尿路上皮はEGFR, FGFR3, やRas-MAPK, PI3K-Aktの恒常的活性化や第9染色体の欠失およびp53-RBタンパク経路の欠失などが起り，浸潤がんへ進行していく．

略語：巻末の略語集参照

んでは60％にみられたとの報告があり，膀胱がん進展と関連するとみられている[32]．

3．E2F

転写因子E2FはRBタンパクと協調して細胞分裂の調節に貢献しており，腫瘍細胞での過剰発現は細胞増殖をもたらすとされている．膀胱がんにおいても高異型度の腫瘍では*E2F*遺伝子の増幅および過剰発現がみられる[33][34]．

おわりに

表在性乳頭状がん，浸潤性がんにおける遺伝子異常やタンパク発現の変化をプロットすると図2のようになる．また，上述の膀胱がんに対するGWASの結果も，3q28（TP63），4p16.3（FGFR3），8q24.21（MYC），8q24.3（PSCA）との関連を支持している[17]．FGFR3や，p53ファミリーのP63タンパクなど，これまでの報告と共通する点もあるが，おのおのの遺伝子異常が膀胱発がんに及ぼす生物学的意義

や発がんの詳細なステップなど不明点も多い．近年のさまざまながんに対する分子標的薬の進歩には，一見臨床とはほど遠い基礎的な分子レベルの知見の蓄積が，大きく貢献していることは間違いない．膀胱がんにおいても，遺伝子レベルでの病因の解明，およびそれをもとにした新しい診断法や，遺伝子変化に応じた新しい治療法の開発などが期待されている．分子標的治療の開発，および臨床試験が進行しており，発がん，浸潤のプロセスの分子生物学的・遺伝学的な解明が，ますます重要となっている．

湯浅　健・羽渕友則

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Tumor Size Is a Potential Predictor of Response to Tyrosine Kinase Inhibitors in Renal Cell Cancer

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OBJECTIVES To investigate the correlations between the initial tumor size and size reduction rate in patients treated with targeted agents. To select the patients who can benefit the most from treatment with targeted agents, it will be necessary to find a tumor characteristic that predicts their effectiveness.

METHODS The data from 139 metastatic and 16 primary lesions treated with the targeted agents were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions in 54 patients with metastatic renal cell carcinoma who had undergone treatment from April 2008 to July 2010. The relationship between the longest tumor diameter at baseline and the rate of reduction in tumor size was assessed using the Spearmancorrelation test.

RESULTS A linear, moderate to strong association between the initial tumor size and tumor size reduction rate was shown (correlation coefficient -0.441, $P < .001$). When these tumors were divided into 2 groups at the threshold value (23.95 mm), which was decided by the receiver operating characteristic curve analysis, the smaller tumors demonstrated a significantly greater size reduction than the larger tumors according to the Mann-Whitney U test ($P < .001$). Both univariate and multivariate linear regression analyses revealed that only the initial tumor size was associated with the rate of reduction in individual tumors ($P < .001$).

CONCLUSIONS The initial tumor size was a good predictor of the tumor size reduction. This simple observation could be useful for physicians who treat patients with metastatic renal cell carcinoma. In addition, in assessing clinical trials of targeted agents for metastatic renal cell carcinoma using the ResponseEvaluation Criteria in Solid Tumors, perhaps this association should be considered. UROLOGY xx: xxx, xxxx. © 2011 Elsevier Inc.

Surgical excision remains the standard and, indeed, the only curative therapy for patients with localized renal cell carcinoma (RCC). However, at the initial diagnosis, one third of patients with RCC will have visceral metastasis, and one half of the remainder will eventually develop distant metastases.[1] Previously, despite its limited clinical activity and significant toxicity, cytokine-based therapy was the mainstay treatment of metastatic RCC (mRCC).[1,2] A better understanding of the molecular biology of RCC has identified signaling pathways related to a hypoxia-inducible factor as rational targets, including the receptors of vascular endothelial growth factor and the mammalian target of rapamycin kinase for anticancer therapy for patients with mRCC.[3]

Because the agents aimed at these molecular targets have demonstrated significant objective responses with moderate and easily manageable toxic effects, a major breakthrough in the treatment paradigm for mRCC has occurred. Among them, sorafenib (Nexavar, Bayer Pharmaceuticals Corporation, West Haven, CT) and sunitinib (Sutent, Pfizer Inc., New York, NY) are tyrosine kinase inhibitors (TKIs), and target vascular endothelial growth factor receptors and platelet-derived growth factor receptors.[4,5]

As other new agents with alternative molecular targets emerge in RCC therapy, to select the patients who can benefit the most from these vascular endothelial growth factor receptor-targeted agents, it is necessary to find a biomarker or tumor characteristic that can predict their effectiveness. Because these agents function as angiogenesis inhibitors,[4,5] the initial tumor size and volume might be important in whether tumors can be expected to shrink using these treatments. Initially, we hypothesized

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that the initial tumor size would be inversely associated with the tumor size reduction rate. In the present study, we investigated the relationship between the initial tumor size and the tumor size reduction rate of patients treated with TKIs.

MATERIAL AND METHODS

Patient and Treatment

The data from 139 metastatic and 16 primary lesions treated with targeted therapeutics were retrospectively analyzed. They consisted of 86 sunitinib-treated and 69 sorafenib-treated lesions from 54 patients with mRCC who had undergone treatment at our hospital from April 2008 to July 2010. Each patient signed an institutional review board-approved protocol-specific informed consent form in accordance withnational and institutional guidelines. Sunitinib was administered orally at a dose of 50 mg/d, consistingof 4 weeks of treatment followed by a 2-week rest period. Sorafenib was administered orally at a continuous dose of 800 mg/d. Dose reductions of sunitinib (to 37.5 mg and then to 25 mg) and sorafenib (to 400 mg/d and then to 400 mg every other day) were performed, depending on the type and severity of the adverse events. All the target lesions were evaluated using multidetector computed tomography (CT) (Lightspeed Pro16, GE Healthcare Japan, Tokyo, Japan), which scans every 5 mm. The tumor measurements were performed by the physicians in charge of the respective patients in clinical practice and calculated separately for the response in the individual primary or metastatic sites. The response was assessed by multidetector CT at least every 2 cycles of treatment, according to the ResponseEvaluation Criteria in Solid Tumors, version 1.0 (RECIST).[6]

Statistical Analysis

To identify an optimal threshold for the prediction of >30% tumor reduction (partial response), receiver operating characteristics analysis was performed by incrementally increasing the cutoff values and recalculating the corresponding true-positive and false-negative rates. The relationship between the longest tumor diameter at baseline the tumor size reduction rate was assessed using the Spearmancorrelation test and the Mann-Whitney *U* test. Independent Student's *t* tests and analyses of variance were used in the univariate analysis for binomial variables, and correlation coefficient analyses were used for continuous variables. Multivariate linear regression analysis was used for the multivariate analysis. Statistical analyses were performed using the Statistical Package for Social Sciences, version 17.0, for Windows (SPSS, Chicago, IL). Two-tailed $P < .05$ was considered significant.

RESULTS

Patient Characteristics

The clinical and pathologic characteristics of the patients treated with TKIs are listed in Table 1. The median follow-up was 12.2 months (range 3.8-29.7). Overall, 16 patients (30%) demonstrated a partial response and 26 (48%) had stable disease according to the RECIST, indicating that 78% of the patients experienced a clinical benefit from these targeted agents. Progression was observed in 9 patients (17%) and early treatment failure before the initial assessment occurred in 3 patients (5%) owing to sorafenib-induced erythema multiforme.[7]

Table 1. Patient characteristics

Characteristic	Patients (n)
Total	54 (100)
Sex	
Male	43 (80)
Female	11 (20)
Age (y)	
Median	62
Range	25-80
ECOG performance status	
0	32 (59)
1	16 (30)
2	6 (11)
Tumor histologic type	
Clear cell	43 (80)
Clear cell plus sarcomatoid components	6 (11)
Papillary	2 (6)
Chromophobe	1 (2)
Xp translocation	1 (2)
Nephrectomy	
Yes	39 (72)
No	15 (28)
Cytokine therapy	
IL-2 and IFN	11 (20)
IFN	20 (37)
None	23 (43)
Tyrosine-kinase inhibitor	
Sunitinib	33 (61)
Sorafenib	21 (39)
Baseline serum laboratory findings	
Hemoglobin (g/dL)	
Median	11.8
Range	6.2-17.7
Corrected calcium (mg/dL)	
Median	9.3
Range	8.5-10.5
Lactate dehydrogenase (U/L)	
Median	173
Range	101-550
C-reactive protein	
Median	0.77
Range	0.03-19.4

ECOG, Eastern Cooperative Oncology Group; IL-2, interleukin-2; IFN, interferon.
Data in parentheses are percentages.

Response to Individual Targeted Lesions

We investigated the objective response of the individual primary or metastatic sites. A total of 155 tumors were examined, including 16 primary kidney lesions and 92 pulmonary, 26 lymph node, 10 liver metastatic, 6 adrenal gland, and 5 soft tissue sites. The mean ± standard deviation tumor size reduction rate was 23.8% ± 56.6%, and the tumor size was reduced by >30% in 103 tumors (66.5%) and >50% in 75 tumors (48.3%).

Correlation Between Initial Tumor Size and Tumor Size Reduction of Individual Targeted Lesions

We investigated the correlation between the initial tumor size and the tumor size reduction rate of the indi-

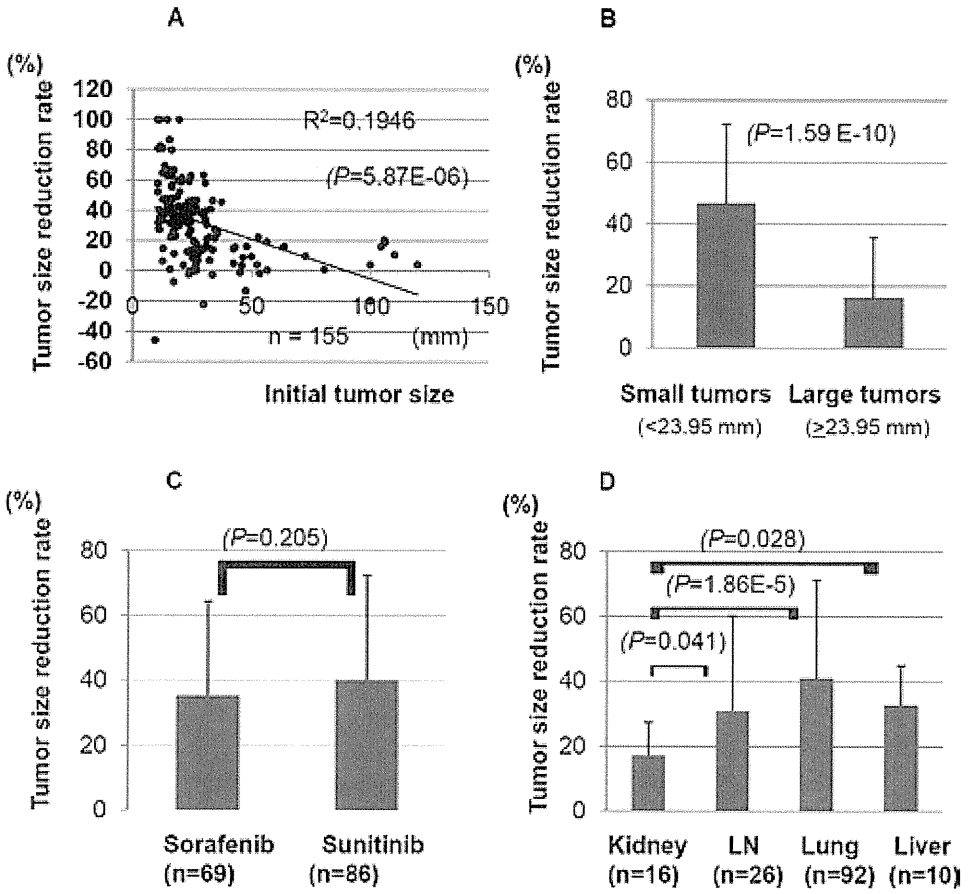

Figure 1. Association between primary tumor size and tumor size reduction. **(A)** Correlations between primary tumor size and tumor size reduction for individual tumor lesions. Smaller tumors demonstrated significant tumor size reduction compared with larger tumors. **(B)** Optimal threshold for prediction of >30% reduction (partial response) was 23.95 mm, as identified by receiver operating characteristics analysis. **(C)** No difference in tumor reduction rate demonstrated between sorafenib and sunitinib. **(D)** Primary kidney tumors demonstrated significantly small tumor size reduction compared with lymph node, metastatic lung, or liver lesions.

vidual targeted lesions. The linear association between the initial tumor size and the tumor size reduction is shown in Figure 1A. The correlation coefficient (r) was −0.441, indicating that a moderate to strong reverse association was confirmed between them ($P < .001$, Fig. 1A). Receiver operating characteristic curve analysis was performed using the clinical criteria of a partial response (>30% reduction) to separate those with and without a response. The area under the receiver operating characteristic curve was 0.814 ± 0.040, and the optimal detection threshold was 23.95 mm, with a sensitivity of 80.0% and specificity of 74.1%. When these tumors were divided into 2 groups at the threshold value (23.95 mm), the smaller tumors demonstrated a significantly greater size reduction than did the larger group ($P < .001$, Fig. 1B). In addition, among these patients, 16 had evaluable primary tumor and metastatic sites, when they were started with TKIs as induction therapy. The initial size of the 16 primary kidney tumor (77.8 ± 27.8 mm) was significantly larger than the metastatic lesions of the same patients (24.0 ± 12.7 mm, $P < .001$). Similarly, the tumor reduction rate of the primary tumor (16.1% ± 17.1%) was also significantly smaller than the metastatic lesions (43.2% ± 26.5%, $P < .001$).

Variables for Tumor Size Reduction

The relationship between the tumor reduction rate and the studied factors was investigated. The studied factors included initial tumor size, disease site, performance status, history of nephrectomy, history of cytokine therapy, TKI used (sunitinib or sorafenib), blood hemoglobin concentration, blood neutrophil count, blood thrombocyte count, serum calcium concentration, and serum lactate dehydrogenase concentration before the administration of TKIs. In the present study, no difference was found between the sorafenib-treated and sunitinib-treated lesions (Fig. 1C). In addition, the tumor reduction rate of the primary kidney was significantly smaller than that of the metastatic lymph node, pulmonary lesion, or liver lesion (Fig. 1D). However, no difference in the reduction rate was seen among the lymph node, lung metastatic, and liver metastatic lesions. On univariate analysis, the initial tumor size and the target organ were associated with the individual size reduction rate (Fig. 1A,B,D). Multivariate linear regression analysis revealed that only

the initial tumor size was also independently associated with the individual size reduction rate ($P < .001$).

COMMENT

It has long been proposed that bulky disease is an adverse prognostic factor during chemotherapy for lymphoma or solid cancer.[8,9] In contrast, the significance of bulky disease when using target therapies has not yet been made clear. For instance, many had assumed that antibody therapy would be ineffective against bulky disease; however, a Phase II study of rituximab (anti-CD20 antibody) for bulky (>10 cm) low-grade lymphoma showed that standard rituximab therapy resulted in a good response rate (43%). In addition, however, they showed that the serum antibody concentration correlated negatively with the baseline tumor bulk.[10] Antiangiogenesis therapy had also been assumed to be ineffective against bulky disease, but this has not been clinically proved. We have demonstrated for the first time that the initial tumor size correlated negatively with the tumor reduction rate in targeted therapy for mRCC.

Up-front cytoreductive nephrectomy, followed by systemic therapy, been established as the standard of care for mRCC in the cytokine era.[1,2] Even for the patients with high-risk and locally advanced RCC, neither preoperative nor postoperative medical treatment has been recommended because of the real lack of effective systemic therapiespreviously available. Therefore, the treatment strategy for RCC must be reconsidered in this targeted therapy era.

According to our results, large tumors will seldom become smaller when TKIs are administered. Therefore, it might be infrequent that an unresectable tumor would become resectable, although no objective criteria exist to define surgical resectability. We believe that the greatest benefit of preoperative approaches in the setting of mRCC is that they can as a litmus test to reserve cytoreductive nephrectomy for only those whowill benefit from the procedure.

Possibly, the resolution of CT scans could affect the tumor size reduction rate. Small lesions might appear to shrink more owing to slice variation and not true size changes. However, we used multidetector CT, which scans every 5 mm. The minimal initial tumor size was 10 mm in the present study, because we excluded tumors that were <10 mm in diameter. Therefore, we believe that the potential issues regarding the resolution of the CT scans did not have a major effect on our conclusions. In addition, when micrometastasis is considered to be of a very small size, adjuvant therapy after radical nephrectomy could meet our expectation of reduced recurrence. Sorafenib and sunitinib have been the focus of adjuvant therapy for patients with resected primary tumors with a high risk of recurrence. Three randomized trials are comparing these agents to placebo in the adjuvant setting: Sunitinib Treatment in Renal Adjuvant Cancer (S-TRAC), Adjuvant Sorafenib or Sunitinib for Unfavorable Renal Carcinoma (ASSURE), and Sorafenib With Placebo in Patients With Resected Primary Renal Cell (SORCE).[11]

Very recently, a study similar to ours was published by Han et al.[12] In their study, the initial tumor enhancement on contrast-enhanced CT could be useful as a clinical predictor during targeted therapy, because it was associated with tumor size reduction of the individual metastases in patients with mRCC who had received targeted therapy.[12] Because of the antiangiogenic therapy, their rationale was quite reasonable. Tumor enhancement was associated not only with tumor size reduction, but also with progression-free survival of the treated patients. However, compared with their study, our study was simpler and could be performed without contrast medium, which can be detrimental to patients with a solitary kidney. In addition, it can be adapted for patients with renal dysfunction, as well as patients who are allergic to contrast medium.

In addition to the treatment paradigm of RCC, our results suggest a weakness in the RECIST, currently the most commonly used system to determine the response in clinical trials and clinical practice. In our study, the longest diameter of large tumor demonstrated a relatively smaller reduction rate than thatof the metastatic small tumor. Therefore, large primary tumors will have an important effect on the overall objective when these are included in the RECIST measurements.Therefore, whether the target lesion includes the large primary and/or metastatic lesions should be considered in calculating the overall response according to the RECIST.

CONCLUSIONS

The initial tumor size was inversely associated with the tumor reduction rate of the individual metastatic sites and primary tumors in patients with mRCC who underwent targeted therapy. Although our small study was preliminary and additional investigations are necessary, we believe that this simple observation might be useful for physicians who treat patients with mRCC, as exemplified by the consideration of the pre- and postoperative approaches. In addition, in assessing clinical trials of targeted agents for metastatic RCC using the RECIST, we might need to consider this association.

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Original Article

Circulating Endothelial Progenitors and CXCR4-Positive Circulating Endothelial Cells Are Predictive Markers for Bevacizumab

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BACKGROUND: Bevacizumab plus chemotherapy is a standard option in the treatment of metastatic colorectal cancer (mCRC). The aim of this study was to investigate the potential of circulating endothelial cell progenitors (CEPs) and phenotypical circulating endothelial cells (CECs) as surrogate markers of clinical outcome in mCRC patients to identify responders to bevacizumab in combination with chemotherapy. **METHODS:** A total of 69 patients with measurable mCRC were enrolled in this prospective study. Whole blood samples were analyzed before initiation of treatment and on days 4 and 14. Phenotypical CECs and CEPs were then isolated and enumerated by using flow cytometry. **RESULTS:** CEP levels of less than 0.04% on day 4 were significantly associated with longer progression-free survival (PFS) and overall survival (OS) ($P < .001$, $P = .002$, respectively) as compared with levels of 0.04% or more. In addition, CXCR4-positive CEC levels of less than 20% at baseline were significantly associated with longer PFS and OS as compared other indicators investigated ($P < .001$, $P = .002$, respectively). **CONCLUSIONS:** Levels of CEPs on day 4 and proportion of CXCR4-positive CECs at baseline were correlated with the prognosis of bevacizumab combination chemotherapy, suggesting that these surrogate markers may play a core role in the selection of candidates for bevacizumab treatment. ***Cancer* 2011;117:4026–32.** © *2011 American Cancer Society.*

KEYWORDS: circulating endothelial progenitors, CXCR4-positive circulating endothelial cells, bevacizumab, metastatic colorectal cancer, chemotherapy.

Antiangiogenic agents such as bevacizumab that target the vascular endothelial growth factor (VEGF) pathway have shown promise in the treatment of a variety of malignancies.[1] However, clinical biomarkers are needed for quantitative evaluation of the effect of bevacizumab.

VEGF is known to promote the mobilization of bone-marrow–derived circulating endothelial progenitors (CEPs) and survival by activating antiapoptotic pathways in circulating endothelial cells (CECs),[2–4] which may subsequently differentiate into mature endothelial cells.[5,6] Recently, CEPs were reported to be involved in tumor angiogenesis in tumor implantation models[7–10] and in clinical studies.[11,12] According to several clinical reports, baseline CEC levels in cancer patients have shown higher values compared with those in healthy controls and were correlated with response and outcome.[13–15]

The aim of this study was to investigate the potential of CEPs and phenotypical CECs as surrogate markers of clinical outcome in metastatic colorectal cancer (mCRC) patients to identify responders to chemotherapy with bevacizumab.

MATERIALS AND METHODS

Patients

Principal inclusion criteria were measurable mCRC and commencement of a new systemic therapy. Other inclusion criteria were Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, adequate organ function, and

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radiographic evidence of disease progression as defined by the Response Evaluation Criteria in Solid Tumors (RECIST). All patients were enrolled on protocols approved by the institutional review board at the Cancer Institute Hospital in The Japanese Foundation for Cancer Research. Written informed consent was obtained from all patients.

Assessment of Biomarkers

Whole blood samples were collected and analyzed at the following times: before initiation of treatment (baseline), immediately after completion of 1 course (day 4), and before commencement of a second cycle (day 14). Blood samples were drawn into 8.5-mL evacuated tubes (BD Biosciences, Franklin Lakes, NJ).

Mononuclear cells isolated by density gradient centrifugation were analyzed using the method established by Duda DG et al.[16] Briefly, Ficoll gradient was used to isolate peripheral blood mononuclear cells (PBMC) and remove red cells and platelets before incubation with antibodies. The following directly conjugated monoclonal antibodies were used for detection of CECs and CEPs by 4-color flow cytometry in peripheral blood: anti-CD31-FITC (BD Pharmingen, San Diego, Calif), anti-CD133-PE (Miltenyi Biotec, Auburn, Calif), anti-CD34-APC (BD Pharmingen), and anti-CD45-PerCP/Cy5.5 (BD Pharmingen). The proportions of CECs (CD31-positive and CD45 negative fractions) and CEPs (CD31-positive, CD34 highly positive, CD133-positive, and CD45 dimly positive fractions) were calculated as percentages of the total number of mononuclear cells after evaluation of at least 50,000 cellular events. Phenotypical CECs expressing VEGFR1, VEGFR2, Tie-2, or CXCR4 were also analyzed. The proportions of these CEC phenotypes were calculated as percentages of the total number of CECs.

Observation of CECs and CEPs

For morphological and immunohistological observation of CECs and CEPs, a small portion of mononuclear cells was fractionated into CXCR4-positive CECs or CEPs by using FACSVantage (Becton Dickinson, Franklin Lakes, NJ). The nuclei of the isolated live CECs and CEPs were stained with DRAQ5 (Alexis, now part of Enzo Life Sciences, Farmingdale, NY) and then observed by confocal laser scanning microscopy (FV1000; Olympus, Center Valley, Penn).

Table 1. Characteristics of Patients Treated With FOLFOX Plus Bevacizumab

Characteristics	Regimen
N=69	FOLFOX+bevacizumab
Median age (range)	61 (27-73)
Sex men/women	38/31
Primary site rectum/colon	24/45
Prior colorectomy +/-	6/63
Metastatic site	
Liver	37
Lung	36
LN	28
Local recurrence	5
Peritoneum	17
Bone	3
Chemotherapy +/-	14/55
5-FU	7
Other	7
CR/PR/SD/PD	2/46/15/6

CR indicates complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Statistical Analysis

Kaplan-Meier survival plots were generated based on CEC levels at each time point of blood sampling, and the curves were compared by using the log-rank test. The Cox proportional hazards regression model was used to determine univariate and multivariate hazard ratios for progression-free survival (PFS) and overall survival (OS).

RESULTS

Patient Characteristics

A total of 69 patients were enrolled. Patient characteristics at baseline are summarized in Table 1. Among 69 patients treated with FOLFOX4 plus bevacizumab assessable for response, we observed complete response in 2 (3%), partial response in 46 (67%), stable disease in 15 (22%), and progressive disease (PD) in 6 (8%) during treatment. Overall response rate was 70%.

Relation Between CEP Levels and Outcome

Univariate Cox regression analysis revealed that CEP levels on day 4 were significantly associated with PFS in 30 of the 69 patients in the training set. To identify the level of CEPs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 0.01%-0.20% of the total number of PBMCs on day 4 were systematically correlated with PFS. Median PFS in patients with levels above or below each threshold differed at 0.04% CEPs of the total number of PBMCs, reaching a plateau at approximately that level. At this level, the Cox

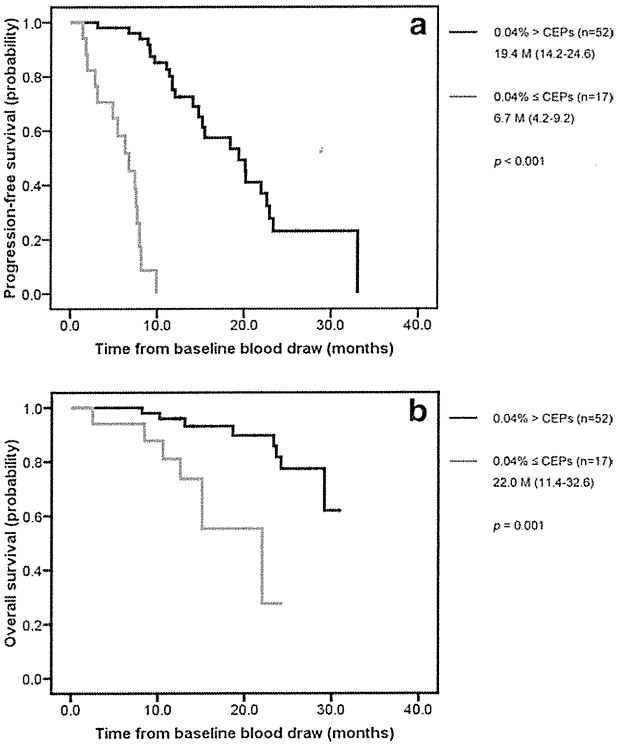

Figure 1. Depicted are (a) Kaplan-Meier plots of progression-free survival (PFS) and (b) Kaplan-Meier plots of overall survival (OS).

proportional-hazard ratio signifying the difference between slow and rapid progression of disease also reached a peak. Therefore, a cutoff of 0.04% CEPs was chosen to distinguish patients. The Kaplan-Meier 0.04% CEP counts were available on day 4 for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 0.04% or more CEPs on day 4 had a shorter median PFS (6.7 months; 95% CI, 4.2-9.2 months) than those with less than 0.04% CEPs on day 4 (19.4 months; 95% CI, 14.2-24.6 months) ($P < .001$) (Fig. 1a). Patients with 0.04% or more CEPs on day 4 had a shorter median OS (22 months; 95% CI, 11.4-32.6 months) than those with less than 0.04% CEPs on day 4 ($P = .001$) (Fig. 1b).

Relation Between CEC Phenotype and Efficacy

Levels of CXCR4 in patients with PD were significantly higher than in those with no PD. Other phenotypes showed no differences between patients with PD and those without (Fig. 2).

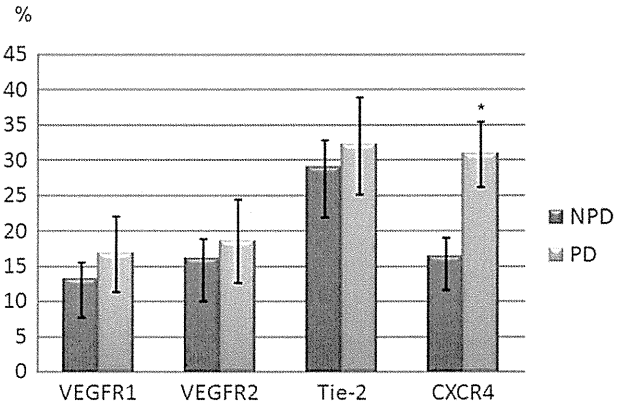

Figure 2. The relation is shown between levels of CEC phenotypes at baseline and bevacizumab efficacy in bevacizumab combination chemotherapy. PD indicates progressive disease; NPD, nonprogression disease. Results are expressed as mean ± standard error of the mean (SE). *$P < .05$

Relation Between CEC Phenotype and Outcome

According to univariate Cox regression analysis, CEC levels at baseline were significantly associated with PFS. To explore the predictive potential of CEC phenotypes at baseline, we analyzed the relation between baseline levels of CEC phenotypes and PFS. Univariate Cox regression analysis revealed that CXCR4-positive CEC levels at baseline were significantly associated with PFS. On the other hand, no correlation was observed between baseline VEGFR1-positive, VEGFR2-positive, or Tie-2–positive CEC levels and PFS. To identify the level of CXCR-positive CECs that most clearly distinguished patients responsive to FOLFOX with bevacizumab, thresholds of 1% to 45% of the total number of CECs at baseline were systematically correlated with PFS. Median PFS in patients with levels of above or below each threshold differed at 20% CXCR4-positive CECs. At this level, the Cox proportional-hazards ratio signifying the difference between slow and rapid progression of disease also reached a plateau. Therefore, a distinguishing cutoff of 20% CXCR4-positive CECs was chosen. The Kaplan-Meier CXCR4-positive CEC count was available at baseline for 30 of the 69 patients in the training set and for 39 of the 69 patients in the validation set. No significant difference was observed in either PFS or OS in either set. Because the 2 sets of data were nearly identical, they were combined to estimate PFS and OS for the entire study population. Patients with 20% or more CXCR4-positive CECs at baseline had a shorter median PFS (6.7 months; 95% CI, 4.1-9.3 months) than those with less than 20% CXCR4-positive

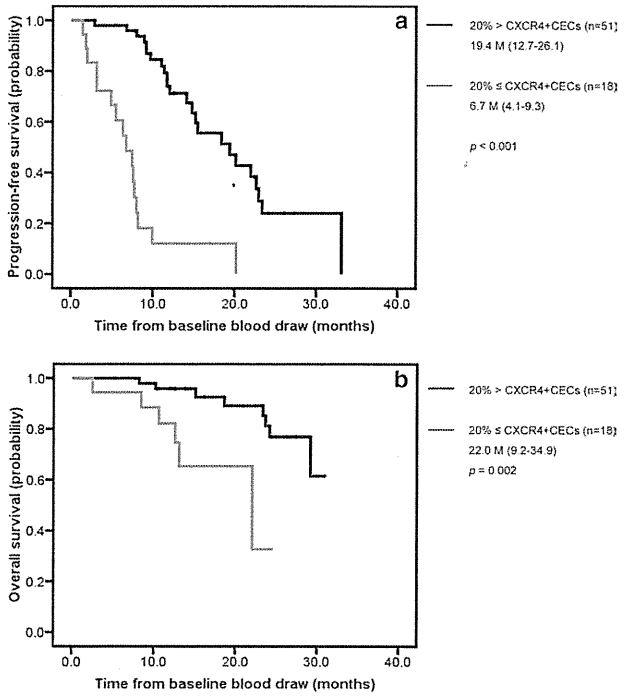

Figure 3. Depicted are Kaplan-Meier plots of (a) progression-free survival (PFS) and (b) overall survival (OS).

CECs at baseline (19.4 months; 95% CI, 12.7-26.1 months) ($P < .001$) (Fig. 3a). Patients with 20% or more CXCR4-positive CECs at baseline had a shorter median OS (22 months; 95% CI, 9.2-34.9 months) than those with less than 20% CXCR4-positive CECs at baseline ($P = .002$) (Fig. 3b).

Univariate and multivariate Cox proportional hazards regression was performed to assess the association between factors of interest and PFS or OS. According to the univariate Cox regression analysis, liver metastasis, lung metastasis, CEP levels on day 4, and CXCR4-positive CEC levels at baseline were associated with PFS; furthermore, peritoneal metastasis, CEP levels on day 4, and CXCR4-positive CEC levels at baseline were associated with OS (Table 2). To evaluate the independent predictive effect of these markers, multivariate Cox regression analysis was carried out (Table 3). Levels of CEP on day 4 and CXCR4-positive CEC levels at baseline were the strongest predictors.

DISCUSSION

Some authors have suggested that CECs are a predictive marker of clinical outcome in cancer patients treated with bevacizumab-based chemotherapy. In breast cancer, most studies[14,17,18] have reported that high CEC levels at baseline indicate a better outcome than low CEC levels. On the other hand, in colorectal cancer, low CEC levels at baseline were reported to indicate a better outcome than high CEC levels.[19,20] These results suggest vascular formation differs according to tumor origin. However, these differences in results between these 2 types of cancer may have resulted from differences in the measurement protocols used. A number of methods and protocols are used to evaluate and count CECs. Two widely used protocols involve the use of flow cytometry. Duda et al[16] reported a cytometry protocol for phenotypic identification and enumeration of CECs and CEPs using 4 surface markers: CD31, CD34, CD133, and CD45. This procedure is believed to allow detection of 0.1% to 6.0% of viable CECs and 0.01% to 0.20% of CEPs from among a blood mononuclear cell population and is mainly used in colorectal cancer. Mancuso et al[21] reported a protocol for the phenotypic identification and enumeration of CECs and CEPs involving 6-color flow cytometry, nuclear staining with Syto16 (Molecular Probes, Eugene, Ore) and 7-AAD (Flow Labs, Irvine, UK) and a panel of monoclonal antibodies, including CD45, CD133, CD31, and CD146. This protocol has been mainly used in breast cancer. In this study, we selected the protocol of Duda et al.

Willet et al[19] reported that CEP levels decreased on day 3 after initiation of bevacizumab with chemoradiation in rectal cancer patients. On the basis of this earlier report, we decided, in this study, to collect samples at 3 days (day 4) after initiation of chemotherapy with bevacizumab. We

Table 2. Independent Predictive Factors by Univariate Cox Regression Analysis for Progression-Free Survival and Overall Survival

Parameter	No. of Patients	HR	95% CI	*P*	X^2
PFS					
CEP	69	7.01	3.5-14.05	<.001	<.001
CXCR4+CEC	69	22.96	8.52-61.87	<.001	<.001
Liver metastasis	69	2.71	1.36-5.38	.004	.003
Lung metastasis	69	2.44	1.22-4.90	.012	.009
OS					
CEP	69	5.45	1.71-17.4	.004	.002
CXCR4+CEC	69	5.26	1.64-16.9	.005	.002
Peritoneal metastasis	69	3.46	1.16-10.33	.026	.018

HR indicates hazard ratio; CI, confidence interval; PFS, progression-free survival; CEP, circulating endothelial progenitor; CEC, circulation endothelial cell; OS, overall survival.