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＜臨床試験に参加しよう＞

ガイドライン診療と臨床試験は何が違うのですか？

七野浩之*
Hiroyuki Shichino

I．ガイドライン診療

ガイドライン，英語表記でguidelineは，「指針」のことです。

ガイドライン診療とは，ある機関が策定した「ガイドラインを基に」して，「一人ひとりの個々の患者」に対して，その患者に最も合った「個別の」診療を行うことです。何かの仮説を証明すること，すなわち，まだ明らかになっていない事柄を明らかにするための試験的内容は含まれません。

近年，各学会・研究会・診療グループなどは，各種の疾患について，診断や治療に関するガイドラインを多数作成し発表しています。それらのガイドラインは，すでに科学的に明らかになっている事柄，すなわち，文献的エビデンスに基づき作成されています。その目的は，ある特定の集団に対し，一定レベルの医療の質の確保・維持・向上を目指すものです。

ガイドラインには，疾患別ガイドライン，治療ガイドライン，診断ガイドライン，疾病予防ガイドラインなどさまざまなものがあります。また，臨床試験を計画立案し実施するためのガイドラインもあります。

ガイドラインは，実地診療のためのものです。大多数の患者に対して参考となる有用な情報を提供します。しかし，その利用に際しては，ガイドラインをそのままその通りに個別の患者に適用するだけでは不十分であり，個別の患者一人ひとりに合うような実際的な利用法が医療者側に求められているものです。

日本小児血液学会では「小児における自己および同種造血幹細胞採取に関する技術指針」や「健常小児ドナーからの造血幹細胞採取に関する倫理指針」などを作成公表していますが，小児白血病リンパ腫をはじめとして各種血液性腫瘍疾患に対する治療ガイドラインは作成していません。日本小児がん学会では，「小児固形悪性腫瘍に対する抗がん剤適正使用ガイドライン」を作成公表していますが，ガイドラインを基に実際の治療を行う目的および形式では作成されていません。仮に白血病リンパ腫に対して，「実際の治療が行える」ような治療ガイドラインを作成しようと試みても，現在の小児白血病リンパ腫医療を取り巻く種々の状況下では，その作成が可能であるとは考えにくいと思います。

II．臨床試験

臨床試験，clinical trialは「試験」です。別の言葉で言い換えれば，治療を行いながら行う「人体に対する実験」です。

一人ひとりの個々の患者に対して，その患者に最も合った個別の実地診療を行うことが臨床試験の目的ではありません。何らかの「仮説を証明する」こと，すなわち「まだ明らかになっていない事柄を明らかにすること」が目的であり，その証明を行えるように厳密にデザインされた「実験」を行うことが，「臨床試験」です。その利用に際しては，被験者の利益を相当に侵すような理由がな

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いかぎり，その実施計画書に記載された手順を厳密に遵守することが求められます。

医療は必ず何らかの形で身体に侵襲を加える行為を伴うものです。臨床試験の最終的な目的は，病気をよりよく治すことであり，そのことにより将来的に人類全体に対して利益をもたらし，人類をより幸福へと導くことです。臨床試験はこのような目的をもってはいますが，実際には，個々の被験者（患者）にとっては，その試験の効果も不利益も未知のものであり，常に実験的要素を大きく含むものです。したがって，試験を受ける人＝被験者（患者）は常に，科学的かつ倫理的に保護されていなければなりません。そのためには，ヘルシンキ宣言・日米欧による International Conference on Harmonization（ICH）の活動・医薬品の臨床試験の実施の基準に関する省令（GCP）等々の遵守が絶対的な要件となります。ガイドライン診療では，こういったガイドラインに基づく必要はありません。

臨床試験は，実験ですので，必ず明らかにしたい仮説が存在し，その仮説に対して必ず何らかの結論を出さなければなりません。目的のない臨床試験は存在しません。結果の出ない臨床試験は存在しません。実験を完遂し必ず結果を出すために，試験開始前に，厳密に試験計画書を作成します。試験計画書では，試験を実施するために必要な，科学的かつ倫理的根拠を明確に記述し，そのほかにも，目的を明確に記述する，対象および登録適格条件を明確に記述する，治療内容や変更基準・中止基準を明確に記述する，エンドポイントを明確に記述する，収集するデータを明確に記述する，検出すべき差の大きさもしくは推定の精度など統計学的手法に基づく事項を明確に記述する，治療法の割り付けの実施法を特定する，サンプルサイズの計算に用いる分布の仮定やエラーの確率を決定するなどなど多数の項目を一つひとつ規定しておく必要があります。ガイドライン診療では，仮説の証明が目的ではありませんので，臨床試験には必要な種々の規定を記載した計画書は必要ではありません。臨床試験とガイドライン診療はまったく別の次元のものといえます。

III．臨床試験に参加しよう

小児がん・小児血液腫瘍疾患では，現時点では，ガイドライン診療は成立しないと考えられます。たとえば，アメリカ National Cancer Institute の PDQ（Physician Data Query）および PDQ 日本語版には，小児急性リンパ性白血病の項目に，以下のように記載があります。

「小児 ALL において治療の進歩が認められるが，全ての小児 ALL の治癒という目的を達成するためには，数々の重要な生物学的かつ治療的な問題が残されている。これらの問題を体系的に研究するためには大規模な臨床試験を実施する必要があり，これらの試験に参加する機会がほとんどの患者および家族に提供されている。一般に，ALL の小児および青年を対象とした臨床試験は，より望ましいと考えられる治療と現在標準と考えられている治療を比較するためにデザインされている。小児 ALL をはじめとする小児癌の治癒的療法の進歩の大部分は，注意深くランダム化された比較臨床試験において検証された，研究者による発見を通して達成されている。」

もし，理想的な治療法が発見され確立されれば，臨床試験は不要になると考えられます。100％の無病生存率と，短期的長期的合併症がまったくない治療法が，理想的な治療法と考えられます。しかしながら，そのような治療法は実現されるとは考えにくいでしょう。母集団も常に新しい集団に変わり，新規薬剤も常に出現し，支持療法も常に改善され，あるいは，まったく異なったパラダイム（疾患に対する考え方）が出現する可能性もあります。新たな仮説は永遠に消滅することなく生まれてくると想像できます。

現在，標準と考えられている治療をもとに，それよりもより優れた治療法をつくりたい，より良く治したいという希望を，医療関係者が失わないかぎり，そして，きちんと作成された臨床試験がその時代でより望ましい治療法と考えられるかぎり，臨床試験に参加しようと考える患者は存在し，臨床試験を実施しようとする医療関係者も存在し，臨床試験がなくなることはないでしょう。

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お知らせ（1）

▨第38回日本小児消化管機能研究会

会　長：嵩原裕夫（徳島大小児外科・小児内視鏡外科）
会　期：2008年2月16日（土）
会　場：ルネッサンスリゾートナルト
（瀬戸内海国立公園内）
主　題：消化管機能検査法の再評価；GERD，ヒルシュスプルング病，高位鎖肛の術後機能評価をどうするか？；（昨年の）慢性便秘に対するコンセンサスカンファレンスの検証
一般演題：小児消化管機能に関する演題を広く募集
演題締切：2007年12月20日（土）
詳細については下記に問合せ
連絡先：徳島大学病院小児外科・小児内視鏡外科
嵩原裕夫
E-mail：takehara@clin.med.tokushima-u.ac.jp

▨第12回ウイルソン病研究会

日　時：2008年5月10日（土）14：00～18：00
会　場：東邦大学医療センター大森病院臨床講堂
（東京都大田区大森西）
代表幹事：青木継稔（東邦大学）
参加費：2,000円
演題募集：Wilson病をはじめMenkes病など銅代謝異常症に関する症例報告，研究報告など。
申込方法：演題名，施設名，演者名（発表者に○印），連絡先（住所，Tel, Fax），及び発表者メールアドレスを記入しE-mailにて下記事務局まで
締　切：2008年2月15日（金）必着
申込先：ウイルソン病研究会事務局
〒153-8515　東京都目黒区大橋2-17-6
東邦大学医療センター大橋病院小児科内
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▨第99回日本小児精神神経学会

会　期：2008年6月13日（金），14日（土）
会　場：米子コンベンションセンターBIGSHIP
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会　長：汐田まどか（鳥取県立総合療育センター）
テーマ：地域発～子どもの心の臨床/新世代の小児精神神経学パート2
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▨第100回記念日本小児精神神経学会

会　期：2008年11月8日（土），9日（日）
会　場：東京医科大学病院6階臨床講堂
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会　長：星加明徳（東京医大小児科）
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＜臨床試験に参加しよう＞

標準治療とはなんですか？

七野浩之*
Hiroyuki Shichino

Ⅰ．臨床試験における標準治療

1．第Ⅰ・Ⅱ・Ⅲ相臨床試験

第Ⅰ相臨床試験とは，新規薬剤の最大耐用量の決定を行う試験です。第Ⅰ相臨床試験の被験者は健康な成人です。健康成人の体における新規薬剤の吸収・分解・排泄・有害作用の出現などを確認し，用量制限毒性を発見することが目的です。通常はフィボナッチの変法が利用されます。

第Ⅱ相臨床試験は，新薬のスクリーニングを行い，どの薬剤の評価を今後さらに続けるかを決定する，言い換えれば，第Ⅲ相臨床試験に進めることができる薬剤かどうかを決定することが目的です。あるいは，単独の薬剤ではなく，いくつかある新しい治療法の中から第Ⅲ相試験に進める治療法を選ぶ場合もあります。第Ⅱ相臨床試験では，実際に患者を被験者とし，その効果と安全性について検討し，用量反応関係がみられるかどうかなども確認します。

第Ⅲ相臨床試験は，複数の治療法を比較し，どの治療法が優れているかを決定することが目的です。治療と結果との因果関係を明らかにし，複数の治療のどれが最も優れているのかを決定するためには，ランダム化比較試験が必須と考えられます。その比較を行う際の「対照」となる治療が「標準治療」です。

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2．臨床試験における標準治療

標準治療とは，臨床試験において使用される用語です。第Ⅲ相臨床試験において，新規治療（試験治療）と比較される対照としての治療法のことです。

通常は，くり返される第Ⅲ相臨床試験によって優れていると結論された治療法が，その臨床試験が終了し結論が出た後の新たな標準治療となります。今度は，この標準治療を対照として，新規試験治療の効果・安全性を比較検討することになります。

現実的には，日本においては小児がん患者の罹患率が少ないことから，小児領域の小児がん血液腫瘍疾患分野では，第Ⅲ相比較臨床試験はほとんど行われていません。今のところ，臨床試験の場で使用する意味での標準治療は，厳密な意味では存在しないと考えられます。第Ⅲ相臨床試験において試験治療と比較される対照としての標準治療は，現在の日本では多くの疾患で見当たりません。

ところが，第Ⅱ相臨床試験を行ううえでも，いわゆる標準治療（標準「的」治療）が必要になります。その時代状況で最も優れている治療法を標準治療として想定する必要があります。そうしないと，臨床試験を行おうとする新規治療法が，その時代の一般的な治療法よりも優れた効果・安全性などが想定されるのかどうかを判断できなくなってしまうためです。そこで，第Ⅱ相臨床試験でも標準治療を策定する必要があるわけです。

通常は，その時代状況の中で，文献的エビデンスなどに基づき最も治療成績が優れ，有害事象が少ないとされていると，各研究者が合意する治療法が選択されることになります。

COG, BFM, JACLS, CCLSG, TCCSG, KYCCSGなどのように，多施設共同臨床試験をくり返し行っている研究グループでは，現在進行している臨床試験のひとつ前の臨床試験の治療法が，そのグループにおける標準治療になるでしょう。では，グループ間の，つまりCOG, BFM, JACLS, CCLSG, TCCSG, KYCCSGなどを横断する標準治療はどう決めるべきでしょうか？　それぞれの標準治療の間でどの治療法が最も標準的なのだろうか？　という質問には，複数の治療を比較するための，第III相臨床試験を行わなければわからないと答えるべきです。したがって，複数の標準治療が存在することは，現実ではやむをえないことでしょう。

II．臨床試験と関係のない標準治療

臨床実地では，臨床試験ではない治療も多数行われます。臨床試験が実施されない疾患や病態も多いと思われます。そのような臨床実地でも，独りよがりではない，科学的倫理的治療を行うためには標準治療という考え方は必須と思われます。たとえば，造血細胞移植が必要な急性リンパ性白血病患者に対する移植前処置の標準治療は何でしょうか？　その答えは，文献的エビデンスや学会レベルでのエビデンスに基づき，その時代状況で，多数の医療関係者が合意する標準治療が自ずと存在するはずです。単一の標準治療ではないかもしれませんが，自ずと標準的な治療法は明らかになると考えられます。しかし，疾患によっては（いわゆる）標準治療の成績は不十分であることも少なくありません。標準治療は必ずしも理想治療ではありません。

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＜臨床試験に参加しよう＞

治療ガイドライン，プロトコール，レジュメの違いはなんですか？

七野浩之*
Hiroyuki Shichino

Ⅰ．ガイドライン

ガイドラインとはguidelineのことで，指針のことです。治療ガイドラインは治療指針を意味します。多くの場合には，詳細な治療規定はなく，大まかな治療の方向性を示すだけのことが多いようです。ガイドラインには，ほかに疾患別ガイドライン，診断ガイドライン，疾病予防ガイドラインなどや，臨床試験の作成のための種々の規定を示したガイドラインがあります。治療ガイドラインは，白血病リンパ腫などの血液腫瘍疾患では，文献的エビデンスに基づき，使用を推奨する薬剤の組み合わせなどを，指針として示すものとなると考えられます。

Ⅱ．プロトコール

プロトコールとはprotocolのことで，プロトコルともいい，通常は手順書のことをいいます。複数の人が使用する決まりごとを，確実に間違いなく実行するための手順について，明確に記述したものをいいます。各専門分野で状況に応じて訳語が使用されています。日本語では，規定，議定書，儀典などと訳されることが多いようです。

臨床試験の場では，臨床試験の手順を記載した「治療計画書」のことをいい，そこから派生して，治療計画書に規定される非常に厳密な治療法のことをプロトコールとよぶこともあります。また，理解がごちゃごちゃしてしまいますが，臨床試験では，種々の治療（化学療法，外科療法，放射線療法，支持療法，後療法など）のなかで，どこからどこまでがその臨床試験で規定した治療なのかを厳密に規定します。この厳密に規定された治療のことを，その臨床試験の「プロトコール治療」とよびます。

プロトコールには，誰もが誤解せず，間違いなく治療が行えるように詳細な記述がなされます。たとえば，「ビンクリスチン1.5 mg/m²（最大投与量は2 mgである）を1週間に1回，ワンショット静注する。患者体重が10 kg未満の場合には0.05 mg/kgを使用する。ビンクリスチン1バイアルは1 mgであり，注射用水または生理食塩液10 mlで溶解する」など詳細な手順が記載されています。

Ⅲ．レジュメ

レジュメはrésumé（フランス語）のことです。英語読みではレジメです。要旨，要約，摘要，概要，梗概，概論の意味です。講演や研究会などで，紙などに発表内容を簡潔にまとめたものをいいます。また最近では，履歴書の意味でも用いられることがあります。

レジメンはregimenのことで，治療計画や投与計画あるいは投与薬剤併用計画の概要を記載したもののことです。多くの場合，レジュメ（レジメ）

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とレジメンはあまり区別をして使われていないようです。

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＜臨床試験に参加しよう＞

臨床試験から脱落した症例はどうしたらよいでしょうか？

七野浩之*
Hiroyuki Shichino

臨床試験から脱落した症例は，その臨床試験治療計画書の規定に従いその後の治療を行います。何も規定がなく，臨床試験担当医の判断に任せられているときには，各担当医が治療法の決定を行うことになります。

臨床試験からの脱落が，原疾患の増悪（PD）の際には，もし，他の臨床試験が存在し，その施設・患者が登録適格条件を満たすのであれば，参加することができるでしょう。たとえば，再発または難治性の小児固形腫瘍の場合には，小児医師主導治験「難治性小児悪性固形腫瘍に対する塩酸イリノテカンの第Ⅰ-Ⅱ相臨床試験」（治験調整医師 国立がんセンター中央病院小児科 牧本 敦）があります。将来的にはこのような再発例や難治例に対する臨床試験が多数並存することが望まれますが，現在では，まだ難治性の小児がん，血液腫瘍性疾患の場合には，それぞれの施設で独自の治療計画や臨床試験が実施されていることが多いようです。今後は，このような患者に対しても，多施設共同の臨床試験の実施が望ましいと考えられます。とくに，新規薬剤の開発・臨床応用をスムーズに早期に進めるためにも，臨床試験体制の構築が必要と考えられます。

もし，臨床試験が存在しない場合には，担当医の判断で，その時点での「標準的治療」と考える治療法を行うか，新規薬剤の使用など実験的な治療法を行うか，治療を終了するか，ということになります。いずれの場合も，患者および親権者による同意を得る必要があることはいうまでもありません。

臨床試験からの脱落が，他の理由による場合でも，その後の治療をどのように行うかは，上記のように，十分な相談と同意のうえで，個々の担当医が判断すべき事柄だと考えられます。

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Ewing Sarcoma/Primitive Neuroectodermal Tumor of the Kidney in a Child

Miho Maeda, MD,[1]* Akio Tsuda, MD,[1] Shingo Yamanishi, MD,[1] Yoko Uchikoba, MD,[1] Yoshitaka Fukunaga, MD,[1] Hajime Okita, MD,[2] and Jun-ichi Hata, MD[3]

A 6-year-old female was admitted with abdominal pain and a mass in the right abdomen. Her lactose dehydrogenase level was 1,200 IU/L, and neuron specific enolase was 120 ng/ml. Computed tomography scan confirmed a large right renal mass with necrosis. A right radical nephrectomy was performed. The tumor was completely encapsulated. Based on small round cell histology, strong MIC-2 (CD99) positive tumor cells, and EWS-FLI-1 fusion transcript, Ewing sarcoma/primitive neuroectodermal tumor of the kidney was diagnosed. Induction and follow-up with seven cycles of chemotherapy were given after surgery. She has had no evidence of recurrence 90 months from diagnosis. Pediatr Blood Cancer 2008;50:180–183. © 2006 Wiley-Liss, Inc.

Key words: electron microscopy; Ewing sarcoma/primitive neuroectodermal tumor; EWS-FLI-1; immunohistochemistry; kidney

INTRODUCTION

Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET) of the kidney is a rare and highly malignant neoplasm. It affects young adults, and only a few pediatric cases (younger than 15 years) have been reported [1–9]. ES/PNET arising in the kidney act aggressively and show poor response to therapy [1]. ES/PNET of the kidney needs to be differentiated from other small round cell tumors of the kidney, because each type of tumor is treated differently. The diagnosis of this neoplasm is currently based on a combination of light microscopy, immunohistochemistry, electron microscopy, chromosomal analyses, and specific chimeric transcripts. Our patient, who was diagnosed by histochemistry and molecular biology analysis of the resected kidney and treated with chemotherapy, has remained alive more than 90 months after diagnosis.

CASE

A 6-year-old female was admitted to our hospital with abdominal pain and an abdominal mass. On physical examination, a large and firm mass was evident in the right abdomen. Laboratory evaluation showed a lactate dehydrogenase level of 1,200 IU/L (normal 218–411 IU/L), a neuron specific enolase level of 120 ng/ml (normal <10 ng/ml), and ferritin level of 160 ng/ml (normal 15–89 ng/ml). Urine catecholamine levels were within normal limits. Abdominal computed tomography (CT) scan confirmed a large right renal mass with areas of necrosis and bleeding. There was no obvious lymphadenopathy and no intra-abdominal metastasis. Bone scintigraphy and CT scan of the thorax did not detect metastasis.

A right radical nephrectomy was performed. The tumor involved a large portion of the lower part of the kidney. The tumor was completely encapsulated and was 5.0 × 4.5 × 4.5 cm. Lymph nodes were negative for malignancy. Histologic examination revealed a small round cell tumor with massive necrosis, but no rosette formations. Periodic acid-Schiff (PAS) staining revealed diastase sensitive material in the tumor cell cytoplasm. Immunohistochemistry revealed that tumor cells were strongly positive for MIC-2 (CD99) as well as vimentin. The tumor cells were negative for chromogranin A, neurofilament, and synaptophysin. Electron microscopic examination showed a high nuclear-cytoplasm ratio and aggregated glycogen granules in the cytoplasm (Fig. 1A). A higher magnification of tumor cells showed neurosecretory-type granules, microtubules, and desmosome-like structures (Fig. 1B). The expression of EWS-FLI-1 fusion transcript was demonstrated by molecular biology (Fig. 2). A single 330 base pair cDNA product was detected by ethidium bromide staining, corresponding to the EWS-FLI-1 as previously reported by Sorensen et al. [10]. Direct DNA sequencing confirmed the presence of a fusion of EWS exon 7 to the FLI-1 exon 6. Unfortunately chromosomal findings failed because proliferation of the tumor cells was poor. According to results on small round cell histology and immunohistochemical profiles, electron microscopic findings, and EWS-FLI-1 fusion transcript, the tumor was diagnosed as an ES/PNET of the kidney. Therapy was initiated with 1.5 gm/m^2 vincristine on days 1, 8, 15, 22, 29, and 36; 500 mg/m^2 cyclophosphamide on days 2, 9, 30, and 37; and 0.45 mg/m^2 dactinomycin on days 16–20 for induction and then a total of seven cycles of 4-drug chemotherapy, consisting of 1.5 gm/m^2 vincristine on days 1, 15, 22, 29, 36, and 43; 0.45 mg/m^2 dactinomycin on days 1–5; 500 mg/m^2 cyclophosphamide on days 16, 23, 30, 37, and 44; and 60 mg/m^2 doxorubicin on day 44 after surgery. She had no serious adverse effects during chemotherapy. She had no evidence of recurrence after 90 months from diagnosis and no late effects have been noted.

DISCUSSION

Though the existence of renal PNET was reported in 1975 in a review of pediatric PNETs [11], only a small number of cases have been reported. Recently, Parham et al. [12] from National Wilms Tumor Study Group Pathology Center reported that 79 of 146 cases of primary malignant neuroepithelial tumors of the kidney in adults and children were considered to be ES/PNET. Follow-up information, however, was only provided for 14 of 146 cases, and it is unclear which, if any, of those were actually ES/PNET [8]. Pediatric cases (younger than 15 years old) of ES/PNET of the kidney are extremely rare, and only ten cases have been reported previously [1–9]. Clinical characteristics, pathologic features, treatments, and outcomes of those cases are summarized in Table I.

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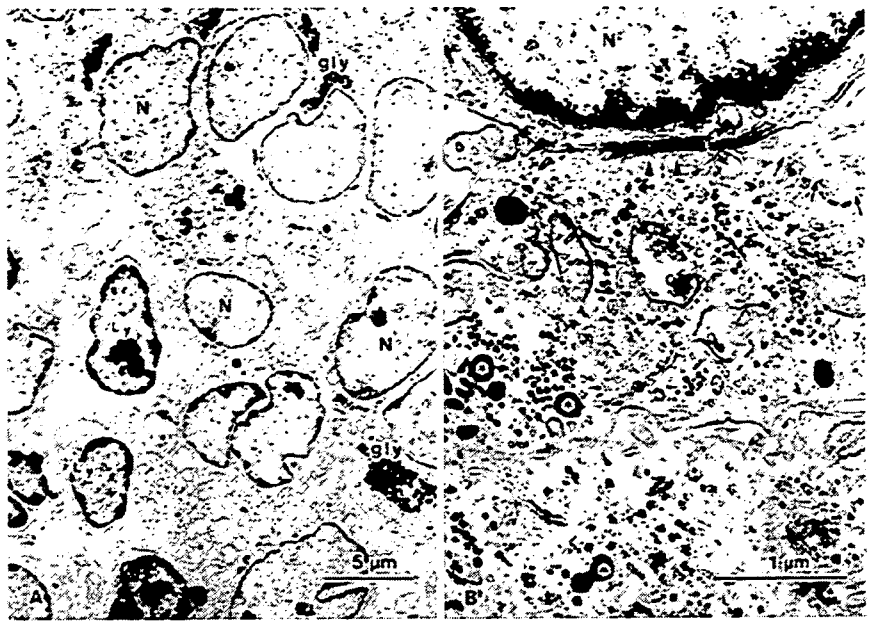

Fig. 1. Ultrastructural findings in the tumor cells. A: Tumor cells are oval and small (about 8–10 μm in a diameter). Nuclear-cytoplasm ratio is high. Nucleus has a few heterochromatin. Aggregated glycogen granules (gly) are observed in the cytoplasm. Ly, lymphocytes; N, nuclei. B: Neurosecretory granules (asterisks), microtubules (arrows), and desmosome-like structures (arrowheads) are observed in the tumor cells under higher magnification.

Several approaches can be used to arrive at a diagnosis of ES/PNET. The first approach is light microscopic examination of tumor tissue including immunohistochemistry. These tumors consist of primitive-appearing round cells with high nucleus to cytoplasmic ratios. The immunohistochemical features of ES/PNET are positive for CD99 (MIC2); however, expression of CD99 is by no means specific for ES/PNET among round cell tumors [13]. Although FLI-1 is a variable histochemical marker for ES/PNET, it is also positive in lymphoblastic lymphoma [14]. In contrast, WT-1 is a positive marker of Wilms tumor and desmoplastic round cell tumors, whereas it is a negative marker for ES/PNET, neuroblastoma and rhabdomyosarcoma. The second approach is electron microscopic examination of tumor tissue. Electron microscopic features include a specific high nuclear-cytoplasm ratio and aggregated glycogen granules in the cytoplasm. Neural differentiation appears on some cells with polar processes, which may contain microtubules or neurosecretory glands [15]. The third approach is chromosomal translocation, such as t(11;22) (q24;q12) which is positive in 88–95% of ES/PNET cases [16]. The final approach involves a molecular biologic examination. In 90–95% of cases of ES/PNET, the chimeric transcript is EWS-FLI-1; the remaining 5–10% are EWS-ERG. Other transcripts, including EWS-ETV1 and EWS-EIAF, have also been reported [16].

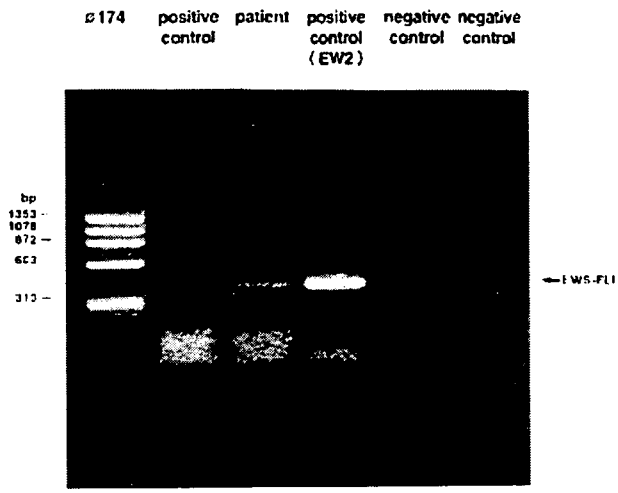

Fig. 2. A single 330 base pair transcript is detected in the patient sample following reserve transcriptase polymerase chain reactor (RT-PCR) performed on RNA extract from tumor tissue.

In terms of prognosis, the 5-year disease-free survival rate of ES/PNET is 45–55% [17], but the prognosis of ES/PNET of the kidney appears worse [1,18]. In pediatric cases (Table I), 5 of 8 patients were alive when the cases were reported; however, 1 patient (no. 6) was alive with disease, 2 patients (no. 3 and no. 5) were followed-up only for 6 and 8 months, and 1 patient was under treatment (no. 9). The follow-up duration was not described in this case. Only 2 patients (no. 8 and our case) were alive after 5 years. For 2 patients, it was not defined whether they were alive or not (Table I). Jimenez et al. [8] described that 3 of 11 patients were alive for 4–64 months, and 5 patients had local recurrence or distance metastasis then died of their disease, and 3 patients were lost to follow-up. Most of the recent therapeutic protocol for children with ES/PNET consists of vincristine, doxorubicin, cyclophsphamide, ifosphamide, and etoposide. Radiation and surgery have been used; some patients have been treated with myeloablative chemoradiotherapy followed by autologous bone marrow rescue. In spite of a lack of radiation therapy and our not using ifosphamide and etoposide for chemotherapy, our patient has survived for a relatively long period with no recurrence. Possible reasons for this good outcome might include the pathologic features of the tumor, the well-encapsulated nature of

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TABLE I. Clinical and Pathological Features of ES/PNET of the Kidney in Pediatric Cases

Case	Ref.	Age	Gender (yr)	Symptoms	Metastasis	Pathology (immunohistochemistries)	Chimeric transcript	Therapy	Outcome (follow-up [Mo])
1	1	4	F	Abdominal pain, fever	RPLN, liver	CD99(+),NSE(+),S-100(+), Ker(+),Act(−),Vim(−),Chro(−)	NS	IFO, CBP, VP-16 radiation	Died (1)
2	1	14	M	Bone pain, weight loss	Lung, bone, bone marrow	CD99(+),NSE(+),Vim(+),Synapto(+) (±)S-100(−),Ker(−),Act(−),Chro(−)	EWS/FLI-1(−) EWS/ERG(−)	CY, VCR, DOX,IFO,VP-16 auto BMT	Alive (under treatment)
3	2	13	NS	Abdominal pain, hematuria	No	MIC2(+),NSE(+),ker(−),Des(−), Act(−)	EWS/FLI-1(+)	Nephrectomy chemotherapy	NS
4	3	10	M	Abdominal mass	No	MIC2(+),NSE(+),Leu7(+), S-100(−),Ker(−), Des(−),Vim(−),Chro(−)	EWS/FLI-1(+)	Nephrectomy chemotherapy	Alive (6)
5	4	5	F	NS	IVC, right heart	NS	NS		NS
6	5	15	F	Abdominal pain, abdominal distention	No	MIC2(+),Vim(+),NSE(−), S-100(−)	NS	Nephrectomy CY, VCR, DOX,IFO,VP-16	Alive (8)
7	6	9	M	Abdominal pain, abdominal mass, weight loss	No	MIC2(+),NSE(−),Vim(−),Ker(−), LCA(−)	NS	Nephrectomy CY, VCR, DOX,IFO,VP-16	Alive (relapse+) (10)
8	7	9	F	Abdominal distention, abdominal mass	No	CD99(+),LCA(−),Ker(−),Act(−), NFM(−)	EWS/FLI-1(+)	Nephrectomy IFO,VP-16,CY,DOX,VCR auto BMT	Died (5)
9	8	11	M	Gross hematuria, abdominal mass	No	CD99(+)	NS	Nephrectomy VCR, DOX, VP-16, CY, DAC	Alive (64)
10	9	14	F	Abdominal pain, abdominal mass	IVC, right heart, liver	NS	NS	Chemotherapy	Died (24)
11	Present case	6	F	Abdominal pain, abdominal mass	No	MIC2(+),Vim(+)NFM(−),Chrom(−)	EWS/FLI-1(+)	Nephrectomy VCR, DAC, CY, DOX,	Alive (90)

RPLN, retroperitoneal lymphonode; IVS, inferior vena cava; NSE, neuron specific enolase; Ker, keratin; Act, actin; Vim, Vimentin; Chro, chromogrn A; MIC2, B microgloblin; Des, desmin; NFM, neurofibrofilament; Synapto, sinaptophilin; IFO, ifomide; CBD, carboplatinum; CY, cyclophosphamide; VCR, vincristine; DOX, doxrubicine; DAC, actinomycin D; BMT, bone marrow transplantation.

the tumor with no involvement beyond the capsule and the accurate diagnosis followed by prompt treatment with chemotherapy. Several approaches including cytogenetical methods are important for early, accurate diagnosis of ES/PNET.

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Cerebellar Medullomyoblastoma With Melanotic Tubular Structures

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This report describes a midline cerebellar primitive neuroectodermal tumor with muscular differentiation, that is, medullomyoblastoma with melanotic tubular structures, which developed in the cerebellar vermis in a 23-month-old male. Rhabdomyoblastic differentiation consisted both of striated muscle fibers and undifferentiated cells showing immunoreactivity for desmin and myogenic transcription factors. The presence of melanotic epithelial structures raised the issue of a teratomatous tumor. This case demonstrates the occurrence of this very rare tumor in early childhood as well as the utility of a careful search for the presence of myogenic and/or melanotic features in medulloblastomas. Pediatr Blood Cancer 2008;50:183–185. © 2006 Wiley-Liss, Inc.

Key words: brain tumors; medullomyoblastoma; pigmented medulloblastoma

INTRODUCTION

Medullomyoblastoma (MMB) is a rare variant of medulloblastoma (MB) characterized by both a primitive neuroectodermal and a striated muscle component [1], in which an additional constituent of pigmented melanocytic cells might be present [2–4]. A midline cerebellar primitive neuroectodermal tumor with muscular differentiation and melanotic tubular structures is described.

CASE

In January 2004, a 23-month-old male was hospitalized with a 4-week history of headache. General physical and neurological

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Inducible Expression of Chimeric EWS/ETS Proteins Confers Ewing's Family Tumor-Like Phenotypes to Human Mesenchymal Progenitor Cells▽†

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Ewing's family tumor (EFT) is a rare pediatric tumor of unclear origin that occurs in bone and soft tissue. Specific chromosomal translocations found in EFT cause EWS to fuse to a subset of ets transcription factor genes (ETS), generating chimeric EWS/ETS proteins. These proteins are believed to play a crucial role in the onset and progression of EFT. However, the mechanisms responsible for the EWS/ETS-mediated onset remain unclear. Here we report the establishment of a tetracycline-controlled EWS/ETS-inducible system in human bone marrow-derived mesenchymal progenitor cells (MPCs). Ectopic expression of both EWS/FLI1 and EWS/ERG proteins resulted in a dramatic change of morphology, i.e., from a mesenchymal spindle shape to a small round-to-polygonal cell, one of the characteristics of EFT. EWS/ETS also induced immunophenotypic changes in MPCs, including the disappearance of the mesenchyme-positive markers CD10 and CD13 and the up-regulation of the EFT-positive markers CD54, CD99, CD117, and CD271. Furthermore, a prominent shift from the gene expression profile of MPCs to that of EFT was observed in the presence of EWS/ETS. Together with the observation that EWS/ETS enhances the ability of cells to invade Matrigel, these results suggest that EWS/ETS proteins contribute to alterations of cellular features and confer an EFT-like phenotype to human MPCs.

AQ: A

Fn2/AQ: B Ewing's family tumor (EFT) is a rare childhood cancer arising mainly in bone and soft tissue. Since EFT has a poor prognosis, it is important to elucidate the underlying pathogenic mechanisms for establishing a more effective therapeutic strategy. EFT is characterized by the presence of chimeric genes composed of EWS and ets transcription factor genes (ETS) formed by specific chromosomal translocations, i.e., EWS/FLI1, t(11;22)(q24;q12); EWS/ERG, t(21;22)(q12;q12); EWS/ETV1, t(7;22)(p22;q12); EWS/E1AF, t(17;22)(q12;q12); and EWS/FEV, t(2;22)(q33;q12) (26). The products of these chimeric genes behave as aberrant transcriptional regulators and are believed to play a crucial role in the onset and progression of EFT (3, 36). Indeed, recent studies have revealed that the induction of EWS/FLI1 proteins can trigger transformation in certain cell types, including NIH 3T3 cells (36), C2C12 myoblasts (12), and murine primary bone marrow-derived mesenchymal progenitor cells (MPCs) (6, 45, 52). However, studies have also indicated that overexpression of EWS/FLI1 provokes apoptosis and growth arrest in mouse normal embryonic fibroblasts and primary human fibroblasts (10, 31), hence hampering understanding of the precise role of EWS/ETS proteins in the development of EFT. The function of EWS/ETS proteins would be greatly influenced by cell type, and thus the cells that can originate EFTs might be more susceptible to the tumorigenic effects of EWS/ETS.

Although the cell origin of EFT is still unknown, the expression of neuronal markers in spite of the occurrence in bone and soft tissues has kept open the debate as to a potential mesenchymal or neuroectodermal origin. As described above, ectopic AQ: C expression of EWS/FLI1 results in dramatic changes in morphology and the formation of EFT-like tumors in murine primary bone marrow-derived MPCs but not in murine embryonic stem cells (6, 45, 52), supporting the notion that MPCs are a plausible cell origin of EFT (45). However, others argue that MPCs cannot be considered progenitors of EFT without further evidence of similarity between human EFT and MPC-EWS/FLI1-induced tumors in mice (29, 46).

The development of experimental systems using murine species is useful for elucidating the mechanisms behind the pathogenesis of EFT. However, several differences between human and murine systems cannot be ignored; these differences include the expression patterns of surface antigens in MPCs, for instance (7, 44, 51, 53). Moreover, human cells are difficult to transform in vitro, and the transformed cells of mice seem to produce a more aggressive tumor than those of hu-

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TABLE 1. Cell lines used in this study and fusion transcript types

Cell line	Diagnosis	Fusion transcript type	Reference
EES-1	EFT	EWS/FLI1 type I	20
SCCH196	EFT	EWS/FLI1 type I	21
RD-ES	EFT	EWS/FLI1 type II	5
SK-ES1	EFT	EWS/FLI1 type II	5
NCR-EW2	EFT	EWS/FLI1 type II	19
NCR-EW3	EFT	EWS/E1AF	19
W-ES	EFT	EWS/ERG	13
NB69	NB		15
NB9	NB		15
GOTO	NB		47
NRS-1	RMS	PAX3/FKHR	40

mans (1). The findings suggest the existence of undefined cell-autonomous mechanisms that render human cells resistant to malignant transformation. Therefore, the use of human cell models is ideal for clarifying how EFT develops. Models of the onset of EFT have been generated using primary fibroblasts (31) and rhabdomyosarcoma cells (23). However, these cell types are not appropriate for studying the origins of EFT, and a model that precisely recapitulates EWS/ETS-mediated EFT formation is required.

UET-13 cells are obtained by prolonging the life span of human bone marrow stromal cells by use of the retroviral transgenes hTERT and E7 (38, 50), retain the ability to differentiate into not only mesodermal derivatives but also neuronal progenitor-like cells, and are considered a good model for studying the cellular events in human MPCs. Therefore, we have examined the biological effect of EWS/ETS in human MPCs by use of UET-13 cells by exploiting tetracycline-inducible systems for expressing EWS/ETS (EWS/FLI1 and EWS/ERG). Here we report that overexpression of EWS/ETS mediates an EFT-like phenotype, including morphology, immunophenotype, and gene expression profile, with enhancement of the Matrigel invasion ability of UET-13 cells.

MATERIALS AND METHODS

Cell cultures and establishment of UET-13TR-EWS/ETS cell lines. UET-13 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% Tet system approved fetal bovine serum (T-FBS) (Takara) at 37°C under a humidified 5% CO_2 atmosphere. EFT cell lines (EES-1 [20], SCCH196 [21], RD-ES and SK-ES1 [5], NCR-EW2 and NCR-EW3 [19], and W-ES [13]) and neuroblastoma (NB) cell lines (NB69 and NB9 [15] and GOTO [47]) were cultured in RPMI 1640 with 10% FBS. A rhabdomyosarcoma cell line, NRS-1 (40), was cultured in Eagle's minimal essential medium with 10% FBS. The cell lines used in this study are listed in Table 1. T1/AQ:D

UET-13 cells were seeded at a density of 5×10^4 cells per well in 24-well tissue culture plates 1 day prior to transfection. For introducing the tetracycline-inducible system, UET-13 cells were transfected with pcDNA6-TR (Invitrogen) by use of Lipofectamine 2000 (Invitrogen). After 72 h, the medium was replaced with fresh medium containing 200 μg/ml of blasticidin S (Invitrogen). Individual resistant clones were selected for a month and designated UET-13TR cells. UET-13TR cells were further transfected with pcDNA4-EWS/ETSs constructed as described below, and individual resistant clones were selected in DMEM containing 10% T-FBS and 200 to 300 μg/ml of Zeocin (Invitrogen). The Zeocin-resistant clones were expanded and tested for the induction of EWS/ETS expression upon the addition of tetracycline by use of reverse transcription-PCR (RT-PCR) as described below.

Plasmid construction. A gateway cassette (bases 1 to 1705) was amplified from pBLOCK-iT3-DEST (Invitrogen) by PCR, and the PCR product was inserted into the EcoRV site of pcDNA4-TO (Invitrogen) (termed pcDNA4-DEST). Since the type II EWS/FLI1 is a stronger transactivator than the type I product (32), we used the type II variant in the present study. EWS/ERG was isolated from W-ES, an EFT cell line, joining EWS exon 7 and ERG exon 9. Full-length EWS/FLI1 type II and EWS/ERG cDNAs were amplified from cDNAs prepared from NCR-EW2 and W-ES cells, respectively, by PCR as described below and cloned into the XmnI-EcoRV sites of pENTR11 (Invitrogen). The resulting pENTR11-EWS/ETSs were recombined with pcDNA4-DEST by use of LR recombination reaction as instructed by the manufacturer (Invitrogen) to construct the tetracycline-inducible EWS/ETS expression vector pcDNA4-EWS/ETSs. AQ: E AQ: F

Western blot analysis. UET-13 transfectants were cultivated with or without 3 μg/ml of tetracycline for 72 h. Western blot analysis was performed as previously described (37). Briefly, the cell lysates were prepared and separated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred onto a polyvinylidene difluoride membrane. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.01% Tween 20 (Sigma) and incubated with primary antibodies. As the primary antibodies, anti-Fli-1, anti-Erg-1/2/3 (Santa Cruz Biotechnology), and anti-actin (Sigma) were used. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibodies (DakoCytomation) were used as secondary antibodies. Blots were detected by chemiluminescence using an ECL Plus Western blotting detection system (GE Healthcare Bio-Science Corp.) and exposed to X-ray film (Kodak) for 5 to 30 min.

MTT assay and detection of apoptosis. Growth curves of UET-13 transfectants were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (18). The apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Biovision) according to the manufacturer's instructions and analyzed by flow cytometry (Cytomics FC500; Beckman Coulter). AQ: G

Immunofluorescence analysis. After 1 week of culture in the absence or presence of tetracycline, UET-13 cells and the transfectants were harvested with 0.25% trypsin plus EDTA (IBL). The cells (2×10^5) were incubated with mouse monoclonal antibodies for 20 min. In the case of fluorescence-labeled antibodies, the cells were washed with PBS and then analyzed. In the case of primary unconjugated mouse antibodies, the cells were washed and then incubated with FITC-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories) for 20 min. Cell fluorescence was detected using a Cytomics FC500 instrument as described previously (27).

Antibodies against the following human antigens were used: CD10, CD13, CD14, CD29, CD34, CD40, CD44, CD45, CD49e, CD54, CD56, CD61, CD90, CD105, CD117, and CD166 from Beckman Coulter; CD73 from BD Biosciences-Pharmingen; CD55 from Abcam; CD59 from Cedarlane Laboratories; and CD133 and CD271 from Miltenyi Biotec GmbH.

Immunocytochemistry. Cells were grown on collagen type I-coated cover glasses (Iwaki). After 72 h with or without tetracycline, cells were fixed for 30 min in 4% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 (Sigma) for 30 min. Subsequently, they were washed with PBS and blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin (Sigma) for 30 min before being incubated with a monoclonal anti-CD99 antibody, i.e., 12E7 (1:100) (DakoCytomation) or O13 (1:200) (Thermo), and polyclonal anti-Fli-1 antibody (1:100) (Santa Cruz) for 1 h. Bound antibodies were visualized with appropriate secondary antibodies, i.e., Alexa Fluor 488 goat anti-mouse IgG (heavy plus light chains) highly cross-adsorbed and Alexa Fluor 546 goat anti-rabbit IgG (heavy plus light chains) highly cross-adsorbed (Invitrogen) for 1 h at 1:300. Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) (Sigma). For the visualization of whole cells, cells were treated with Celltracker Blue (Invitrogen) for 30 min and then fixed. Fluorescence was observed and analyzed using a confocal laser scanning microscope and image software (either FV500 from Olympus or LSM510 from Carl Zeiss). Precise measurements of cell size, nuclear size, and the nucleus-to-cytoplasm (N/C) ratio were performed using Image J (16).

RT-PCR analysis. Total RNA was extracted from cells by use of an RNeasy kit (Qiagen) and reverse transcribed using a first-strand cDNA synthesis kit (GE Healthcare Bio-Science Corp). RT-PCR was performed with a HotstarTaq master mix kit (Qiagen). As an internal control, human GAPDH cDNA was also amplified. The sequences of gene-specific primers for RT-PCR were as follows: for EWS/FLI1 (forward), 5′-ATGGCGTCCACGGATTACAGTACCT-3′; for EWS/FLI1 (reverse), 5′-GGGTCTTCTTTGACACTCAATCG-3′; for EWS/ERG (forward), 5′-ATGGCGTCCACGGATTACAGTACCT-3′; for EWS/ERG (reverse), 5′-TTAGTAGTAAGTGCCCAGATGAGAA-3′; for GAPDH (forward), 5′-CCACCCATGGCAAATTCCATGGCA-3′; and for GAPDH (reverse), 5′-TCTAGACGGCAGGTCAGGT/CCACC-3′. PCR products were electrophoresed with a 1% agarose gel and stained with ethidium bromide.

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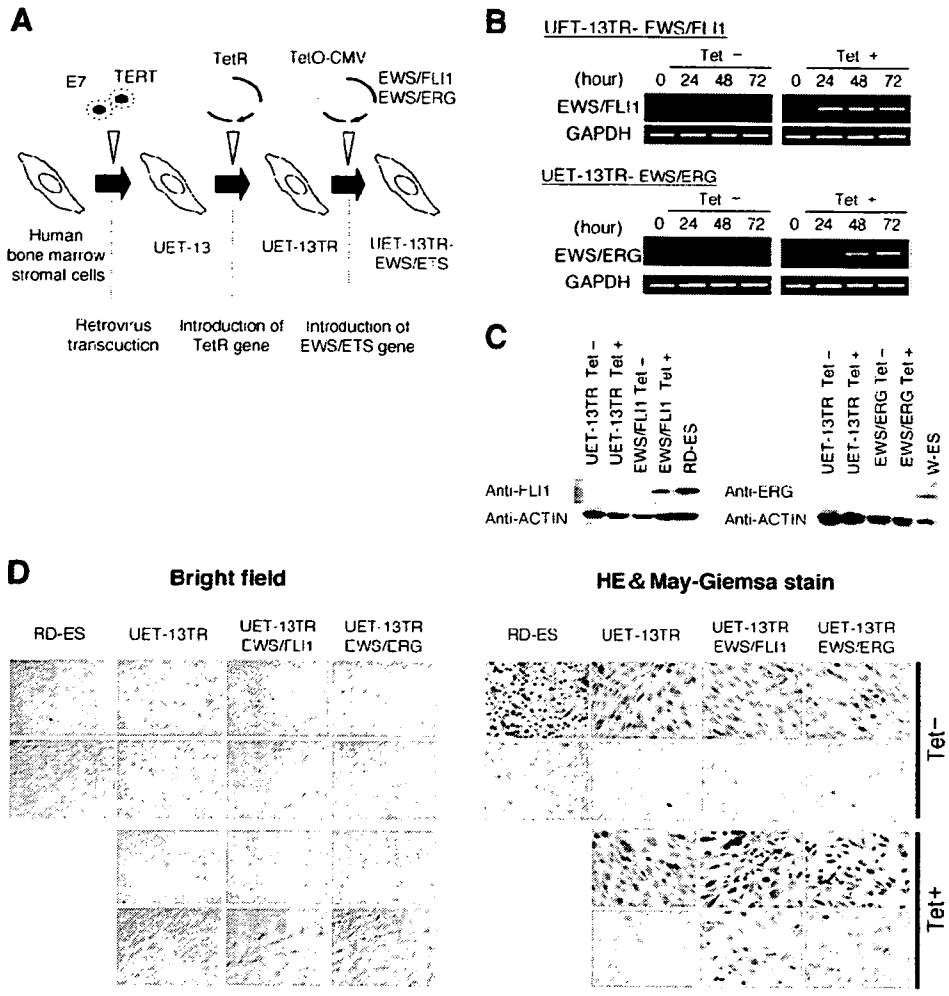

FIG. 1. The effect of EWS/ETS on the morphology of UET-13 cells. (A) The establishment of a tetracycline-inducible EWS/ETS expression system in UET-13 cells. CMV, cytomegalovirus. (B) Analyses for confirming the inducible expression of EWS/ETS genes. EWS/ETS mRNAs were detected in UET-13 transfectants UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG by RT-PCR. These cells were treated with or without 3 μg/ml of tetracycline (Tet) for the indicated periods. As an internal control, a human GAPDH gene was used. (C) Analyses for confirming the inducible expression of EWS/ETS proteins. The cells were treated as described for panel B and subjected to Western blotting for the detection of EWS/ETS proteins. The extracts of RD-ES and W-ES cells were also examined as positive controls. Membranes were reprobed with anti-actin antibody as a loading control. (D) Morphological change after tetracycline treatment of UET-13 transfectants. UET-13 cells and the transfectants were cultured in the absence or presence of tetracycline for 72 h and observed by light microscopy. Magnification, ×40 (top); ×200 (bottom). Cells were also examined using hematoxylin-eosin (HE) (top) and May-Giemsa (bottom) staining (magnification, ×200).

Real-time RT-PCR. Real-time RT-PCR was performed using TaqMan universal PCR master mix and TaqMan gene expression assays and an inventoried assay on an ABI Prism 7900HT sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The human GAPDH gene was used as an internal control for normalization.

DNA microarray analysis. Total RNA isolated from cells was reverse transcribed and labeled using one-cycle target labeling and control reagents as instructed by the manufacturer (Affymetrix). The labeled probes were hybridized to the human genome U133 Plus 2.0 array (Affymetrix). The arrays were performed in single experiments and analyzed using GeneChip operating software, version 1.2 (Affymetrix). Background subtraction, normalization, and principal component analysis (PCA) were performed by GeneSpring GX 7.3 software (Agilent Technologies). Signal intensities were prenormalized based on the median of all measurements on that chip. To account for the difference in detection efficiencies between the spots, prenormalized signal intensities on each gene were normalized to the median of prenormalized measurements for that gene. The data were filtered using the following steps. (i) Genes that were scored as absent in all samples were eliminated. (ii) Genes for which the signal intensities were lower than 100 were eliminated. (iii) Performing cluster analysis using filtering genes, we selected the genes that exhibited increased expression or decreased expression in tetracycline-treated cells. Accession numbers for the microarray data are given below.

Invasion assay. The invasion assay was performed using Matrigel (BD Bioscience) according to the previous description (34) with some modification. Polycarbonate filter inserts containing 8-μm pores (BD Falcon) were coated with 50 μl of a 6:1 mixture of culture medium and Matrigel and placed into 24-well culture plates containing DMEM supplemented with 10% T-FBS as chemoattractants. Cells (2.5×10^4) treated with or without tetracycline for 72 h were suspended in DMEM containing 0.01% T-FBS and plated on top of each filter insert. After 20 h in culture in the presence or absence of tetracycline, noninvading cells were removed from upper surface of the filter with a cotton swab. The invading cells on the lower surface of the filter were fixed with formalin, stained with hematoxylin-eosin, and counted in five fields per membrane with light microscopy. As a control, cells were also cultured on uncoated filter inserts. The invasion efficiency was presented as the ratio of the number of invading cells on Matrigel-coated inserts to that on uncoated inserts. Experiments were performed in triplicate, and the means with standard deviations of the values are shown in the graphs in the figures. AQ: H

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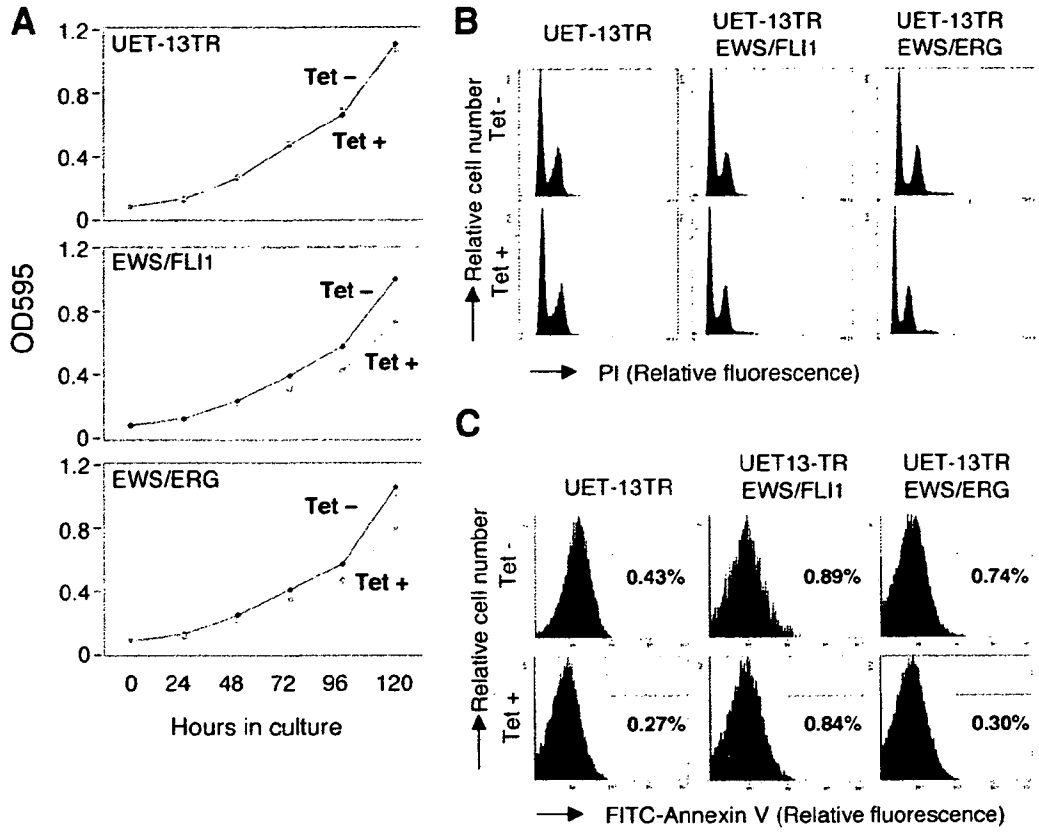

FIG. 2. Effects of EWS/ETS on cell growth in UET-13 cells. (A) Growth curve for UET-13 transfectants. Cells were seeded at 10^3/well and cultured as described for Fig. 1. The increase in cell number was analyzed by MTT assay. Values are means with the standard errors (SE) from three independent experiments. Diamond symbols indicate UET-13 transfectants in the absence of tetracycline (Tet); box symbols indicate UET-13 transfectants in the presence of tetracycline. (B) Cells were cultured as described for panel A in the absence or presence of tetracycline for 3 days and then stained with PI, and DNA contents were analyzed by flow cytometry (*x* axis, relative intensity of fluorescence; *y* axis, relative cell number). (C) Cells treated as described for panel B were stained with FITC-annexin V and analyzed.

AQ: 1 **Microarray data accession numbers.** Microarray data have been deposited in the Gene Expression Omnibus database GEO (www.ncbi.nlm.nih.gov/geo) (accession numbers GSE8665 and GSE8596).

RESULTS

EWS/ETS expression results in morphological changes in UET-13 cells. To investigate how the expression of EWS/ETS affects human MPCs, we used UET-13 cells as a model of human MPCs and expressed EWS/FLI1 (UET-13TR-EWS/FLI1) and EWS/ERG (UET-13TR-EWS/ERG) in a tetracycline-inducible manner (Fig. 1A). As shown in Fig. 1B and C, we confirmed that the tetracycline treatment could induce EWS/ETS expression by RT-PCR analysis and Western blotting. The inducibility upon the addition of doxycycline was comparable to that upon the addition of tetracycline. F1

Using these cell systems, first we examined the effect of EWS/ETS expression on morphology in UET-13 transfectants. When tetracycline was added to the culture, the morphologies of both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were dramatically changed (Fig. 1D). Tetracycline-treated UET-13TR-EWS/ETS cells consisted of a mixture of small round-to-polygonal cells and short spindle cells. The cell morphology resembled that of EFT cell lines. To assess the reproducibility of this phenotypic change, other UET-13TR-EWS/ETS clones were examined, and similar morphological changes were observed. Since tetracycline treatment did not affect the morphology of UET-13TR cells (Fig. 1D), it was suggested that the morphological alteration in UET-13 cells from a mesenchymal cell shape to small round cells, one of the characteristics of EFT, can be attributed to EWS/ETS expression.

EWS/ETS expression inhibits cell growth in UET-13 cells. Next, the effect of EWS/ETS expression on the growth of UET-13 cells was analyzed. As shown in Fig. 2A, an MTT assay revealed that the addition of tetracycline had no effect on the growth of UET-13TR cells but slightly inhibited that of UET-13TR-EWS/ETS cells. We also assessed the cell growth of UET-13 transfectants after tetracycline addition by cell counting and obtained results well in accord with those from the MTT assay (data not shown). To determine the mechanism of this inhibition, DNA content and the binding of annexin V to UET-13 transfectants were examined. No significant increase in either sub-G_1-phase cells (Fig. 2B) or annexin V binding cells (Fig. 2C) was detected, suggesting that EWS/ETS-mediated growth inhibition in UET-13 cells was not due to the activation of an apoptotic pathway. Moreover, no significant decrease in S-G_2-phase cells was observed (Fig. 2B). F2

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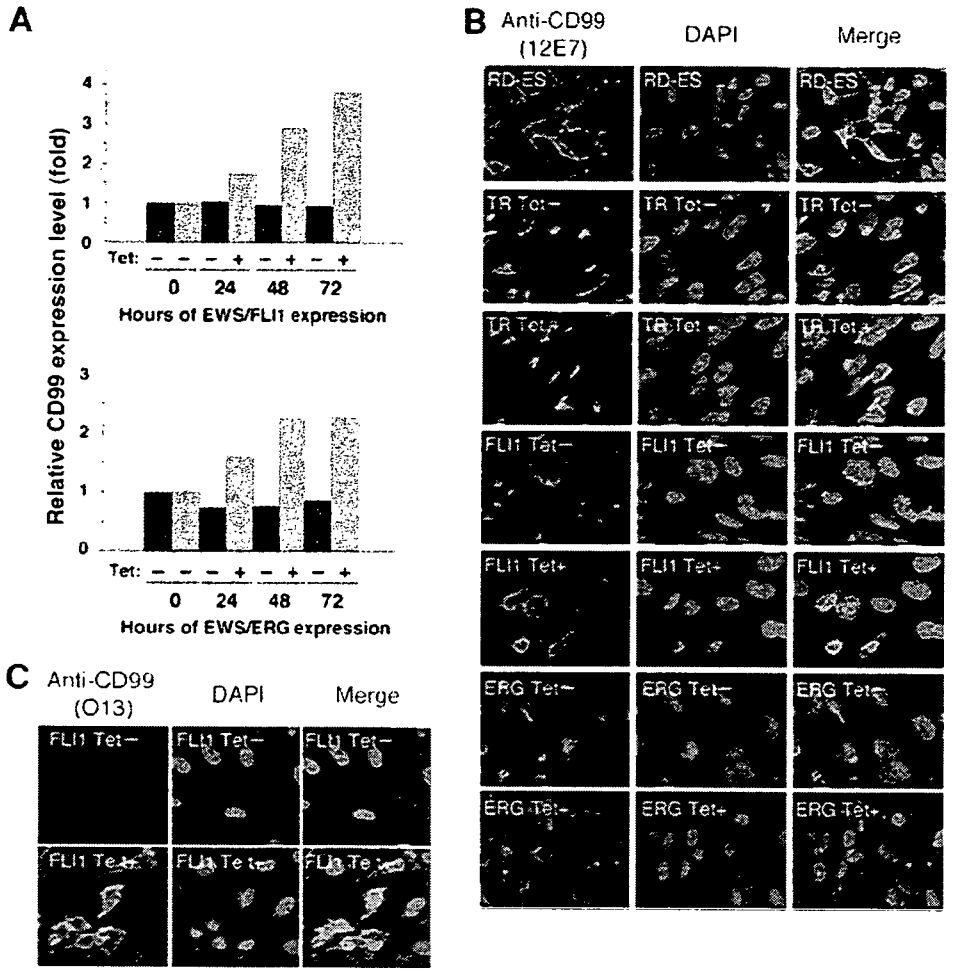

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FIG. 3. Effects of tetracycline-mediated EWS/ETS expression on the expression and distribution of CD99 in UET-13 cells. (A) Relative CD99 levels in UET-13 transfectants in the absence or presence of tetracycline (Tet). UET-13 transfectants were treated with or without 3 μg/ml of tetracycline for the indicated periods. Real-time RT-PCR was performed to investigate the expression pattern of CD99. Signal intensities of CD99 were normalized using those of a control housekeeping gene (human GAPDH gene). Data are relative values with standard deviations from triplicate wells and are normalized to the mRNA level at 0 h, which is arbitrarily set to 1 in the graphical presentation. (B and C) Immunocytostaining of CD99 in UET-13 transfectants. Cells were cultured on coverslips in the absence or presence of tetracycline for 72 h and then stained with anti-CD99 antibody 12E7 (B) or O13 (C) as described in Materials and Methods. RD-ES cells were also examined as a positive control. For the staining of nuclei, DAPI was used.

Effect of EWS/ETS on CD99 expression in UET-13 cells. The p30/32MIC-2 gene product, CD99, is a cell surface glycoprotein expressed in EFT with a strong membranous staining pattern and thus constitutes a useful marker for EFT (2, 30). Knowing the dramatic change of morphology in UET-13 cells, we next investigated the mRNA level of CD99 in tetracycline-treated and untreated UET-13 transfectants by quantitative real-time RT-PCR. CD99 levels were clearly elevated by tetracycline treatment in both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells in a time-dependent manner (Fig. 3A).

We also examined the protein expression of CD99 by immunostaining using 12E7 antibody, which is most widely used as an anti-CD99 antibody. An EFT cell line, RD-ES, showed strong membranous staining of CD99 (Fig. 3B), while neither UET-13TR cells nor UET-13 cells had such a staining. Of note is the fact that although 12E7 reactivity was observed only in the cytoplasm in perinuclear regions in both UET-13TR (Fig. 3B) and UET-13 (data not shown) cells, this antibody is well known to cross-react with a cytoplasmic protein not yet characterized. Since another anti-CD99 antibody, O13, did not react with either UET-13TR (Fig. 3C) or UET-13 (data not shown) cells, we concluded that the perinuclear staining of 12E7 mentioned above was a cross-reaction with unrelated proteins.

In the absence of tetracycline, both UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were also negative with anti-CD99 antibodies (a pattern designated $CD99^-$), similar to UET-13 cells. Surprisingly, however, tetracycline induced a membranous staining pattern (designated $CD99^+$) in UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells, and some $CD99^+$ cells had irregularly contoured nuclei (Fig. 3B). The same results were observed with another anti-CD99 antibody, O13 (Fig. 3C), indicating that the membranous staining observed for UET-13 transfectants with the anti-CD99 antibodies

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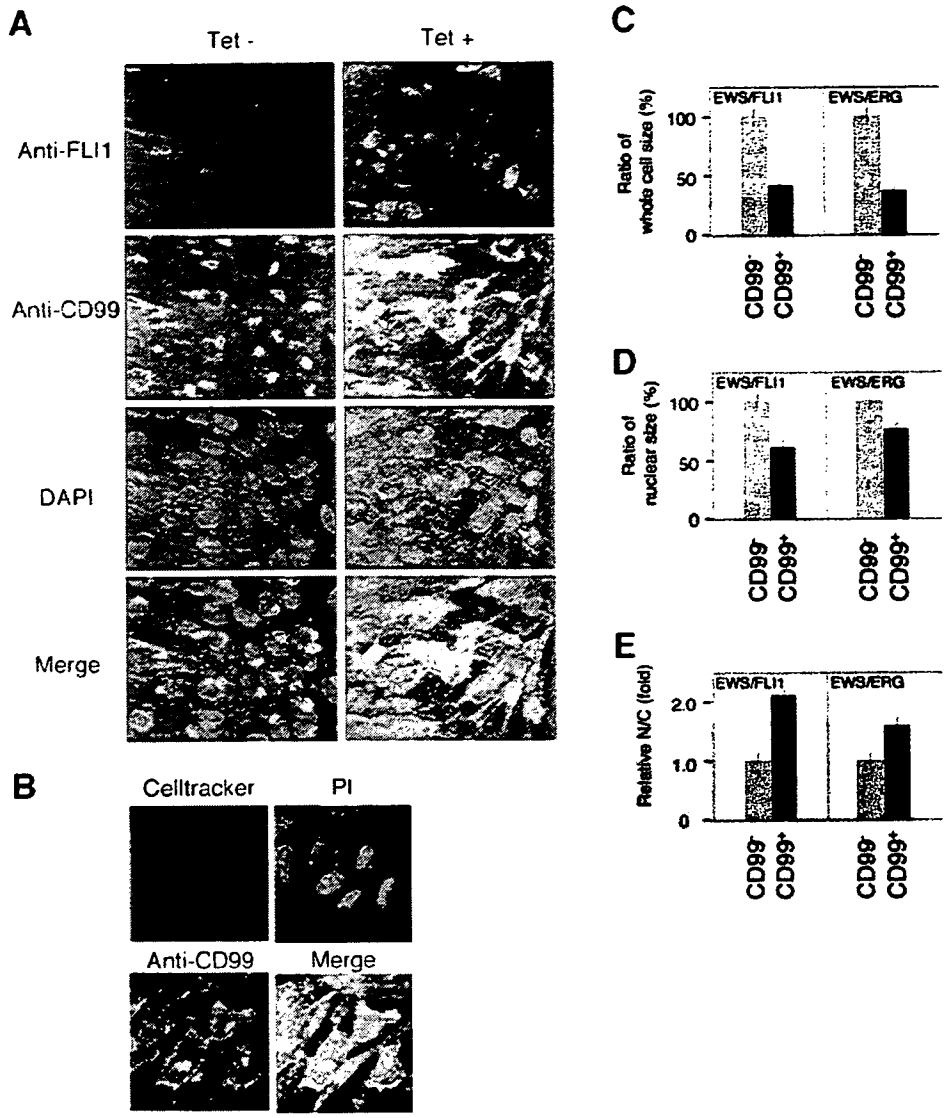

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FIG. 4. EWS/ETS expression, alteration of CD99 distribution, and cell morphological changes in UET-13 cells. (A) Immunofluorescence studies using anti-Fli1 (red), anti-CD99 (green), and DAPI (blue). UET-13TR-EWS/FLI1 cells were cultured on coverslips in the absence or presence of tetracycline (Tet) for 72 h and then stained as described in Materials and Methods. White arrowheads indicate mb-CD99 cells that have a strong staining pattern with anti-Fli1 antibodies and also have remarkable CD99 expression and morphological features. (B) Immunofluorescence analysis by triple staining with whole cells (Celltracker; blue), CD99 (anti-CD99; green), and nuclei (PI; red). UET-13TR-EWS/FLI1 cells were cultured as described for panel A and then stained as described in Materials and Methods. (C to E) Measurements of whole-cell size (C), nuclear size (D), and N/C ratio (E) in tetracycline-treated UET-13 transfectants. UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells were cultured on coverslips in the presence of tetracycline for 72 h and then stained as described in Materials and Methods. These samples were analyzed by the image analysis software Image J (n = 50). (C and D) Data are relative values with the SE and are normalized to the size of cp-CD99 cells, which is arbitrarily set to 100. (E) Data are relative values with the SE and are normalized to the size of cp-CD99 cells, which is arbitrarily set to 1.

was really CD99 derived. Despite the fact that cells were single colony derived, there was a heterogeneous response to tetracycline treatment in UET-13TR-EWS/FLI1 and UET-13TR-EWS/ERG cells, but most of the CD99$^+$ cells had a small round morphology, one of the characteristics of EFT. To assess the correlation between EWS/FLI1 expression and the change of the CD99 expression pattern, we performed immunofluorescence studies using anti-Fli1 and anti-CD99 antibodies. As F4 shown in Fig. 4A, tetracycline treatment induced a marked enhancement of nuclear staining with anti-Fli1 antibodies in a large number of UET-13TR-EWS/FLI1 cells, indicating the induction of EWS/FLI1 proteins. Furthermore, we observed that the cells with a strong signal for Fli1 tended to reveal a membranous staining pattern with anti-CD99 antibodies and a small round morphology (Fig. 4A). To further verify the correlation between CD99 expression pattern and cell morphology, we estimated the size of cells by triple staining using Celltracker Blue, PI, and anti-CD99 antibody (Fig. 4B). As