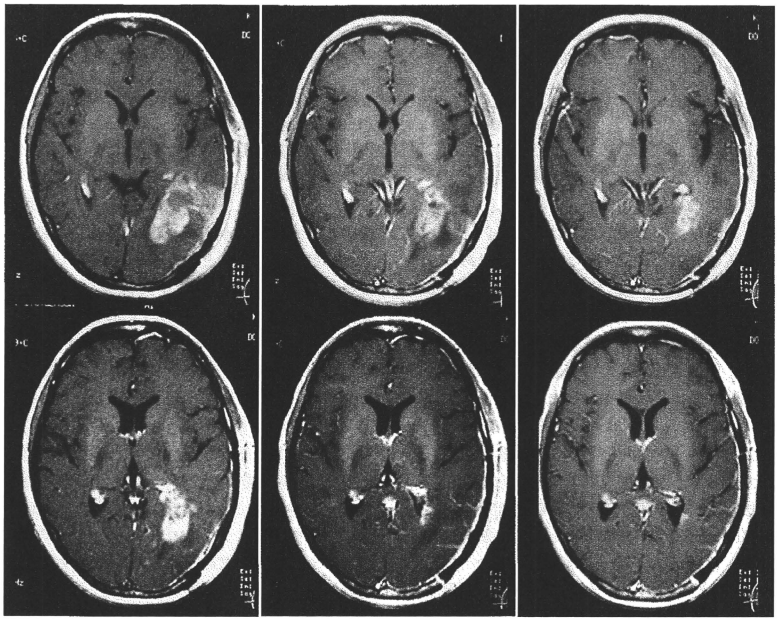

図2 ホウ素中性子捕捉療法(BNCT)による治療例
(新規診断膠芽腫, Gd造影T1強調MRI)
熱外中性子を用いた非開頭BNCTに, 集積機序の異なる2種類のホウ素化合物(BPA, BSH)を併用したプロトコールでの治療例. 治療後早期から画像上増強効果を示す腫瘍の縮小効果を認め, 非常に良好な局所制御が得られた.

23.5カ月(n=11)と延長することを示した[10].

また最近では, YamamotoらがBNCTの自験例から, 新規診断膠芽腫における開頭・術中照射群と非開頭・外照射群の治療成績を比較し報告している. これによれば, 開頭・術中照射群ではBSH単剤を用い, 非開頭・外照射群では著者らと同様, BSH, BPA(250 mg/kg)の併用にX線分割外照射を組み合わせ, MSTがそれぞれ23.3(n=7), 27.1(n=8)カ月と非常に良好である[11].

BNCTの治療成績は, 膠芽腫という比較的均質な対象患者においても, 治療グループによりばらつきがある. これは, BNCTが単一の治療法ではなく, '放射線治療'や'化学療法'と並ぶ'BNCT'という治療法の中に無数のプロトコールがあり, いまだ無限の可能性を秘めていることで説明がつく. つまりBNCTは, ホウ素化合物の組み合わせや投薬量・投与法によっても異なった治療法・成績になりうるのである.

b. 既放射線治療例に対する有効性

再発悪性神経膠腫の予後は極めて不良であり, 特に既放射線治療例では治療に難渋する. 手術や放射線追加照射も行われてきたが, 生存期間は約6カ月である. 再発例においても, BNCTの治療効果は良好で, 他の治療法では得られがたい画像上の縮小効果も多くの例で経験する[12].

最近の再発膠芽腫を対象とした報告によると，BNCT後の生存期間は8.7カ月(n=12)であり[13]，著者らの9.6カ月(n=19)と同様に良好である[14]．

3. BNCTとpositron emission tomography(PET)検査

非開頭BNCTでは，従来の開頭照射のようにホウ素化合物投与後に組織を採取し，腫瘍内のホウ素濃度を測定することはできない．そこで，著者らのBNCTでは，ホウ素化合物であるBPAをトレーサーとした^{18}F-BPA-PETを導入し，個々の患者ごとに腫瘍への薬剤集積を計算し，治療に反映している[9]．本検査は非開頭BNCTを大きく前進させ，また再発悪性神経膠腫や悪性髄膜腫，頭頸部腫瘍といった新規適応拡大の根拠にもなっている．また，BNCTでは^{18}F-BPA-PETで高集積を示す部分に高線量が'当たる'のであって，空間的な照射計画から'当てる'照射法とは一線を画する．著者らのBNCTにおけるBPA-PETは，診断薬としての役割をもちながら，治療薬の挙動をも評価可能で，'見える薬'と考えられ，この治療での効果を担保するものでもある．

おわりに

BNCTが医療として認知されるには，原子炉から脱却しなければならない．最近，脳腫瘍での成績の向上や他臓器への応用など多方面からの注目もあり，加速器中性子源の開発研究に拍車がかかっている．加速器が実現すれば，医療機器としての申請が可能となり，BNCTが医療承認を目指す'治験'という枠組みに参入できるようになる．現在，世界中で医療用加速器が開発研究されているが，国内ではKURRIに設置されており，2010年秋の治験開始を目指し準備が進められている．著者らはその準備段階として，これまでの臨床経験をふまえた新規治療プロトコールを立案し，原子炉BNCTによる多施設共同第2相臨床試験(UMIN000002385, NCT00974987)を立ち上げ，症例登録を開始した．また将来的には，創薬シーズが刺激され，分子標的薬やナノテクノロジー，ドラッグデリバリーシステムなどの手法を用いたホウ素化合物の投入も期待される[15]．

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先端医療シリーズ41

臨床医とコメディカルのための
最新クリニカルPET

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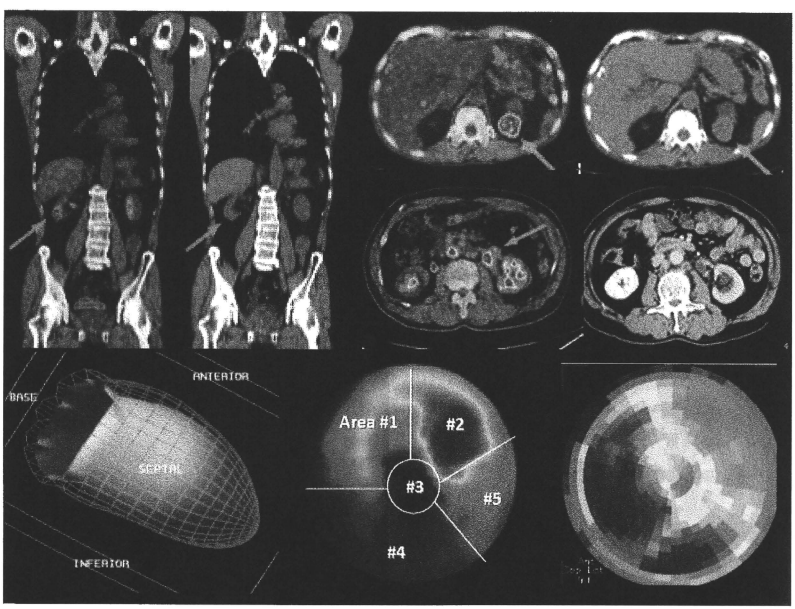

先端医療技術研究所

3. 硼素中性子捕捉療法による悪性脳腫瘍、頭頚部腫瘍の治療

要約

硼素中性子捕捉療法（Boron Neutron Capture Therapy; BNCT）は、局所高線量による放射線治療という性格を有しながら、腫瘍を細胞レベルで標的とし、正常脳に浸潤した腫瘍細胞をも選択的に治療できるという“細胞選択的粒子線治療”である。我々は2002年以降、熱外中性子および集積機序の異なる2種類の硼素化合物（BSH, BPA）を併用した改良型BNCTを用い、再発例を含む50例以上の悪性神経膠腫を治療してきた。我々の改良型BNCTは非開頭で行い、照射前には治療薬であるBPAをトレーサーとしたPET検査を行い、個々の患者で硼素化合物の腫瘍内集積を考慮し線量計画を行っている。新規診断神経膠芽腫全体での治療成績では従来治療に比較し、生存期間中央値には有意な延長がみられた。2004年以降、新規診断例では、選択的照射であるBNCTにX線外照射を加えることで、治療成績はさらに向上した。またBNCTは、既放射線治療再発例に対しても生命予後の改善が得られ、一般に予後不良とされる治療困難例においても、BNCTは有効性が高いと考える。また最近では、BPA-PETの導入による治療適応拡大も積極的に行われ、難治性頭頚部腫瘍に対しても良好な成績が報告されている。現在は医療用原子炉でしか行えないBNCTであるが、加速器中性子源による病院内BNCTの開発研究がすすめられており、今後飛躍的な進歩が期待される。

3.1　はじめに

硼素中性子捕捉療法（boron neutron capture therapy; BNCT）は、画像誘導下に線量計画を行う局所高線量放射線療法とは異なり、生物学的に腫瘍を細胞レベルで標的とする粒子線治療である。硼素の同位体である^{10}B（硼素-10、boron-10）が中性子と核反応を生じ、そこから生じたヘリウム原子核（アルファ粒子）とリチウム反跳核により腫瘍細胞を破壊するBNCTの理論は、1932年に中性子が発見されたわずか4年後（1936年）に、すでにアメリカのLocherにより提唱されていた。この反応は非常に弱いエネルギーの中性子で得られ、しかも生じるこれらの粒子の飛程がほぼ腫瘍細胞一つ分に相当するため、腫瘍選択性のある^{10}B化合物をあらかじめ患者に投与しておき、化合物が十分に腫瘍に集積した時点で患部に中性子を照射すれば、腫瘍細胞のみが選択的に破壊されるわけである（図1）。従って、本治療法が最も威力を期待できるのが、浸潤性の悪性神経膠腫である。

悪性神経膠腫、特に膠芽腫は治療抵抗性を示すきわめて予後不良の原発性脳腫瘍であり、その平均生存期間は約1年とされる。予後不良の原因として、血液脳関門や薬剤耐性機構の存在などが指摘されているが、最も大きな原因は腫瘍の浸潤性性格にある。悪性神経膠腫は明瞭な辺縁を持たず、細胞レベルでは画像上の造影域を越え、最低でも周囲脳2cmまでは腫瘍細胞が存在するとされる。BNCTはこのような浸潤部腫瘍細胞をも標的とする細胞選択的治療法であり、理論上は正常脳組織を温存しつつ腫瘍細胞を破壊することが可能である。

3.2　BNCTによる脳腫瘍の治療

BNCTは広く普及するには至っていないが、米国では1951年に臨床応用が開始された。本邦でも1960年代から日本原子力研究開発機構（Japan Atomic Energy Agency; JAEA）、京都大学原子炉実験所（Kyoto University Research Reactor Institute; KURRI）等において医療照射が開始された[1]。これらの初期の臨床研究では、治療効果がみられたものの、従来の治療法を格段に上回る成績を得ることは出来ず、治療が原子炉に限られる点、硼素化合物が高価かつ入手が困難である点や原子炉内での開頭手術を要したことなどから広く普及しなかった。我々はこれらの問題点を克服するため、従来のBNCTを改良した新しい治療

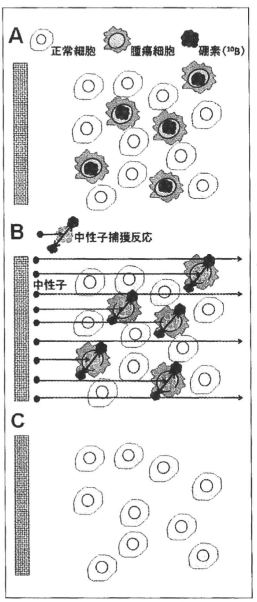

図1　BNCTの理論図

悪性グリオーマは非常に強い浸潤性性格を有し、腫瘍細胞は腫瘍塊（MRI上の造影域）を超えて周囲正常脳へ浸潤し、外科的切除によっても浸潤部細胞の治療は行い得ず、この部からの再発が必至である。

硼素中性子捕捉療法（BNCT）では、あらかじめ腫瘍選択性を有するホウ素（^{10}B）化合物の投与を行う。(A)

^{10}B化合物投与後に、低エネルギーの中性子を照射することで、^{10}Bが中性子と核反応を生じ、そこから生じたヘリウム原子核（アルファ粒子）とリチウム反跳核で、腫瘍細胞を選択的に破壊する。(B)

C：選択的に腫瘍に集積し、かつ正常脳・血中の濃度が低下した時点で患部に中性子を照射すれば、^{10}Bが集積した腫瘍細胞のみが浸潤部においても選択的に破壊される。(C)

プロトコールを立案し、臨床応用してきた[2,3]。BNCTの大きな変遷となったのは、組織深達性に優れた熱外中性子の利用であり、これによって非開頭BNCTが実現可能となった。従来の熱中性子の線量ピークは皮膚表面であり、腫瘍線量の不足と同時に皮膚障害も問題であったが、熱外中性子はピークが皮膚から2cm深部であり、皮膚線量を軽減したまま、深部腫瘍線量を増加させることが可能となった。また近年、臨床で用いられてきた硼素化合物は、BSH（sodium borocaptate）とBPA（boronophenylalanine）のみであり、従来はどちらか一方を単剤で使用してきた。どちらの化合物にも一長一短があり、我々のBNCTでは双方の欠点を補うため、これら2種類の化合物を併用する新たな試みで治療成績の向上を目指した[4]。現在我々は、前述の如く従来行われてきた全身麻酔下・開頭術中BNCTに代わり、非開頭により外照射でBNCTを施行している[4]。非開頭BNCTが行えるようになった背景として、熱外中性子の導入のみならず硼素化合物であるBPAを^{18}Fでラベルしトレーサーとしたアミノ酸（フェニルアラニン）PET（^{18}F-BPA-PET）の果たす役割は大きい[5]。

2004年以後は2種類の化合物の併用プロトコールに改良を加え、BPAを増量し持続的に点滴静注することで腫瘍内の硼素濃度をより均一化し、BNCTの照射線量を高めるとともに、新規診断例に対しては、X線分割外照射を併用している。新規診断の膠芽腫全体では、平均生存期間（MST）は診断後15.6ヵ月（n=21）で、X線分割外照射による標準治療と比べ、有意な延長がみられ、ハザード比は0.40であった。さらにBNCTにX線分割外照射を併用した群（n=11）でのMSTは、23.5ヵ月（ハザード比0.32）となった。選択的照射であるBNCTに、X線外照射を加えることで、新規診断例の治療成績は向上し、局所制御は良好であったが、随腔内播種や遠隔部での再発が問題となった[6]。

BNCTは細胞選択性を有するため、高線量照射が躊躇される再発性の悪性神経膠腫（既放射線治療例）に対しても極めて有効な手段である。再発例においても、BNCTの治療効果は強力で、他の治療法では得られ難い、画像上の縮小効果も多くの例で経験する。22例の悪性神経膠腫（膠芽腫19例）に対しBNCTを用いた治療を行い、生存期間中央値は10.8ヵ月であり、膠芽腫で9.6ヵ月と良好であった[7]。

このようにBNCTは、X線分割外照射との併用や既放射線治療例に対する照射が可能であることも、他の局所高線量治療とは異なる有利な点である（図2）。

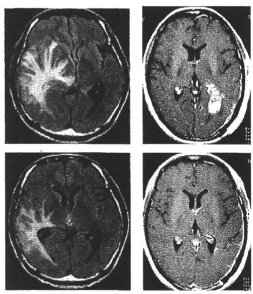

図 2　BNCT による治療経過
(左) MRI (FLAIR 像)、上) 治療前・下) BNCT 5 週後、神経膠芽腫、再発 (既放射線治療) 例。造影域縮小効果 (40.5%) に加え、周囲の高信号域の軽減および正中構造変位の改善が見られる。
(右) MRI (造影 T1 像)、上) 治療前・下) BNCT 5 週後、神経膠芽腫、新規治療例。照射 48 時間後から縮小効果が観察され、5 週間後には 80.3%の縮小効果を示した。

3.3　BNCT と PET のかかわり (頭頸部腫瘍への応用)

非開頭 BNCT では、従来の開頭術中照射のように硼素化合物投与後に組織を採取し、腫瘍内の硼素濃度を測定することはできない。そこで、硼素化合物である BPA をトレーサーとした BPA-PET から、個々の患者毎に腫瘍への薬剤集積を計算し治療に反映するテーラーメード型の照射計画を実現している[6]。BPA-PET が施行可能となり、BNCT 用治療薬であった BPA は、"見える薬" として個々の患者の治療に応用可能となった[8]。他の照射法においては、メチオニン PET をもとに照射計画を立て、成績の向上を目指す報告が散見されるが、BNCT では治療薬である BPA の PET で、高集積を示す部分に高線量が "当たる" のであって、空間的な照射計画をもって "当てる" 照射法とは一線を画する。我々の BNCT における BPA-PET は、診断薬としての役割を持ちながら、治療薬の挙動をも評価可能で、"見える薬" と考えられ、この治療での効果を担保するものでもある (図 3)。

我々は、非開頭 BNCT の線量計算に反映させるべく BPA-PET を行っているが、PET を用いることで BNCT の適応が判断可能になるともいえる。我々は近年、この手法を用いて、有効な治療法がない浸潤性悪性腫瘍である悪性髄膜腫にも適応を拡大し、BNCT の有効性を報告してきた[8]。また BPA-PET を行うことで、頭頸部や肺・肝臓など他の腫瘍への適応拡大も積極的に取り組まれており、まとまった報告ではないが、いずれも治療成績は良好である[9,10]。本手法による頭頸部腫瘍への適応拡大は、世界の BNCT 治療をリードしており、最近欧米では、本邦に追随する形で頭頸部腫瘍に対する臨床試験の準備が開始されている。

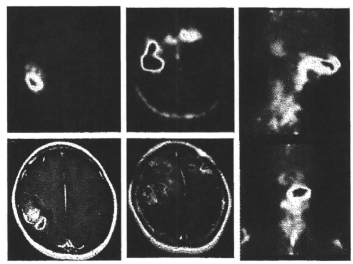

図 3　膠芽腫 (左) と悪性髄膜腫 (中)、頭頸部腫瘍 (右) の BPA-PET
BNCT では硼素化合物の取り込みがない部分には治療効果が及ばず、安全に治療が可能である。治療薬である BPA の PET で、高集積を示す部分に高線量が照射されるため、本画像から BNCT での治療効果を担保でき、適応拡大の可能性も探索できる。頭頸部腫瘍 (右) は硼素化合物 (BPA) の高集積から実際に BNCT により治療がなされた例である。

3.4　BNCT の今後の展望について

有効な治療手段の確立していない難治性腫瘍に対して、BNCT の積極的な適応拡大が望まれるが、原子炉の有するマシンタイムの問題や、化合物の供給・費用などの面から、今後は加速器中性子源を中心に、医療機器・治療薬として認可を目指す試みがある。国内では京都大学原子炉実験所内での設置準備が進められており、2010 年末から 2011 年初めを目指し、再発悪性神経膠腫、再発頭頸部癌および胸膜中皮腫を対象とした臨床治験が計画されている。

加速器 BNCT が実現すれば、医療機器としての申請が可能となり、いよいよ BNCT は医療承

認を目指すことになる。我々はその準備段階として、これまでの臨床経験をふまえた新規治療プロトコールを立案し、原子炉BNCTによる多施設共同第2相臨床試験（UMIN000002385、NCT00974987）を立ち上げ、症例登録を開始した。加速器BNCTが臨床応用されれば、照射の自由度は増し、現時点では困難な、分割照射や複数回照射、多門照射など様々な応用も可能となる。

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（川端信司、宮武伸一）

先端医療シリーズ 41
臨床医とコメディカルのための最新クリニカルPET

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Phase II study of ifosfamide, carboplatin, and etoposide in patients with a first recurrence of glioblastoma multiforme

Clinical article

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Object. The prognosis of recurrent glioblastoma multiforme (GBM) remains unsatisfactory. The authors conducted a Phase II study of ifosfamide, carboplatin, and etoposide (ICE) for a first recurrence of GBM to determine whether it prolonged a patient's good-quality life.

Methods. This trial was an open-label, single-center Phase II study. Forty-two patients with a first GBM relapse after surgery followed by standard radiotherapy (60 Gy) and first-line temozolomide- or nimustine-based chemotherapy were eligible to participate. The primary end point was progression-free survival at 6 months after the ICE treatment (PFS-6), and secondary end points were response rate, toxicity, and overall survival. Chemotherapy consisted of ifosfamide (1000 mg/m^2 on Days 1, 2, and 3), carboplatin (110 mg/m^2 on Day 1), etoposide (100 mg/m^2 on Days 1, 2, and 3), every 6 weeks.

Results. Progression-free survival at 6 months after ICE treatment was 35% (95% CI 22–50%). The median duration of PFS was 17 weeks (95% CI 10–24 weeks). The response rate was 25% (95% CI 9–34%). Adverse events were generally mild and consisted mainly of alopecia.

Conclusions. This regimen was well tolerated and has some activity and could be one of the options for patients with recurrent GBM. *(DOI: 10.3171/2009.5.JNS081738)*

Key Words • **glioblastoma multiforme** • **chemotherapy** • **ifosfamide** • **carboplatin** • **etoposide**

Glioblastoma multiforme is one of the most devastating malignancies, destroying important cognitive functions in the brain, altering the personalities of humans, and leading to death. Standard treatment of a newly diagnosed GBM usually consists of cytoreductive surgery followed by conventional radiotherapy concomitant with temozolomide chemotherapy.[1,22] Gliadel wafers also have been used in patients with GBM.[29] Despite the administration of multimodal treatments, this tumor always recurs no more than 1 or 2 years later. Unfortunately, we have no established second-line treatments once the tumor progresses.[9]

As shown in a published database of Phase II trials that included 225 patients with recurrent GBM, the benefit from chemotherapy in such patients is very limited.[31] The median duration of PFS is 9 weeks, and PFS at 6 months (PFS-6) is 15%, meaning that 15% of patients survive without tumor progression by 6 months after treatment. Several chemotherapeutic or biological agents may palliate patients but produce only a minimal increase in survival, although dose-intensified temozolomide or bevacizumab has shown promising effects.[24,26]

Carboplatin has demonstrated activity against malignant brain tumors.[33] Etoposide, a semisynthetic derivative of podophyllotoxin, has shown synergistic activity with cisplatin.[16] Ifosfamide is an alkylating agent that has

Abbreviations used in this paper: CR = complete response; GBM = glioblastoma multiforme; ICE = ifosfamide-carboplatin-etoposide; KPS = Karnofsky Performance Scale; MGMT = O^6-methylguanine-DNA methyltransferase; NCI = National Cancer Institute; NR = no response; PFS = progression-free survival; PR = partial response.

demonstrated an increased therapeutic effect in a variety of refractory solid tumors.[34] The ifosfamide-carboplatin-etoposide (ICE) combination has demonstrated a therapeutic effect in a broad range of malignancies, even after the failure of previous chemotherapy.[19]

A Phase II study of the ICE regimen in patients with recurrent malignant GBMs has been conducted with interesting results.[20] Carboplatin and etoposide were both given at a dose of 75–100 mg/m^2/day for 3 days, whereas a dose of ifosfamide ranging from 750 to 1500 mg/m^2/day was administered for 3 days. This regimen was repeated every 4 weeks. The PFS-6 was ~ 30% and the response rate was 28%, but the hematological toxicity was not acceptable. Recurrent malignant GBMs are refractory and ultimately incurable. Long-term tumor stabilization with low toxicity is important in the management of malignant lesions.[23] We performed a feasibility study of a lower-dose ICE regimen to confirm whether its safety and efficacy were acceptable. A feasibility study in 15 patients with malignant GBMs showed a response rate of 45% and Grade 3 or 4 toxicities of 8% (unpublished data); therefore, we initiated a Phase II study of a lower-dose regimen for further evaluation.

Methods

Eligibility for Study Participation

Patients enrolled in the study had a first relapse of supratentorial GBM previously treated with surgery, conventional radiotherapy (60 Gy), and a first-line nitrosourea-based[2] or temozolomide-based chemotherapy. Tumor progression was confirmed on Gd MR imaging. If radiation necrosis or pseudoprogression was suspected, spectroscopic MR imaging was performed to confirm the presence of viable cells. If mainly necrosis was noted in those who underwent repeat surgery for a recurrence, adjuvant chemotherapy was continued. Patients were required to be at least 18 years old, have a KPS score ≥ 60, and have a life expectancy > 12 weeks. An interval > 12 weeks from the completion of radiotherapy, > 4 weeks from prior chemotherapy (6 weeks from a nitrosourea-based regimen), and > 2 weeks from surgery had to have elapsed for the patient to be eligible for study enrollment. Adequate laboratory values were also required, including a neutrophil count ≥ 1500/ml^3, platelet count ≥ 100,000/mm^3, transaminase and alkaline phosphatase levels < 3 times the upper limit of laboratory normal, and serum creatinine and bilirubin levels < 1.5 times the upper limit of laboratory normal. In addition, patients could not be pregnant, have an uncontrolled infection, or have had any prior malignancy. All patients signed informed consent according to the principles of the Declaration of Helsinki and the rules of good clinical practice. The institutional review board of Kitano Hospital, Japan, approved the protocol.

Patient Characteristics

Between March 2002 and February 2005, 42 patients were prospectively enrolled in the study. Their median age was 55 years (range 21–70 years), and 57% (24 of 42 patients) were men (Table 1). Fifty-five percent (23 of 42) of the patients had a KPS score ≥ 80. All patients had undergone surgical interventions at the time of the initial diagnosis of GBM. Furthermore, all patients had undergone conventional radiotherapy (2.0 Gy per fraction 5 times a week, total dose 60 Gy), followed by a first-line chemotherapy regimen consisting of a nimustine (ACNU)-based regimen[2] in 24 patients and temozolomide in 18 patients. The median time from the initial diagnosis to first relapse was 39 weeks (range 12–98 weeks). Between the patients who had been on a temozolomide regimen and those on a nimustine regimen, there was no significant difference in the prognostic factors (age, sex, and KPS score) and the time from first diagnosis to study enrollment. Salvage surgery at the first recurrence before ICE treatment was performed in 14 patients (33%); on the basis of MR imaging results, the extent of resection was considered gross total in 10 patients and partial in 4. At enrollment 35 of 42 patients were taking antiepileptic prophylaxis without enzyme-inducing properties (valproic acid); 7 patients were on antiepileptic prophylactic enzyme-inducing drugs (phenytoin in 4 cases, phenobarbital in 2, and carbamazepine in 2).

Treatment Regimen

Chemotherapy consisted of ifosfamide (1000 mg/m^2 on treatment Days 1, 2, and 3), carboplatin (110 mg/m^2 on treatment Day 1), and etoposide (100 mg/m^2 on treatment Days 1, 2, and 3). This combination was intravenously administered and repeated every 6 weeks until tumor progression, provided that all hematological toxicities from the previous course had resolved to a Grade 2 or lower (NCI common toxicity criteria, version 3.0) and all nonhematological toxicities had recovered to either Grade 0 or 1. If enough recovery had not occurred, the subsequent course was delayed until these criteria were met; a delay of up to 2 weeks was allowed. No dose escalation was allowed. Dose reduction for toxicity was allowed—a 30% reduction for all drugs—but only 2 dose reductions were permitted. Patients having Grade 3 toxicity of any type after 2 dose reductions were excluded from the study. Ondansetron was given to all patients before administration of the chemotherapy regimen to prevent nausea and vomiting.

Schering-Plough Pharmaceuticals kindly supplied the temozolomide through a compassionate use program, as the Japanese administrative agency only approved temozolomide in September 2006.

Response Evaluation

Response was assessed using a modification of the Macdonald criteria.[18] We compared baseline contrast-enhanced MR images obtained in a week before every course of chemotherapy, while also considering any changes on the neurological examination and the dose of steroids. In brief, CR was defined as the disappearance of all enhanced tumors at least 1 month after they had appeared on the last MR image obtained, with no corticosteroids and no neurological deterioration. Partial response was defined as a > 50% reduction in lesion size

TABLE 1: Summary of characteristics in 42 patients with recurrent GBM

Parameter	No. (%)
age in yrs	
median	55
range	21–70
<50	14 (33)
≥50	28 (67)
sex	
M	24 (57)
F	18 (43)
KPS score	
100	4 (12)
90	9 (26)
80	10 (33)
70	6 (19)
60	3 (10)
time from 1st diagnosis to study enrollment (wks)	
median	39
range	12–98
antiepileptic prophylaxis w/ enzyme-inducing drug	7 (17)
prior treatment at 1st relapse	
salvage surgery	
gross-total resection	10 (24)
partial resection	4 (10)
no surgery	28 (66)
chemotherapy regimens	
temozolomide	18 (43)
nimustine-based regimen	24 (57)

(the product of the largest perpendicular diameters). This response had to be maintained for at least 1 month without either neurological deterioration or an increased dose of corticosteroids. No response was defined as no change in tumor size for a minimum interval of 4 weeks or a change in tumor size after 1 month that did not qualify as a CR, PR, or progressive disease. Progressive disease was defined by the following: any new tumor or a > 25% increase in lesion size, a deterioration in the patient's neurological status, or a stable neurological status on an increased dose of steroids. Unequivocal evidence of recurrence or progression of the disease on Gd MR imaging was also required; acceptable evidence was disease progression on 2 subsequent MR images separated by at least 1 month. A multidisciplinary team consisting of a neurosurgeon, a neuroradiologist, a neurooncologist, and a radiotherapist evaluated the images.

Treatment Toxicity

Toxicity monitoring was performed in patients on all treatment cycles, according to the NCI common toxicity criteria (version 3.0) (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf). A physical examination, complete blood count, urinalysis, and biochemistry profile were performed every cycle. Weekly hematological tests and serum chemistries were also required.

Statistical Considerations

The primary end point of this study was the percentage of patients alive and progression free at 6 months (PFS-6). Secondary end points included the percentage of patients who were progression free at 3, 12, and 18 months; tumor response to treatment; overall survival; and time to disease progression. The regimen would be considered a success if at least 30% of enrolled patients responded to it, whereas the regimen would be considered ineffective if a success rate ≤ 10% was observed. In the North Central Cancer Treatment Group (NCCTG) database of patients with recurrent GBMs, the PFS-6 was 10%.[31] Our design used 33 patients and had an alpha level of 0.04 and a power of 0.89 for detecting a true success probability of 30%. The original sample size of the trial was 37 (33 patients with 4 additional patients in case of drop-outs), and 42 patients were accrued initially.

The time to progression was defined as the time from study entry to disease progression. Patients who died were considered to have disease progression at the time of death until there was documented evidence that no progression had occurred before death. Overall survival was defined as the time from study entry to death from any cause. Patients who did not die or whose tumor did not progress were censored at the last known follow-up.

Results

The median follow-up period was 13.2 months (range 1.5–22.4 months).

Treatment Responses

Only 36 patients could be assessed for a treatment response because 5 patients underwent almost total resection at a salvage surgery and 1 patient died of a pulmonary embolism during the 1st month of treatment before any response could be evaluated. There was 1 CR (3%) and 8 PRs (22%) to the ICE treatment (Table 2). The overall response rate (CR + PR) was 25% (9 of 36 patients, 95% CI 12–37%). The median duration of disease stabilization in 18 patients was 21 weeks (range 6–69 weeks). The median duration of the overall response rate (CR + PR) was 25 weeks (range 8–71 weeks). All responding patients were taking either a stable dose of or no corticosteroids at the time of the best response. The rate of stable disease was 50% (95% CI 39–62%).

Disease Progression

Considering all 42 enrolled patients (Fig. 1), the median time to tumor progression was 16.2 weeks (95% CI 12.4–27.3 weeks). Progression-free survival at 6 and 12 months after ICE treatment were 35% (95% CI 22–43%) and 7% (95% CI 3–15%), respectively. On univariate analysis, there was no difference in the possibility of progression based on surgical treatment at relapse ($p = 0.55$), the extent of surgery ($p = 0.31$), prior nitrosourea-based chemotherapy ($p = 0.46$), patient age ($p = 0.33$), or KPS

TABLE 2: Response rate following ICE treatment*

Level of Response	No. (%)
complete	1 (3)
partial	8 (22)
stable	18 (50)
progression	9 (25)
overall (CR + PR)	9 (25)

* Measurable enhanced masses on MRI (36 cases).

score (p = 0.44). On multivariate analysis, the factors predictive of disease progression were treatment response or disease stabilization attained with the ICE therapy (p = 0.003) and the number of treatment cycles (1 or 2; p = 0.005). At the second relapse—that is, the relapse after ICE treatment—nobody received bevacizumab, 4 patients received interferon-β, 5 patients underwent radiosurgery, and 3 patients underwent salvage surgery. None of these treatments showed a significant effect on overall survival.

Overall Survival

The median survival time in the population studied, calculating from the start of the ICE chemotherapy, was 10.7 months (95% CI 9.7–15.2 months), and 81% (95% CI 67–96%) and 37% (95% CI 27–63%) of the patients were alive at 6 and 12 months after ICE treatment, respectively (Fig. 1). On univariate analysis, there was no difference in the possibility of overall survival based on surgical treatment at relapse (p = 0.55), the extent of surgery (p = 0.28), prior nitrosourea-based chemotherapy (p = 0.46), patient age (p = 0.33), or KPS score (p = 0.44). On multivariate analysis, factors predictive of progression were treatment response or disease stabilization attained with the ICE therapy (p = 0.003) and the number of treatment cycles (1 or 2; p = 0.006).

Safety and Tolerability of the ICE Regimen

Toxicities, which were graded based on the NCI common toxicity criteria (version 3.0), were recorded for all enrolled patients. The ICE regimen was generally well tolerated (Table 3).

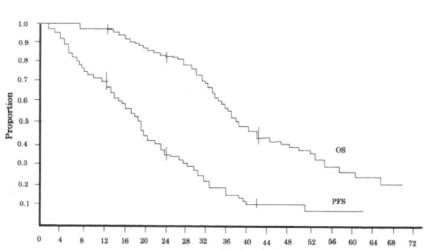

FIG. 1. Kaplan-Meier estimates of PFS and overall survival (OS) in patients with GBMs at first relapse. *Tick marks* represent censored patients.

TABLE 3: Toxicities per patient following an ICE regimen

Parameter	No. of Patients (%)	
	Grade 1/2	Grade 3/4
patients w/ toxicities	42 (100)	9 (21)
anemia	25 (59)	2 (5)
thrombocytopenia	30 (71)	3 (7)
neutropenia	24 (57)	4 (10)
lymphocytopenia	9 (2)	1 (2)
nausea/vomiting	12 (29)	1 (2)
transaminase	13 (31)	2 (5)
creatinine	9 (22)	1 (2)
alopecia	42 (100)	0 (0)
constipation	11 (26)	2 (4)
thrombosis	1 (2)	1 (2)
fatigue	15 (36)	1 (2)
convulsion	3 (7)	1 (2)
diarrhea	3 (7)	0 (0)
anorexia	4 (10)	0 (0)
pulmonary	2 (4)	1 (2)
rash	3 (7)	1 (2)

Grade 3 or 4 neutropenia occurred in 4 patients (10%), and Grade 3 or 4 thrombocytopenia occurred in 3 patients (7%). A lack of appetite or nausea sometimes persisted for 1 or 2 days, although the best antiemetic agents were available to patients.

Grade 3 or 4 hepatic dysfunction, indicated by transaminase levels, occurred in 2 patients (5%).

Grade 3 or 4 renal dysfunction, indicated by creatinine levels, was observed in 1 patient, and hemorrhagic cystitis was not observed even though ifosfamide can sometimes cause this inflammation. No one had encephalopathy or auditory toxicity. Although none of the patients had *Pneumocystis carinii*, 1 patient had a Grade 3 or 4 pulmonary infection. All patients had Grade 1 or 2 alopecia. Thirteen patients were alive on follow-up > 12 months.

The number of treatment cycles per patient ranged from 1–12. Four patients received only 1 cycle, 6 received 2, 9 received 3, 6 received 3.8, 7 received 5, and 10 received ≥ 6. The median number of cycles per patient was 4.1. Treatment was delayed for a median time of 1 week (recovery after 1–2 weeks) in 3 patients (7%) due to a Grade 3 thrombocytopenia, Grade 2 infection, or Grade 3 neutropenia. The most common reason for drug discontinuation was disease progression, which occurred in 83% of patients. Treatment was discontinued in 1 patient because of a pulmonary embolism, in 1 because of surgery for an unrelated condition, in 4 because of Grade 2 fatigue (they withdrew consent), and in 1 because of pulmonary infection (Table 4).

Discussion

The aim of chemotherapy in patients with recurrent GBM is to offer a palliative benefit and maintain a useful neurological status with the prolongation of survival.

TABLE 4: Reasons for discontinuing treatment and exiting the study

Reasons	No. of Patients (%)	Duration in Study (wks)
disease progression	35 (83)	6–52
thromboembolic complication	1 (2)	24
surgery for unrelated condition	1 (2)	19
Grade 2 fatigue, w/drew consent	4 (10)	6, 12, 24, 42
pulmonary infection	1 (2)	54

Hopefully, these goals are achieved by delaying tumor progression with less treatment toxicity.

Recently, temozolomide has been used as standard therapy in patients with newly diagnosed GBM.[20] Once the tumor recurs, however, there is no established regimen.[7] Temozolomide has shown limited activity against recurrent GBM in patients who have received no chemotherapy or a nitrosourea-based regimen.[29] Authors of several studies have generally reported response rates < 20% and a PFS-6 < 30% in patients with recurrent GBM (Table 5).

The methylation status of the MGMT promoter is associated with a favorable outcome after temozolomide chemotherapy for patients with newly diagnosed GBM.[12] Since temozolomide is an alkylating agent like carmustine, the possible development of resistance depends on the expression of MGMT.[12] The concept of enhanced MGMT depletion with an alternative temozolomide-intensified dosing regimen has been rigorously tested.[22] Among the various tested regimens, the efficacy and tolerability of temozolomide in an alternating weekly regimen seems to be one of the most promising. This regimen showed a PFS-6 of 43.8% with less toxicity in patients with recurrent GBMs.[27]

Among the prevailing molecular targeted therapies, the regimen of bevacizumab plus irinotecan seems promising.[23,24] Bevacizumab is a humanized immunoglobulin monoclonal antibody that binds to and inhibits the activity of vascular endothelial growth factor. Irinotecan, a topoisomerase I inhibitor, has a cytotoxicity mechanism different from that of alkylating agents including temozolomide and nitrosoureas. This regimen showed a high response rate > 40% and a good PFS-6 > 40% in patients with recurrent GBM.[24] Recently, a regimen of bevacizumab alone was shown to be comparable to a combination of bevacizumab and irinotecan.[9] At present, regimens of dose-intensified temozolomide and those including bevacizumab are most promising.

Carboplatin is a platinum compound, and etoposide is an inhibitor of the enzyme topoisomerase II.[14,30] Both have mechanisms of action different from those of alkylating agents like temozolomide. Ifosfamide is a nitrogen mustard alkylating agent.[31] The mechanism of the ICE regimen against tumors is different from that of temozolomide. The ICE regimen is expected to be effective in patients with recurrent GBM after the failure of temozolomide or nitrosourea-based regimens. Sanson et al.[20] have reported on a Phase II study of the ICE regimen in patients with recurrent malignant gliomas. The hematological toxicity of ICE was not well tolerated, although the response rate was favorable. Tumor stabilization for an extended period of time with low toxicity is important in the management of an incurable malignant tumor.[21] We selected a lower dose of the ICE regimen against recurrent GBM. To test the safety and efficacy of this regimen, we performed a feasibility study in 15 patients with malignant recurrent gliomas; the response rate was 38% and the PFS-6 was 38%. The rate of Grade 3 or 4 toxicities was 8% (unpublished data). Therefore, we initiated this Phase II study of patients with GBM at the first relapse.

Grade 3 or 4 toxicities in the present study were minor and rare. Alopecia was a common mild toxicity. Although

TABLE 5: Literature survey of Phase II chemotherapy trials on recurrent GBM*

Authors & Year	Regimen	No. of Patients	% Chemotherapy-Naïve Patients	Response Rate (%)	PFS-6	
					%	95% CI
Wong et al., 1999	studies	225	NR	6	15	21–28
Kappelle et al., 2001	PCV	63	68.3	11	29	NR
Fine et al., 2003	BCNU + thalidomide	38	50	24	27	15.9–45.9
Yung et al., 2000	TMZ: 5 days on/23 days off	112	35	5.4	21	23–29
Brada et al., 2001	TMZ: 5 days on/23 days off	138	71	8	18	11–26
Wick et al., 2007	TMZ: 7 days on/7 days off	64	36	15	43.8	NR
Brandes et al., 2006	TMZ: 3 wks on/1 wk off	33	100	9	30.3	18–51
Groves et al., 2002	TMZ + marimastat	44	43	13.6	39	NR
Jaeckle et al., 2003	TMZ + 13-cis-retinoic acid	40	NR	NR	32	21–51
Brandes et al., 2004[4]	TMZ + cisplatin	50	100	20.4	34	23–50
Brandes et al., 2004[5]	BCNU + irinotecan	42	0	21.4	30.3	18–51
Cloughesy et al., 2008	bevacizumab	85	NR	20	35.1	23.2–47.0
Vredenburgh et al., 2007[27]	bevacizumab + irinotecan	35	0	57	46	32–66
present study	ICE	42	0	27	35	22–50

* BCNU = carmustine; NR = not reported; PCV = procarbazine, vincristine, lomustine; TMZ = temozolomide.

TABLE 6: Comparison of drug costs

Regimen	Acquisition Cost/ Mo	Reference
TMZ: 5 days on/23 days off	~ €2,206	Wasserfallen et al., 2005
bevacizumab + irinotecan	$28,000–39,000	Chamberlain, 2008
low-dose ICE	~ ¥50,000 ($500)	present study

myelosuppression was a dose-limiting factor of side effects on this regimen, its toxicity was not cumulative and resolved before the next treatment cycle in most cases, and usually could be resolved with one dose-level reduction or a treatment delay of up to 1 or 2 weeks. A Grade 3 or 4 pulmonary toxicity was rare. Hemorrhagic cystitis, which is sometimes observed with ifosfamide-containing regimens, did not appear in this study. The lower doses of the drugs in this study were thought to be the main reason for these safety profiles. The percent of patients with Grade 3 or 4 toxicities in the present study is almost comparable to the 25% reported for temozolomide.[3] The toxicity of cisplatin and etoposide is increased by valproic acid (enzyme-inhibiting drug). The activity of ifosfamide is reduced by phenobarbital (enzyme-inducing drug). Note, however, that the influence of antiepileptic drugs on the toxicity and activity of the ICE regimen has not been determined.[25]

So-called pseudoprogression can occur in up to 20% of patients who have been treated using radiotherapy concurrent with temozolomide.[7,8] Because temozolomide had not been approved in Japan during the time of our study (2002–2005), our supply was very limited; the Schering-Plough company provided the temozolomide through a compassionate use program. The 18 patients who had received a previous temozolomide regimen were treated daily for 5 days during the adjuvant phase before the present study. All of them underwent radiotherapy alone, not concurrent with temozolomide; therefore, the incidence of pseudoprogression was < 10%. Five patients were enrolled in the study 6 months after radiotherapy. If pseudoprogression was misdiagnosed as progression, then the estimated number of misdiagnoses seems to be very small. Therefore, we think the PFS-6 in the present study is not so much influenced by pseudoprogression.

The PFS-6 in the present study was 35%, and the rate of Grade 3 or 4 toxicity was 9%. In comparison with the findings in a Phase II trial of bevacizumab and irinotecan or a Phase II trial of an alternating weekly regimen of temozolomide, our results indicated that the ICE regimen showed comparable efficacy and tolerability.[24,27]

As new drugs or treatment modalities are introduced into the clinic, attention should focus not only on efficacy or safety, but also on cost-effectiveness.[19] Another challenging aspect with regimens of bevacizumab or dose-intensified temozolomide is pharmacoeconomic (Table 6). Both irinotecan and bevacizumab are expensive. The pharmacy cost for irinotecan is ~ $4000 for 125 mg/m^2 to $9000 for 340 mg/m^2 per dose. Bevacizumab is ~ $9000 per dose. An average treatment over 6 months would cost between $169,000 and $234,000 for pharmacy-incurred charges only.[8] Temozolomide acquisition costs €2206 per cycle.[25] On the other hand, ICE acquisition costs ~ ¥50,000 (~ $500). Considering these costs, the ICE regimen could be one of the options for patients with recurrent GBM. Further evaluation of various aspects of the regimen, including cost, is warranted.

Conclusions

The combination of ifosfamide, carboplatin, and etoposide in a low-dose setting was effective against recurrent GBM. Progression-free survival at 6 months after ICE treatment was 35%. The toxicity of the regimen was acceptable and mild, consisting mainly of alopecia. Although further evaluation is necessary, this regimen could be one of the options for patients with recurrent GBM.

Disclosure

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nature
cell biology

Ciliary transition zone activation of phosphorylated Tctex-1 controls ciliary resorption, S-phase entry and fate of neural progenitors

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Primary cilia are displayed during the G_0/G_1 phase of many cell types. Cilia are resorbed as cells prepare to re-enter the cell cycle, but the causal and molecular link between these two cellular events remains unclear. We show that Tctex-1 phosphorylated at Thr 94 is recruited to ciliary transition zones before S-phase entry and has a pivotal role in both ciliary disassembly and cell cycle progression. However, the role of Tctex-1 in S-phase entry is dispensable in non-ciliated cells. Exogenously adding a phospho-mimic Tctex-1^{T94E} mutant accelerates cilium disassembly and S-phase entry. These results support a model in which the cilia act as a brake to prevent cell cycle progression. Mechanistic studies show the involvement of actin dynamics in Tctex-1-regulated cilium resorption. Tctex-1 phosphorylated at Thr 94 is also selectively enriched at the ciliary transition zones of cortical neural progenitors, and has a key role in controlling G_1 length, cell cycle entry and fate determination of these cells during corticogenesis.

Primary cilia are microtubule-based, hair-like organelles that extend from the plasma membrane to sense and transduce extracellular signals. Primary cilia are displayed on G_0/G_1 cells, and are resorbed as the cells re-enter the cell cycle[1]. The biological significance of these temporally coupled cellular events and the molecular mechanisms underlying the transition between these processes are poorly understood.

Radial glial cells are ciliated neural progenitors in the developing neocortex[2]. Radial glia preferentially undergo proliferative division to expand the progenitor population during early corticogenesis, whereas later progenitors preferentially undergo neurogenic division (differentiation). Proliferation and differentiation are modes of division that are characterized by short and long G_1 durations, respectively[3]. Shortening G_1 accelerates cell cycle entry and expands the progenitor population[4,5], whereas lengthening G_1 drives cell cycle exit and differentiation into neurons[6]. The mechanisms that drive the molecular switch between self-renewal and neuronal differentiation in neural progenitors of the developing neocortex remain unclear.

Tctex-1 (or DYNLT) was originally described as a light chain subunit of cytoplasmic dynein[7,8]. However, Tctex-1 can be uncoupled from the dynein complex to perform dynein-independent functions[9]. Tctex-1 is selectively enriched in proliferating neural progenitors of both embryonic (this paper) and adult brains[10]. However, its function in these cells is unknown. Here we show that Tctex-1, when phosphorylated at Thr 94 (phospho(T94)Tctex-1), regulates ciliary resorption and S-phase entry. In the developing neocortex, phospho(T94)Tctex-1 has an important role in the cell cycle regulation of radial glia and maintenance of the proliferating progenitor population.

RESULTS

Tctex-1 has a key role in cilia-dependent S-phase entry

We tested the role of Tctex-1 in cell cycle control by performing loss-of-function analysis in diploid human hTERT-RPE-1 (herein RPE-1) cells[11]. Shortly before serum starvation, cells were transfected with a plasmid encoding Tctex-1 short hairpin RNA (shRNA) and green fluorescent protein (GFP; plasmid labelled as Tctex-1-sh), or a plasmid encoding GFP alone (vector; Fig. 1a). Following 48 h serum starvation, cell cycle re-entry was induced by serum addition. As predicted, a significant increase in the levels of Rb phosphorylated at Ser 795, Rb phosphorylated at Ser 807/811 and cdc2 phosphorylated at Tyr 15 were observed in control cells 24 h after serum treatment (Fig. 1b, c). Rb phosphorylation is required for G_0/G_1–S transition[12], whereas cdc2 phosphorylated at Tyr 15 is a G_2 marker. Serum treatment failed to induce both phosphorylated Rb and phosphorylated cdc2 in cells with suppressed Tctex-1 (Fig. 1b, c), indicating that Tctex-1 is required for cell cycle re-entry.

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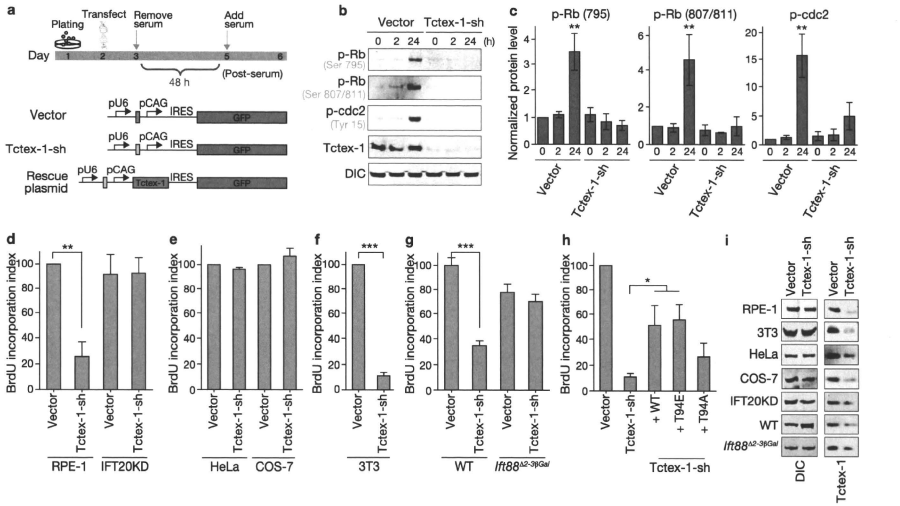

Figure 1 Tctex-1 is involved in cilium-dependent cell cycle re-entry. (**a**) Schematic representations of the timeline of transfection experiments (top) and the plasmids used for transfection (bottom). IRES; internal ribosome entry site. Grey and yellow boxes represent control and *Tctex-1* shRNA sequences, respectively. (**b**) Representative immunoblots of lysates from control- or Tctex-1-sh-transfected RPE-1 cells probed with antibodies against the indicated proteins. Cells were treated as indicated in **a** and the times at which cells were harvested after serum re-addition are shown. p-Rb and p-cdc2, phosphorylated Rb and cdc2, respectively; DIC, dynein intermediate chain. (**c**) Quantification of results from **b**; the signal levels were normalized to the DIC signal. Data are means ± s.e.m.; $n = 3$ experiments, ** $P < 0.01$, *t*-test. (**d**–**h**) BrdU incorporation indices (fraction of BrdU-labelled GFP$^+$ cells) of synchronized RPE-1 or IFT20KD-RPE-1 cells (**d**), HeLa or COS-7 cells (**e**), 3T3 cells (**f**, **h**), and wild-type (WT) or *Ift88* mutant MEF cells (**g**) after serum release. Cells were treated as in **a** and transfected with control vector or Tctex-1-sh plasmid; in **h** cells were additionally transfected with plasmids encoding the indicated Tctex-1 proteins. Incorporation of BrdU in the control cells was taken to be 100%. Data are means ± s.e.m.; $n = 5, 4, 7, 3$ and 7 experiments for figures **d**, **e**, **f**, **g** and **h**, respectively; an average of 400 cells was counted in each experiment; ** $P < 0.01$, *** $P < 0.001$, *t*-test for **d**–**g**; * $P < 0.05$, one-way ANOVA test for **h**. (**i**) Immunoblotting assays of lysates from the indicated cell types transfected with control vector or Tctex-1-sh plasmid. There was a reduction of endogenous Tctex-1 levels in various cell types transfected with Tctex-1-sh plasmid. Uncropped images of blots are shown in Supplementary Fig. S6.

To examine S-phase entry, cells were pulse-labelled with BrdU after serum release, and the fraction of BrdU-labelled GFP$^+$ transfected cells (BrdU incorporation index) was scored (Supplementary Fig. S1a). The BrdU incorporation index of Tctex-1-suppressed cells was significantly lower than that of control cells, indicating that reduced Tctex-1 inhibited S-phase entry (Fig. 1d). S-phase entry was also blocked in unsynchronized cells transfected with either Tctex-1-sh or *Tctex-1* siRNA (short interfering RNA; data not shown). In addition, most control cells were positive for the proliferation marker Ki67, whereas only ~30% of cells transfected with Tctex-1-sh were Ki67 labelled (Supplementary Fig. S1b). These results imply that Tctex-1 suppression induces cell cycle exit (that is, G_0 arrest).

Although a decrease in BrdU incorporation was also seen in 3T3 cells transfected with Tctex-1-sh (Fig. 1f), it was not seen in HeLa and COS-7 cells transfected with Tctex-1-sh (Fig. 1e). As both RPE-1 and 3T3 are ciliated, but HeLa and COS-7 cells are not, we postulated that the role of Tctex-1 in cell cycle progression is cilia-dependent. Two additional 'cilia-free' cell models were employed: a stable RPE-1 cell line in which IFT20 is silenced (that is, IFT20KD-RPE-1; ref. 13), and *Ift88* mutant mouse embryonic fibroblasts (MEF) derived from *Ift88*$^{\Delta 2-3\beta Gal}$ mice[14,15] (Supplementary Fig. S1c–e). Although Tctex-1 knockdown reduced BrdU incorporation in control RPE cells (Fig. 1d) and wild-type MEF cells (Fig. 1g), it did not affect BrdU incorporation in IFT20KD-RPE-1 (Fig. 1d) and *Ift88*-mutant (Fig. 1g) cells. Similarly, a significantly smaller fraction of Ki67-labelled cells was found in wild-type, but not *Ift88*-mutant MEF cells in which Tctex-1 was suppressed (Supplementary Fig. S1f). Quantification of Tctex-1 level with immunoblots (Fig. 1i) and cell-based immunofluorescence microscopy (Supplementary Fig. S1g, h) showed that the degree of Tctex-1-sh-mediated knockdown was comparable among all cell types tested, confirming that differences were not due to differential gene silencing efficacy. Taken together, these results demonstrate that Tctex-1 has an important role in cilia-dependent S-phase entry.

Transfection of cells with Tctex-1-sh had no effect on the expression levels of several components of the dynein complex, such as dynein intermediate chain (Fig. 1i) and dynein heavy chain[9]. To further test whether the impaired cell cycling mediated by Tctex-1 knockdown was due to its dynein-independent role, we examined whether or not the phosphomimic mutant Tctex-1^{T94E} could rescue the phenotype. Previous studies have shown that Tctex-1^{T94E} fails to be incorporated into the dynein complex, and therefore represents a dynein-free pool of Tctex-1 (ref. 9). A counterpart construct encodes the non-phosphorylatable mutant Tctex-1^{T94A}, which binds dynein[9] and is used here as a control. Thr94 of

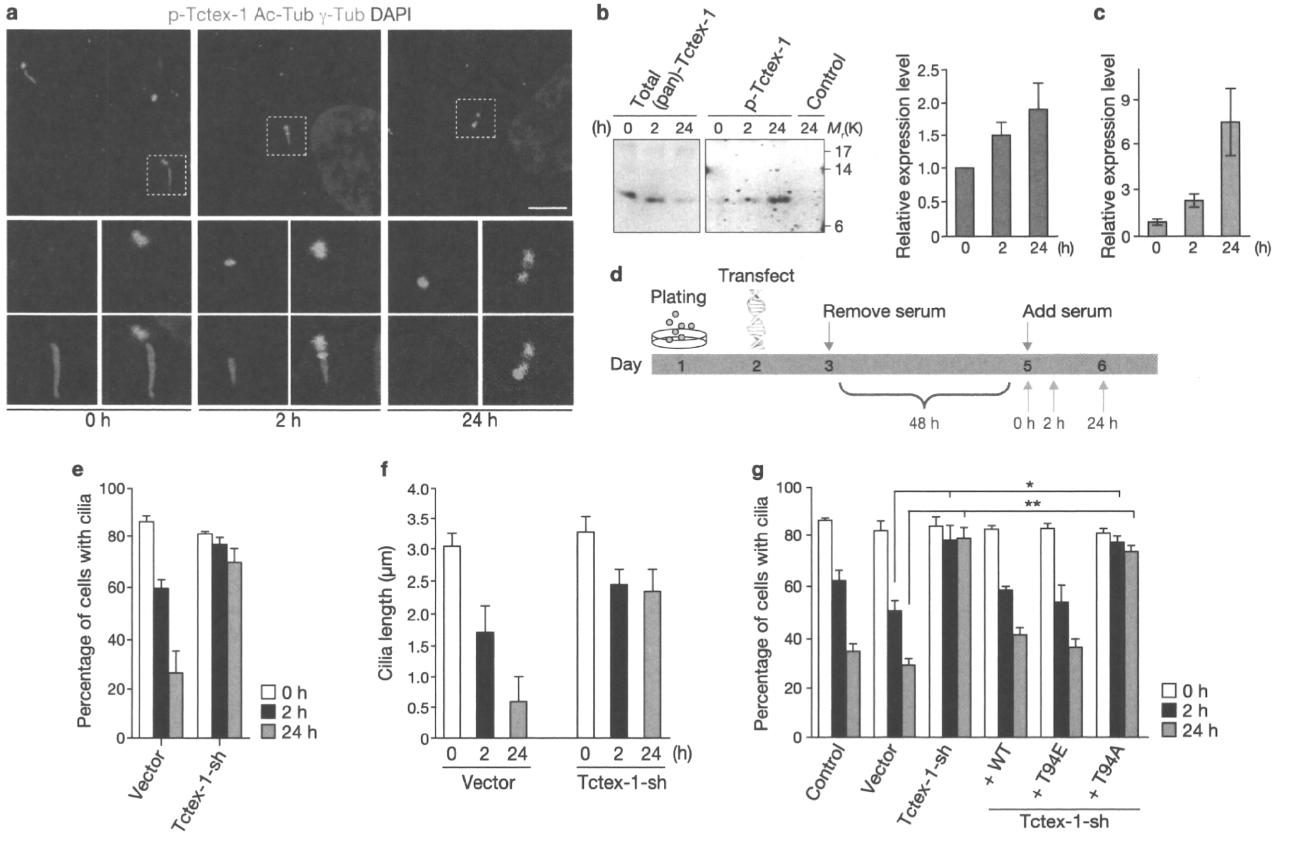

Figure 2 Temporal activation of phospho(T94)Tctex-1 at the transition zone and its function in ciliary disassembly. (**a**) Immunofluorescence microscopy of phosphorylated Tctex-1 (p-Tctex-1; green), acetylated α-tubulin (Ac-Tub; red) and γ-tubulin (γ-Tub; cyan) in quiescent RPE-1 cells or cells treated with serum for 2 h or 24 h. DAPI; nuclei (blue). Insets are higher magnification images of indicated regions. Scale bar, 5 μm. (**b**) Left: representative immunoblots show the levels of (pan)Tctex-1 and phosphorylated Tctex-1 in cells harvested at the indicated times after serum re-addition. To improve the detection of phosphorylated Tctex-1, immunoprecipitation was first carried out using a saturated amount of anti-(pan)Tctex-1, the immunoprecipitates were resolved by electrophoresis and immunoblotted with antibody against phospho(T94)Tctex-1. Anti-HA (haemagglutinin) was used as an immunoprecipitation antibody control. Right: the relative expression level of phospho(T94)Tctex-1 level was normalized to the total amount of immunoprecipitated Tctex-1. (**c**) The relative intensity of phospho(T94)Tctex-1 in immunofluorescence microscopy images of RPE-1 cells at the indicated times after serum re-addition. The ratio of phospho(T94)Tctex-1:γ-tubulin intensity was quantified by MetaMorph software (data are means ± s.e.m.; $n = 3$ experiments). (**d**) Schematic representation of the timeline of cilium disassembly experiments used in **e**–**g**. (**e**) Fraction of transfected RPE-1 cells containing a cilium were scored in cells harvested at the indicated times after serum addition. An average of 500 cells were counted for each experiment (data are means ± s.e.m.; $n = 3$ experiments). (**f**) Quantification of the cilia lengths in transfected 3T3 cells harvested after serum addition (data are means ± s.e.m.; $n = 3$ experiments). (**g**) The fractions of transfected cells containing a cilium. Cells were harvested at indicated times after serum addition and scored and statistically analysed (data are means ± s.e.m.; $n = 5$ experiments; * $P < 0.05$, ** $P < 0.01$; one-way ANOVA). Uncropped images of blots are shown in Supplementary Fig. S6.

Tctex-1 is a conserved residue, and resides within a consensus motif for protein kinase C and integrin-linked kinase (http://www.cbs.dtu.dk/services/NetPhosK/; http://gps.biocuckoo.org/). T94E and T94A variants of Tctex-1 were generated in bovine Tctex-1, which is insensitive to shRNA against mouse Tctex-1, allowing us carry out the rescue experiments in mouse 3T3 cells. Impaired S-phase entry caused by Tctex-1 knockdown can be rescued by the re-introduction of wild-type Tctex-1 and the Tctex-1^{T94E} mutant, but to a much lesser extent by the T94A mutant (Fig. 1h). These results not only suggest that the phenotypes caused by Tctex-1-sh were not an 'off-target' effect, but also that Tctex-1^{T94E} mimicked the functionally active form of Tctex-1.

Temporal and spatial activation of phospho(T94)Tctex-1 at the transition zone is required for ciliary resorption

The kinetics of cilium assembly/disassembly, as well as the temporal relationship between cilium dynamics and cell cycle progression, have been characterized in RPE-1 and 3T3 cells[11,16,17]. In both cell types, serum starvation induces quiescence and cilium formation. The return of serum triggers biphasic ciliary resorption, which peaks at 2 h and 24 h post-serum treatment. The first wave of cilium shortening occurs at mid/late G_1-phase preceding S phase entry, whereas the second wave occurs as cells are preparing to enter the G_2/M phase [16]. Immunolabelling using an antibody specifically recognizing phospho(T94)Tctex-1 showed that phospho(T94)Tctex-1 was rarely seen in quiescent cells (0 h), whereas prominent phospho(T94)Tctex-1 signals were consistently detected at the ciliary base immediately adjacent to the shortened cilia in cells treated with serum for 2 h (Fig. 2a). Phospho(T94)Tctex-1 was specifically distributed to the transition zone between the γ-tubulin-labelled basal bodies and acetylated α-tubulin-labelled cilia. Consistent with the cilia loss and cell cycle re-entry in cells after 24 h serum treatment, phospho(T94)Tctex-1 signals were found on centrosomes in interphase cells (Fig. 2a) and mitotic poles in dividing cells (Supplementary

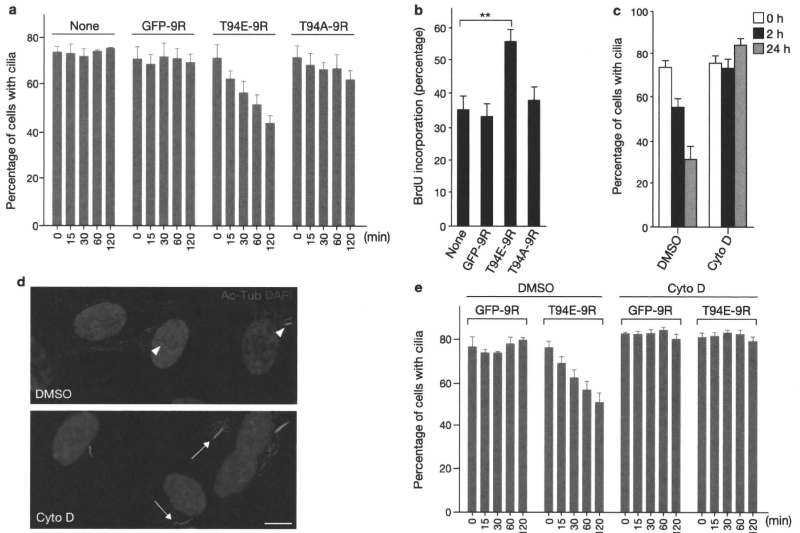

Figure 3 Phospho(T94)Tctex-1 and actin dynamics participate in ciliary resorption. (**a**) Fractions of cells displaying cilia. RPE-1 cells were serum starved and left untreated (none) or treated with GFP-9R, Tctex-1^{T94E}-9R or Tctex-1^{T94A}-9R peptides, and harvested at indicated times after the treatments. Data are means ± s.e.m.; $n = 4$ experiments, except untreated control, where $n = 3$ experiments. (**b**) Cells in serum-free medium were treated with the indicated peptides followed by incubation with BrdU for 16 h. Fractions of BrdU-labelled cells are shown (data are means ± s.e.m.; $n = 7$ experiments; ** $P < 0.01$; one-way ANOVA). (**c**) Cells were treated with DMSO (as a control) or Cyto D (0.5 μM) after serum addition and the fraction of ciliated cells was quantified (data are means ± s.e.m.; $n = 3$ experiments). (**d**) Representative image of cilia displayed in control cells versus Cyto D-treated cells 24 h after serum addition. Arrowheads and arrows indicate shortened and long cilia in the DMSO- and Cyto D-treated cells, respectively. Scale bar, 5 μm. (**e**) Fractions of cells that had cilia after the addition of GFP-9R or Tctex-1^{T94E}-9R for indicated time in the presence of DMSO ($n = 3$ experiments) or Cyto D ($n = 5$ experiments). Data are means ± s.e.m.

Information Fig. S2a). Quantitative immunoblotting and immunofluorescence microscopy assays showed that the level of phospho(T94) Tctex-1 gradually increases with serum re-addition (Fig. 2b, c).

Pre-absorption of the antibody with the antigen peptide, but not the control peptide (corresponding to the antigen except lacking the phospho(T94) modification), effectively removed the phospho(T94) Tctex-1 signals (Supplementary Fig. S2b). The signal from phospho(T94) Tctex-1 in immunofluorescence microscopy images was greatly reduced in the RPE-1 cells transfected with Tctex-1-sh (Supplementary Fig. S2c). Furthermore, phospho-Tctex-1 signal on immunoblots was sensitive to the treatment of alkaline phosphates (Supplementary Fig. S2d). These results confirm the specificity of phospho(T94)Tctex-1 immunolabelling.

The appearance of phospho(T94)Tctex-1 at the ciliary base shortly preceding the disassembly of cilia indicates its functional involvement in this cellular event. To directly test the role of Tctex-1 in ciliary disassembly, serum-starved cells transfected with Tctex-1-sh were harvested at 0 h, 2 h and 24 h after serum re-addition (Fig. 2d). The fraction of GFP$^+$ cells displaying a cilium and the length of the cilia in transfected cells were scored. In RPE-1 (Fig. 2e), 3T3 (Fig. 2f, g), and MEF (Supplementary Fig. S2e) cells, controls and Tctex-1-suppressed cells had similar abilities to form cilia after serum starvation. The lengths of their cilia were also similar (Fig. 2f). However, serum-induced cilium disassembly was significantly blocked by Tctex-1 suppression, suggesting that Tctex-1 is not required for ciliogenesis, but is critical for cilium disassembly. Furthermore, co-transfection of Flag-tagged wild-type Tctex-1 or Tctex-1^{T94E}, but not Tctex-1^{T94A}, with Tctex-1-sh effectively reversed the inhibition of cilium disassembly caused by Tctex-1 silencing (Fig. 2g).

To further investigate the causal relationship between ciliary disassembly and S-phase entry, we investigated whether or not exogenously added Tctex-1^{T94E} can trigger rapid cilium disassembly of RPE-1 cells, and thus accelerate their S-phase entry. We employed a high-efficiency protein transduction system[18] to deliver Tctex-1 peptides into RPE-1 cells with pre-formed cilia. Purified recombinant Tctex-1 (or GFP) containing a nine-arginine sequence (9R) entered cells effectively, as confirmed by both immunoblotting and immunostaining assays (Supplementary Fig. S2f, g). The addition of control peptides GFP-9R and Tctex-1^{T94A}-9R had no effect on cilia disassembly (Fig. 3a). In contrast, cells treated with Tctex-1^{T94E}-9R underwent significantly faster cilium resorption even when cultured in the absence of serum (Fig. 3a). Cells treated with Tctex-1^{T94E}-9R, but not control peptides, also exhibited significantly higher BrdU incorporation (Fig. 3b).

Phospho(T94)Tctex-1 has a reported ability to modulate actin dynamics[9]. To investigate the role of actin in cilium resorption, we