要　旨

転移性脳腫瘍治療の問題点と手術適応

成田　善孝

癌患者の死亡者数は年間 32 万人にも上り，年間少なくとも 5 万人以上の癌患者が転移性脳腫瘍を合併すると考えられる．転移性脳腫瘍は癌患者の予後に影響を与え，神経症状出現による QOL の低下を招く．直径が 3 cm 以上の単発の転移性脳腫瘍や神経症状が進行している症例に対しては，手術＋全脳照射が標準治療として行われる．直径が 3 cm 以下で 3〜4 個までの腫瘍に対しては，ガンマナイフなどの定位放射線照射単独による治療が広く行われているが，これまでの臨床研究から定位放射線照射＋全脳照射が標準治療になりえると考えられる．一方，全脳照射による痴呆などの神経障害などの有害事象を考慮し生存率・QOL を検討する臨床研究が，日本臨床腫瘍グループ（JCOG）など国内外で進行中である．転移性脳腫瘍の治療にあたっては EBM に基づいた治療法を選択する必要がある．

脳外誌　16：820-827，2007

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脳神経外科学

悪性脳腫瘍に対する新しい治療

Current advances in treatment for malignant brain tumors

悪性脳腫瘍の代表ともいえる悪性神経膠腫(グリオーマ)は，いまなお治療困難な疾患のひとつであり，治療のスタンダードも確立していない．1970年代から欧米では第Ⅲ相試験を含む臨床試験が実施され，その結果，予後を改善する因子として手術による摘出，放射線治療などがあげられてきた．化学療法については有意に予後の改善につながる薬剤はなく，BCNU (carmustine)などの nitrosurea 系薬剤が，そのなかでも生存期間延長の傾向があり，20年来補助療法の薬剤として用いられてきた．2002年に発表されたメタアナリシスでは，悪性神経膠腫に対し nitrosourea 系抗癌剤を併用した放射線治療群と放射線単独療法群の比較を，過去に発表された12の無作為化試験に登録された3,004例の症例について行った[1]．その結果，抗癌剤併用群の1年生存率が46%，放射線単独群が40%であり，有意に前者の生存率が勝っているという結果であり，nitrosourea 併用の有効性が証明された．

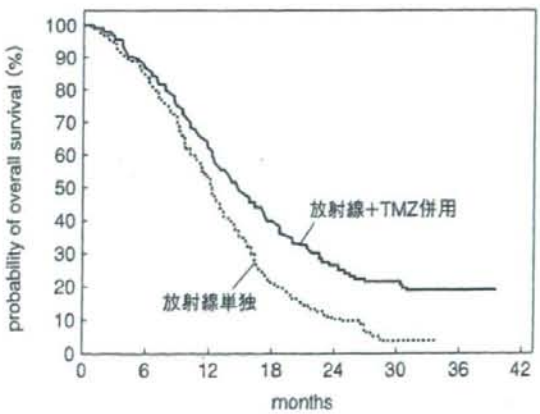
日本の現状

一方，国内で確立されたエビデンスはほとんどなく，前述のメタアナリシスに加えられた論文も Takakura らの1編のみであった．これは，国内で開発された nitrosourea 系抗癌剤である ACNU (nimustine hydrochloride)を併用した放射線治療群と放射線単独群の効果を比較した第Ⅲ相比較試験で，その結果，生存率では両者の差は認められなかったが，奏効率で前者が勝っているというものであり，これらの結果を踏まえ，国内では ACNU＋放射線治療が悪性神経膠腫治療のスタンダードとして用いられるようになった[2]．しかし，その効果は不十分であり，実際には各脳神経外科施設が独自の治療を行ってきたというのが実情である．

国内でのスタンダード確立のために，日本臨床腫瘍研究グループ(JCOG)内の脳腫瘍グループが第Ⅱ/Ⅲ相試験を実施している．これは術後の化学放射線治療として ACNU 併用治療と procarbazine および ACNU を併用する治療を比較するものである．後者は procarbazine を ACNU に先行して投与し，ACNU に対する薬剤耐性を発揮する O^6-methylguanine DNA-methyltrasferase (MGMT)という酵素を阻害して，その効果を高めようという治療法である[3]．現在，第Ⅱ相段階が終了し，その有効性，安全性について検討中である．

Temozolomide(TMZ)併用放射線治療

2005年，European Organisation for Research and Treatment of Cancer (EORTC)から悪性神経膠腫の治療について画期的な報告がなされた．これは悪性神経膠腫のなかでもっとも悪性度の高い膠芽腫を対象とし，手術後に temozolomide (TMZ)併用放射線治療を行い，さらに6コースの同剤による化学療法を行う群と放射線単独群とを比較した第Ⅲ相試験であり，両群とも280例を超える登録がなされ



放射線単独	286	240	144	59	23	2	0
放射線+TMZ併用	287	246	174	109	57	27	4

図1 膠芽腫に対する放射線単独照射と放射線＋Temozolomide (TMZ)の比較試験結果[4]
生存期間中央値は前者が12.1カ月に対し，後者は14.6カ月で，有意に生存期間の延長がみられた．

た[4]．その結果，併用群の生存期間中央値が14.6カ月，放射線単独群が12.1カ月であり，有意に前者の生存期間の延長がみられた．2年生存率も前者が26.5%，後者が10.4%であり，膠芽腫を対象とした臨床試験ではじめて有意な差をもって，放射線単独治療に対し，より有効な治療法として証明された(図1)．TMZは経口投与で髄液移行もよく，他の抗癌剤と比べ骨髄抑制も軽微であり，欧米ではすでに標準治療薬として用いられている．これらの試験結果を受け，国内でも第Ⅱ相試験が行われ，2006年9月保険治療薬として認可された．

治療法は，放射線治療開始時より6週間，75 mg/m^{2}を連日服用し，放射線終了後は28日ごとに最初の5日間に150 mg/m^2，減量基準に該当しなければ，2回目より200 mg/m^{2}を6コース服用するというものである．国内でも認可後6カ月で2,000例あまりに使用されているといわれ，有効例も数多く報告されている．有害事象として嘔気，嘔吐，便秘などがめだつほか，リンパ球減少との関連性も考えられるニューモシスチス肺炎(カリニ肺炎)による死亡が3例報告されており，ST(sulfamethoxazole/trimethoprim)合剤の使用が推奨されている．

おわりに

国内でも今後，TMZが悪性神経膠腫に対する標準治療薬として使用されると考えられるが，本剤の膠芽腫に対する効果も平均生存がたかだか14カ月であり，まだまだ十分なものとはいえない．とくに前述のMGMTの発現がみられる腫瘍については発現のない腫瘍に比べ，その効果が半減する(生存期間中央値12.7カ月 vs. 21.7カ月)といわれ，併用薬の工夫などが必要である．

TMZの出現により，30年来変化のなかった悪性神経膠腫の治療成績も若干向上することが期待できる．しかし，その治療成績はまだまだ満足できるものではなく，今後さらなる治療薬・治療法の開発が望まれる．

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＊＊次号の特集予告(222巻13号)＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊＊

◆がん外来化学療法コンセプトシート

(企画：畠　清彦／癌研究会癌研有明病院化学療法科・血液腫瘍科，外来治療センター)

＊＊

がんに対する副作用の少ない新規分子標的薬などの抗腫瘍薬剤の進歩，副作用に対する支持サポート療法の進歩などによる抗がん剤の安全な使用法の確立に伴い，がん治療も外来で行われる時代が到来している．各地の基幹病院には外来治療センターが新設され，専任スタッフも置かれるようになっているが，その運営マネジメントにはまだ課題も多いという声が聞かれる．本特集では，各癌腫別の最新治療エビデンス項目も多く紹介するとともに，有害事象と対策，大学・一般病院施設別の特色，各職種の役割，各コメディカルごとの役割，サポート・医療環境など，今後のわが国がん外来化学療法の方向性を示す"コンセプトシート"としての構成による最新情報を提供する．

特集2 グリオーマの新しい治療薬 temozolomide（TMZ，テモダール®）の最近の話題

temozolomide（TMZ）を長期間投与することの有効性と危険性

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SUMMARY

膠芽腫においては術後放射線照射に加えてtemozolomideを投与することが標準治療となったが，報告されたランダム化第III相比較試験におけるtemozolomideの維持療法としての投与期間は6ヵ月である．6コースを越える長期間投与の安全性と有効性について我が国における再発退形成性星細胞腫に対する治験のデータを解析した．6コースを越えての投与により初めてCRに到達する症例が認められ，temozolomideがゆっくりと効果を発揮することが示され，長期投与の有用性が示唆された．一方，有害事象の蓄積は認められなかったが，文献的には二次癌，とくにmyelodysplastic syndromaの報告が散見され今後注意を要するものと考えられた．

KEY WORDS

temozolomide
グリオーマ
化学療法
二次癌
骨髄異形成症候群

はじめに

2005年に発表されたEORTC/NCICのrandomized phase 3 studyによって，膠芽腫に対する標準治療は，手術後放射線照射にtemozolomideを併用・維持療法として使用する方法となった[1]．この臨床試験で注意しなければならないのは年齢上限が70歳であることと，維持療法が6コースまでに制限されていることである．前者については，すなわち高齢者膠芽腫におけるtemozolomideの有効性と安全性はまだ証明されていないということであり現在複数の臨床試験が行われている．本稿では後者の問題を取り上げてみたい．temozolomideの高い安全性と有効性から考えると，6コースよりも更に長期間に渡って投与したいというのは，脳腫瘍を治療しているもの誰もが考えることであろう．しかし投与期間に関する高いレベルのエビデンスは未だ存在しない．

I. 我が国における治験によるtemozolomide長期投与のデータ

我が国におけるtemozolomide承認のためのデータパッケージの一つとなった初回再発退形成性星細胞腫患者における治験では，6コースまでをSTEP 1として密に経過を観察したが[2]，CR/PR/SDである患者においては6コース以降の投与もSTEP 2としてこれを許可

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した．STEP 1におけるMRIは1コース毎であるのに対してSTEP 2では2コース毎，血液検査もSTEP 1においては1週間毎であるがSTEP 2では2週間毎である．また治験薬投与は初回から2年間継続できるが，治験責任医師がこれを有益であると認めた場合は最長3年までの投与も可とした．

まず本試験に登録された32症例の背景は**表1**の通りである．登録された32症例中14例はSTEP 1で投与を終了し，18例が7コース以降のSTEP 2に移行した．STEP 2に移行した症例の投与コース数中間値は12.5，最多投与数は35コースであった．6コースまでに投与が中止となった症例の中止理由の11/14は原疾患の悪化によるものであった．同様にSTEP 2において18コース以前に投与を中止した主たる理由も原疾患の悪化であったが，一方19コース以上投与された症例においては，2年間が経過したため，あるいはtemozolomide市販のためといった，原病の状態とは無関係な理由により投与が中止された（**表2**）．

有害事象に関するデータは**表3**の通りである．6コース以降に新たに重度の有害事象が出現したことはほとんどなかった．中等度以上の有害事象に関しても，臨床的に問題となる事象は観察されていない（**表4**）．文献上も，128例の12コース以上投与症例で観察された有害事象は，grade 3以上の血小板減少10％，白血球減少7％，消化器症状5％，感染4％と報告されている[3]．

一方腫瘍縮小効果をみると，6コース以降にCRになった症例が3例存在する（**表5**）．MRI上で測定した腫瘍の大きさの推移を図1に示す．3例は6コース終了時点ではPRであるが，その後もゆっくりと腫瘍が縮小し最終的にはCRに至っていることが分かる．

また一時的には腫瘍が増大し，しかしその後も臨床的判断により薬剤投与を継続した結果，徐々に腫瘍縮小に転じた症例が5例みられた（**図2**）．これらはtemozolomideの効果が6コース以降も徐々に継続的に発現していくという特徴を示している．典型例を**図3**に示す．

表1　我が国における治験，初回再発の退形成性星細胞腫患者に対するtemozolomide単剤投与の有効性および安全性の検討－多施設共同第II相試験－，の患者背景

年齢	中央値	52.5歳
性別	男性	18 (56%)
	女性	14 (44%)
中央病理判定	退形成性星細胞腫	22 (69%)
	退形成性乏突起星細胞腫	6 (19%)
	膠芽腫	2 (6%)
	乏突起膠腫	1 (3%)
	Rosetted glioneuronal tumor	1 (3%)
治験薬投与前のKPS	中央値	90
初発時の手術内容	全摘出	8 (25%)
	部分摘出	15 (47%)
	生検	9 (28%)
初回再発時の手術内容	無し	27 (84%)
	全摘出	0 (0%)
	部分摘出	4 (13%)
	生検	1 (3%)

表2　我が国における治験，初回再発の退形成性星細胞腫患者に対する temozolomide 単剤投与の有効性および安全性の検討ー多施設共同第 II 相試験ー，における temozolomide 投与の中止時期とその理由

投与中止理由	投与中止時期（投与コース数）				
	≦6	7-12	13-18	19-24	≧25
有害事象の発現	2	0	0	0	0
原疾患の悪化	11	7	2	0	0
Temozolomide 市販のため	0	0	0	1	2
治療開始から2年経過	0	0	0	3	0
その他*	1	2	1	0	0
合計	14	9	3	4	2

*その他とは転居，プロトコール遵守困難，パートナーの妊娠，残存腫瘍摘出手術を理由とする．

表3　初回再発の退形成性星細胞腫患者に対する temozolomide 単剤投与の有効性および安全性の検討ー多施設共同第 II 相試験ー，において6コース以前と6コース以降を含む全治験期間においてみられた重度の有害事象の頻度の比較

有害事象	6コース以前	全サイクル
リンパ球数減少	8 (25%)	10 (31%)
好中球数減少	1 (3%)	1 (3%)
白血球数減少	0 (0%)	1 (3%)
血小板数減少	2 (6%)	2 (6%)
ALT 増加	1 (3%)	1 (3%)
便秘	1 (3%)	1 (3%)
食欲不振	0 (0%)	1 (3%)

表4　初回再発の退形成性星細胞腫患者に対する temozolomide 単剤投与の有効性および安全性の検討ー多施設共同第 II 相試験ー，において7コース以降に新たに発現した中等度以上の有害事象

7-8コース	9-10コース	11-12コース	13コース以降
眼乾燥	舌炎	毛包炎	季節アレルギー
変色便	麦粒腫		白癬感染
季節アレルギー	インフルエンザ		
高血糖	蛋白尿		
不規則月経			
嚥下性肺臓炎			

表5　初回再発の退形成性星細胞腫患者に対するtemozolomide単剤投与の有効性および安全性の検討ー多施設共同第II相試験ー，において6コース以前における腫瘍縮小効果と，6コース以降も含む全期間における腫瘍縮小効果

腫瘍縮小効果と奏効割合	6コースまで	全期間
CR	3/32 (9%)	6/32 (19%)
PR	8/32 (25%)	8/32 (25%)
SD	18/32 (56%)	15/32 (47%)
PD	2/32 (6%)	2/32 (6%)
判定不能	1/32 (3%)	1/32 (3%)
奏効割合	11/32 (34%)	14/32 (44%)

表6　Temozolomideが関与していると考えられる二次癌（myelodysplastic syndrome and leukemia）の報告の一覧

文献	年齢	性別	脳腫瘍	Temozolomideサイクル数
Noronha V, et al.	66	F	AO	25
Dufour C, et al.	11	M	O	3
Su Y-W, et al.	44	F	AA	6
Chamberlain MC, et al.	44	F	LGA	12
	69	F	AA	6
	38	M	AA	6
	34	M	LGA	24
	34	F	AOA	28
	59	F	AO	6

AO : anaplastic oligodendroglioma, O : oligodendroglioma, AA : anaplastic astrocytoma, LGA : low-grade astrocytoma, AOA : anaplastic oligoastrocytoma.

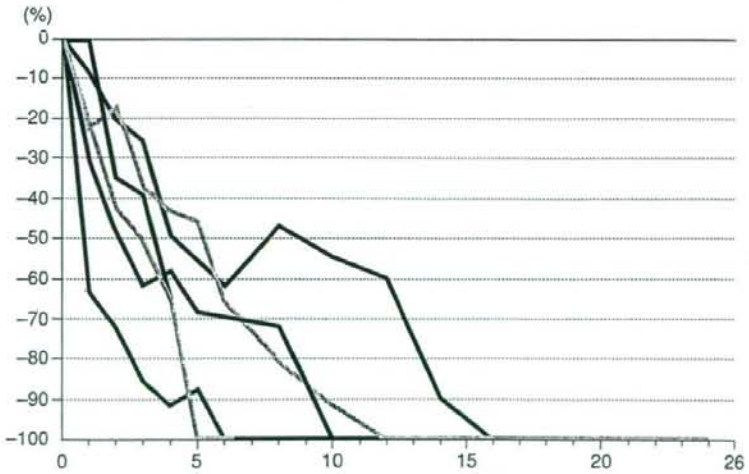

図1　CR 6例の腫瘍サイズの推移

横軸は治療コース数，縦軸は腫瘍縮小割合（%）．6例のうち3例は6コース終了の時点ではPRであるが，その後も腫瘍縮小効果が進行してCRに至っている．

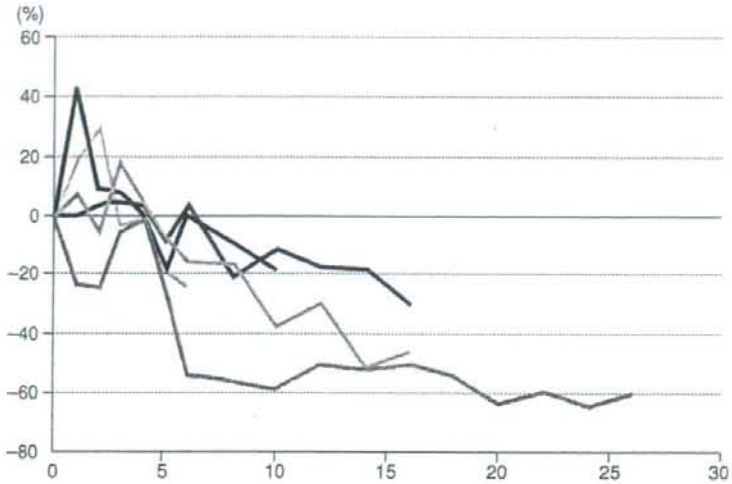

図2　一時的に腫瘍が増大し，その後腫瘍縮小に転じた5症例の腫瘍縮小率の推移
横軸は治療コース数，縦軸は腫瘍増大・縮小割合（%）．

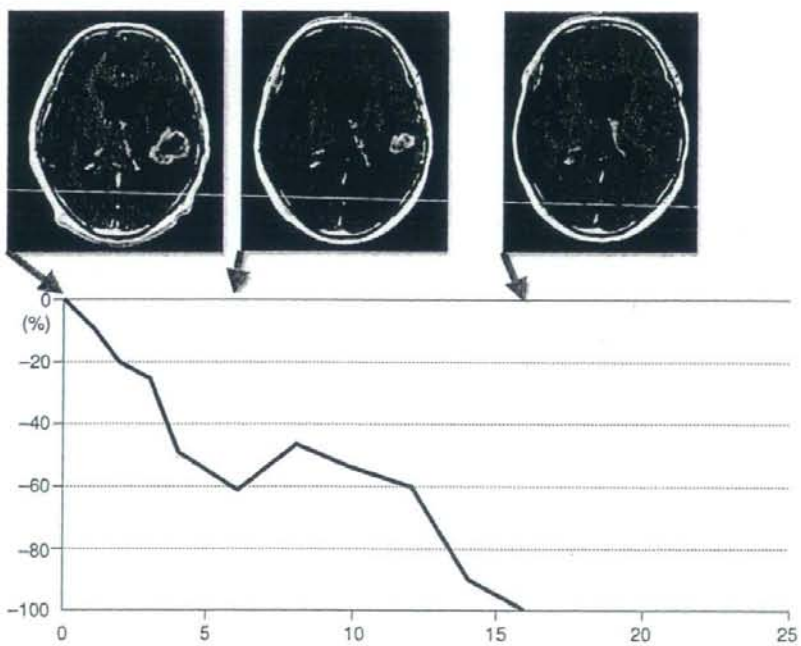

図3　ゆっくりと腫瘍が縮小し16コース後にCRとなった症例
横軸は治療コース数，縦軸は腫瘍縮小割合（%）．

II. 二次癌

temozolomideの長期投与において有害事象の有意の蓄積がみられないことは上記の通りであるが，実は問題となるのは二次癌である．これまでに報告された二次癌の一覧は表6の通りである．従来のニトロソウレア製剤によるmyelodysplasia/AMLよりも薬剤投与からの経過が短い傾向が指摘されているが，今後の症例の蓄積と解析が必要である[4-7]．

謝辞

治験データの解析はシェリング・プラウ株式会社臨床開発第二部辻文人，池田菜穂子両氏に多大なる御指導を頂いた．本論文の前半部分は第25回日本脳腫瘍学会（東京，2007年）において発表した．

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Acta Neuropathol (2006) 111: 483–488
DOI 10.1007/s00401-006-0063-y

ORIGINAL PAPER

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Increased expression of podoplanin in malignant astrocytic tumors as a novel molecular marker of malignant progression

Received: 30 January 2006 / Revised: 22 February 2006 / Accepted: 6 March 2006 / Published online: 5 April 2006

Abstract Podoplanin (aggrus) is a mucin-like transmembrane sialoglycoprotein that is expressed on lymphatic endothelial cells. Podoplanin is putatively involved in cancer cell migration, invasion, metastasis, and malignant progression and may be involved in platelet aggregation. Previously, we showed upregulated expression of podoplanin in central nervous system (CNS) germinomas, but not in non-germinomatous germ cell tumors, except for parts of immature teratomas in limited numbers. However, little information exists about its role in CNS astrocytic tumors. In this study, 188 astrocytic tumors (30 diffuse astrocytomas, 43 anaplastic astrocytomas, and 115 glioblastomas) were investigated using immunohistochemistry with an anti-podoplanin antibody, YM-1. In 11 of 43 anaplastic astrocytomas (25.6%) and in 54 of 115 glioblastomas (47.0%), podoplanin was expressed on the surface of anaplastic astrocytoma cells and glioblastoma cells, especially around necrotic areas and proliferating endothelial cells. However, the surrounding brain parenchyma was not stained by YM-1. On the other hand, podoplanin expression was not observed in diffuse astrocytoma (0/30: 0%). Furthermore, we investigated the expression of podoplanin using quantitative real-time PCR and Western blot analysis in 54 frozen astrocytic tumors (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). Podoplanin mRNA and protein expression were markedly higher in glioblastomas than in anaplastic astrocytomas. These data suggest that podoplanin expression might be associated with malignancy of astrocytic tumors.

Keywords Podoplanin · Astrocytoma · Glioblastoma · YM-1

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Introduction

Astrocytic tumors are the most common tumors of the central nervous system (CNS) and are categorized into diffuse astrocytomas (World Health Organization (WHO) Grade II), anaplastic astrocytomas (AA; WHO Grade III) and glioblastomas (GBM; WHO Grade IV) [11]. Among them, GBMs are the most frequent and most malignant type of astrocytic tumor. Despite advances in surgical techniques, radiation therapy, and adjuvant chemotherapy, their prognosis remains poor: the median survival time for patients with GBMs is only 1 year [2]. Glioblastoma may occur de novo or may result from progression of low-grade astrocytomas [4]. Molecular mechanisms of tumorigenesis and malignant progression are associated with the inactivation of tumor suppressor genes such as p53-Rb pathway or the overexpression of oncogenes such as epidermal growth factor receptor [10]. However, the mechanisms of tumorigenesis and progression of astrocytic tumors have not been resolved. Identification of genes that are expressed differentially in high-grade astrocytomas, low-grade tumors, or normal brain tissues is important to elucidate the molecular mechanisms of tumorigenesis and to develop novel therapeutic strategies.

Podoplanin was reported to be expressed in lymphatic endothelium and in tumor-associated lymphangiogenesis; also, podoplanin deficiency resulted in congenital lymphedema and impaired lymphatic vascular patterning [16]. Furthermore, expression of podoplanin has been

shown to be upregulated in skin squamous cell carcinoma [12], lung squamous cell carcinoma [9], malignant mesothelioma [14], Kaposi's sarcoma, angiosarcoma [1], hemangioblastoma [15], testicular seminoma [7, 8], and dysgerminoma [17].

We have recently shown that podoplanin is overexpressed in CNS germinomas, but not in non-germinomatous germ cell tumors, except in a limited number of immature teratomas with partial positive reactivity [13]. In adult non-neoplastic CNS, podoplanin was evident in the subependymal areas, the leptomeninges, choroid plexus, ependyma, and Purkinje cells [15, 17]. However, podoplanin expression in CNS astrocytic tumors has not been studied intensively. In this study, we investigated podoplanin expression in 188 astrocytic tumors.

Materials and methods

Tissue samples

Tumor specimens were obtained during surgery from eight patients with diffuse astrocytomas, 14 patients with anaplastic astrocytomas, and 34 patients with glioblastomas. Informed consent had been obtained previously from patients or their guardians. The tumor specimens were routinely fixed in 10% buffered formalin for 18–20 h at room temperature and processed to paraffin. Sections (5 μm thick) were cut and attached to poly-L-lysine-coated glass slides. Hematoxylin-eosin was used for routine staining. Tissue microarrays of 132 astrocytic tumors (22 diffuse astrocytomas, 29 anaplastic astrocytomas, and 81 glioblastomas) were purchased from Cybrdi, Inc. (Frederick, MD). The histology of these tissue samples was confirmed by experienced neuropathologists.

Immunohistochemical analysis

Specimens were deparaffinized, rehydrated, and incubated first with YM-1 (1/100 diluted; Medical Biological Laboratories Co., Ltd, Nagoya, Japan) at room temperature for 1 h, then with biotin-conjugated secondary anti-rat IgG antibody (DakoCytomation, Glostrup, Denmark) for 1 h, and finally with peroxidase-conjugated biotin–streptavidin complex (Vectastain ABC Kit; Vector Laboratories, Inc., Burlingame, CA) for 1 h. Color was developed using 3,3-diaminobenzidine tetrahydrochloride tablet sets (DakoCytomation) for 3 min. Podoplanin expression was assessed semi-quantitatively from the percentage of tumor cells with cytoplasmic/membrane staining: 0, no staining; +, $<10\%$; ++, 10–50%; and +++, $>50\%$.

Western blot analysis

Tissues were solubilized with lysis buffer (25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% Na deoxycholate, 2% nonidet P-40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, and 50 mg/ml aprotinin). They were then electrophoresed under reducing conditions on 10–20% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd, Tokyo, Japan). The separated proteins were transferred to a nitrocellulose membrane. After blocking with 4% skim milk in PBS, the membrane was incubated with YM-1 (1/500 diluted) or anti-β-actin antibody (1 μg/ml; Sigma Chemical Co., St. Louis, MO), and then with peroxidase-conjugated secondary antibodies (1/1,000 diluted; Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). The proteins were subsequently developed for 3 min using ECL reagents (Amersham Pharmacia Biotech) using X-Omat AR film (Eastman Kodak Co.).

Quantitative real-time PCR

Total RNAs were prepared from frozen sections that have been obtained from astrocytic tumor patients, employing an RNeasy mini prep kit (Qiagen, Inc., Hilden, Germany). The initial cDNA strand was synthesized using SuperScript III transcriptase (Invitrogen Co., Carlsbad, CA) by priming nine random oligomers and an oligo-dT primer according to the manufacturer's instructions. We performed PCR using oligonucleotides: human podoplanin sense (5′-GGAAGGTGTCAGCT CTGCTC-3′) and human podoplanin antisense (5′-CG CCTTCCAAACCTGTAGTC-3′). Real-time PCR was carried out using the QuantiTect SYBR Green PCR (Qiagen, Inc.). The PCR conditions were 95°C for 15 min (1 cycle), followed by 40 cycles of 94°C for 15 s, 53°C for 20 s, 72°C for 10 s. Subsequently, a melting curve program was applied with continuous fluorescence measurement. A standard curve for podoplanin templates was generated through serial dilution of PCR products (1×10^8–1×10^2 copies/μl). The expression level of podoplanin was normalized by total RNA weights. The statistical significance of podoplanin mRNA expression in astrocytic tumor tissues was determined using paired *t* tests.

Results

Immunohistochemical staining for podoplanin in malignant astrocytic tumors

The cellular distribution of podoplanin in astrocytic tumors was examined immunohistochemically using anti-podoplanin antibody, YM-1, which can strongly recognize podoplanin [6, 13]. In this study, we used 56 surgical tissues (8 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). Podoplanin immunoreactivity was detected in 5 of 14 (35.7%) anaplastic astrocytomas and in 18 of 34 (52.9%) glioblastomas; staining was graded as +++ in 16 glioblastomas and as ++ in two glioblastoma cases. We also stained other astrocytic tumors of tissue microarrays. Podoplanin was detected in 6 of 29 (21%) anaplastic astrocytomas and in 36 of 81 (44%) glioblastomas. In all, 11 of 43 anaplastic

astrocytomas (25.6%) and 54 of 115 (47.0%) glioblastomas were stained using YM-1 (χ^2, $P<0.05$; Table 1). Representative staining for podoplanin in glioblastoma samples is shown in Fig. 1. Immunostaining for podoplanin demonstrated predominantly cell surface patterns in glioblastoma cells (Fig. 1). In anaplastic astrocytoma, the tumor cell surface was stained using YM-1 (Fig. 1c, d). In glioblastomas, podoplanin-positive tumor cells were prominent around microvascular proliferations (Fig. 1e, f) and necrotic tissues (Fig. 1g). Proliferating endothelial cells were negative for podoplanin (Fig. 1e, f). Podoplanin was detected strongly in the plasma membrane of highly anaplastic multinucleated giant cells (Fig. 1h). In non-neoplastic areas of the brain (Fig. 1a) and in diffuse astrocytoma (Fig. 1b), podoplanin immunostaining was absent.

Podoplanin expression in malignant astrocytic tumors using Western blot analysis

To confirm immunohistochemical findings from astrocytic tumors, lysates of frozen tumor specimens from 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas) were analyzed using Western blotting. As shown in Fig. 2, an antibody to podoplanin, YM-1, detected about 36-kDa proteins in extracts of malignant astrocytomas. Using YM-1, 6 of 14 anaplastic astrocytomas (42.8%) and 22 of 34 glioblastomas (64.7%) showed strong labeling (χ^2, $P<0.05$), while all diffuse astrocytomas were negative. Podoplanin expression detected by Western blot analysis was closely correlated with the results of immunohistochemistry.

Differential expression of podoplanin mRNA in astrocytic tumors

To quantify the expression of podoplanin mRNA in human astrocytic tumors of different grades, we performed quantitative real-time PCR analyses of astrocytic tumors from 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas). The relative podoplanin mRNA expression levels of each tumor grade are shown in Fig. 3. Average copies of podoplanin mRNA/μg total RNA in diffuse astrocytomas (Grade II), anaplastic astrocytomas (Grade III), and glioblastomas (Grade IV) were 21.7±30.6, 16.1±35.4, and 411.2±511.7, respectively. Podoplanin transcript levels were significantly higher in glioblastomas than those in diffuse astrocytomas, anaplastic astrocytomas, or non-neoplastic human brain tissues ($P<0.01$).

Discussion

Immunohistochemical, Western blot, and real-time PCR analyses demonstrate that the expressions of podoplanin mRNA and protein are correlated with the malignant progression from anaplastic astrocytoma (Grade III) to glioblastomas (Grade IV). Of particular interest, 47.0% of highly invasive glioblastomas express podoplanin, whereas 25.6% of anaplastic astrocytomas and 0% of the less invasive diffuse astrocytomas (Grade II) express podoplanin by immunohistochemical staining on surgically resected and microarray tissues (Table 1). On the other hand, 6 of 14 anaplastic astrocytomas (42.8%) and 22 of 34 glioblastomas (64.7%) were strongly labeled by Western blot analyses using YM-1 (Fig. 2). Of all 188 astrocytic tumors analyzed immunohistochemically, 132 cases were derived from tissue microarrays whose tissue spots are small and, therefore, the percentage of podoplanin-positive tumors might have been underestimated. Furthermore, YM-1 detected podoplanin strongly by Western blot analysis, as described previously [6]. For these reasons, podoplanin-positive ratios in immunohistochemical analysis are inferred to be smaller than those of Western blot analysis, although the expression of podoplanin detected by immunohistochemistry was closely correlated with that by Western blot or real-time PCR analyses. The distribution of podoplanin-positive tumor cells was prominent around necrotic tissue and proliferating endothelial cells in glioblastomas (Fig. 1). Normal brain tissue surrounding the tumor bed was negative for podoplanin. Therefore, podoplanin expression was correlated with high tumor grades and aggressive histological behavior.

The biological functions of podoplanin remain largely unknown. In vascular endothelial cells, overexpression of T1α/podoplanin induces elongated cell extensions and considerably increases cell adhesion, migration, and tube

Table 1 Results of podoplanin immunostaining in 188 patients with astrocytic tumors

Tumor type	No. of cases	Podoplanin immunostaining				Positive rate (%)
		+++	++	+	−	
Diffuse astrocytoma	30	0	0	0	30	0
Surgical resection samples	8	0	0	0	8	0
Tissue microarray	22	0	0	0	22	0
Anaplastic astrocytoma	43	6	3	2	32	25.6
Surgical resection samples	14	3	1	1	9	35.7
Tissue microarray	29	3	2	1	23	20.7
Glioblastoma	115	39	10	5	61	47
Surgical resection samples	34	16	2	0	16	52.9
Tissue microarray	81	23	8	5	45	44

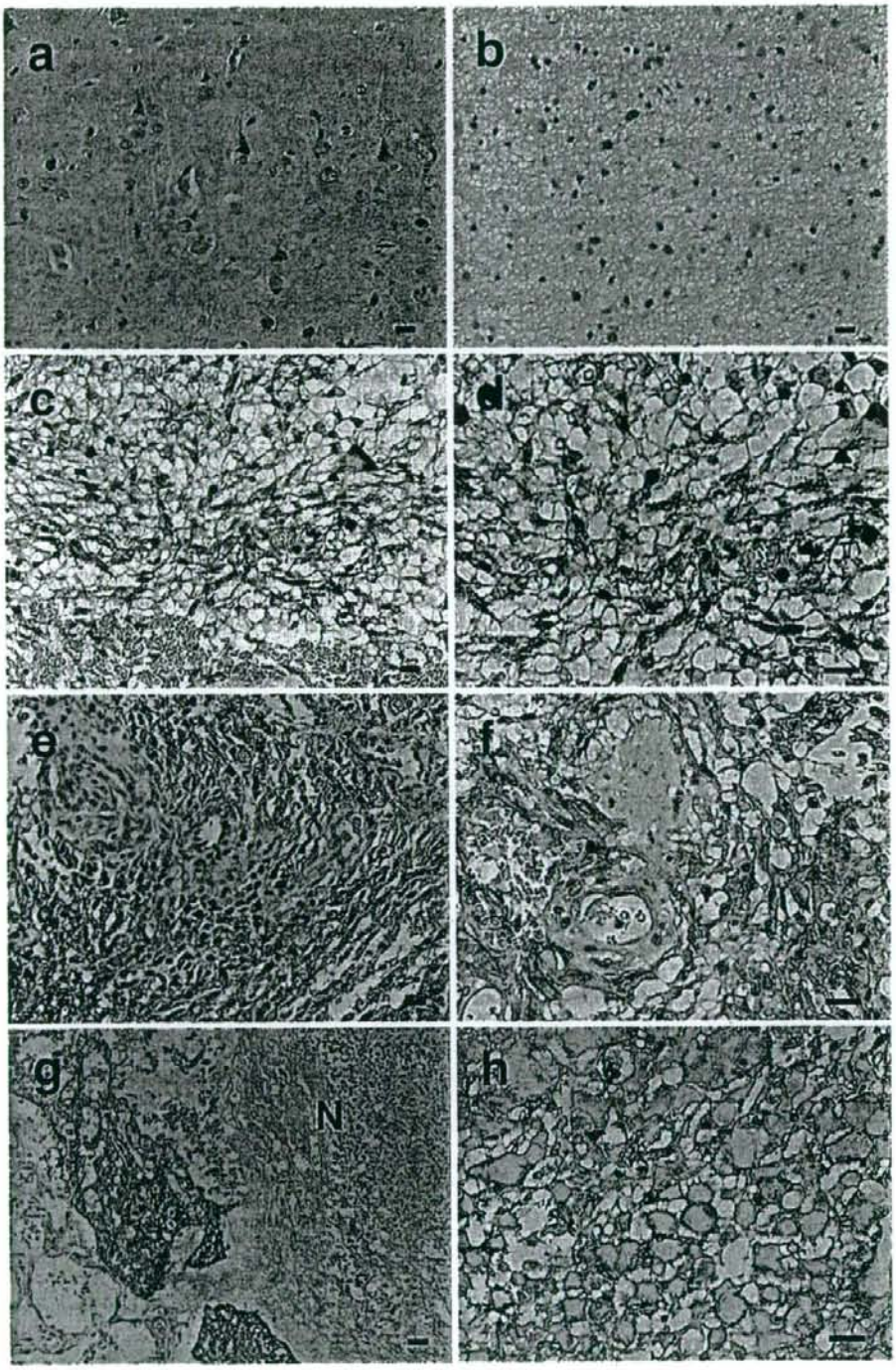

Fig. 1 Immunohistochemical detection of podoplanin in astrocytic tumors. No staining is apparent in a normal brain (**a**, ×200) and in diffuse astrocytoma (**b**, ×200). In anaplastic astrocytoma, the tumor cell surface was stained positively (**c**, ×200; **d**, ×400). Accentuated staining is visible around an area of microvascular proliferation in glioblastoma (**e**, ×200; **f**, ×400). Podoplanin immunostaining of glioblastoma cells at the necrotic area (*N*) (**g**, ×200) and in the plasma membrane of highly anaplastic multinucleated giant cells (**h**, ×400). *Bar* = 10 μm

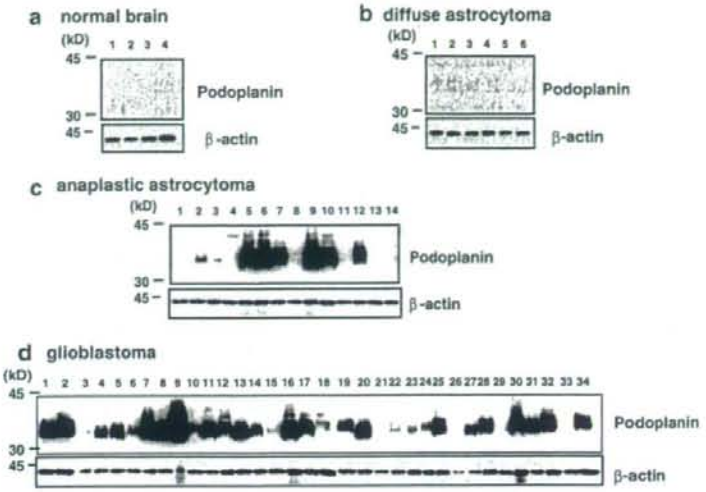

Fig. 2 Western blot analyses of podoplanin expression in astrocytic tumors. Tissues from normal brain (**a**), diffuse astrocytomas (**b**), anaplastic astrocytomas (**c**), and glioblastomas (**d**) were solubilized and immunoblotted using anti-human podoplanin monoclonal antibody YM-1 (*upper panel*) or anti-β-actin antibody (*lower panel*)

formation by promoting the rearrangement of the actin cytoskeleton [16]. PA2.26/podoplanin was identified as a cell surface protein induced in epidermal carcinogenesis and skin remodeling [18, 19]. Expression of PA2.26/podoplanin in pre-malignant keratinocytes induces a fully transformed and metastatic phenotype. Furthermore, human PA2.26/podoplanin has been found in the invasive front of oral squamous cell carcinomas, consistent with a role in tumor cell migration and invasion [12]. Moreover, a monoclonal antibody against gp44/aggrus/podoplanin inhibits pulmonary metastasis of a highly metastatic clone of mouse colon adenocarcinoma in vivo [21, 22]. In this study, we showed upregulated expression of podoplanin in CNS malignant astrocytic tumors. Recently, Shibahara et al. [20] also reported podoplanin expression in subsets of CNS tumors. However, the results obtained so far showed only associations between podoplanin expression and malignancy of astrocytic tumors, while its direct biological function in malignant astrocytomas remains to be established.

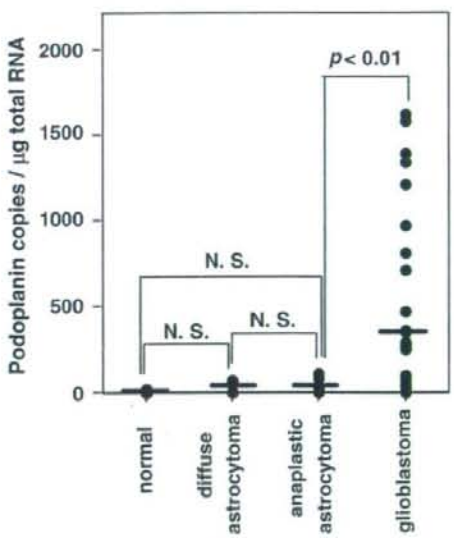

Fig. 3 Quantitative real-time PCR analysis of podoplanin transcripts in astrocytic tumors. First-strand cDNA samples derived from astrocytic tumor tissues of 54 patients (6 diffuse astrocytomas, 14 anaplastic astrocytomas, and 34 glioblastomas) and four normal brain tissues were used as real-time PCR templates. The respective expression levels of podoplanin were normalized to μg or total RNA, as described in Materials and methods

PA2.26/podoplanin was co-localized with ezrin, radixin, moesin family proteins, which are concentrated in cell surface projections, where they link the actin cytoskeleton to plasma membrane proteins [18]. Consistent with the association of podoplanin with ezrin, the latter's immunoreactivity is also associated with increasing malignancy of astrocytic tumors [3, 23]. The combination of podoplanin and ezrin might thus represent a possible tool for grading of astrocytic tumors.

Platelets play an important role in hemostasis and thrombosis and are also involved in tissue repair and tumor metastasis [5]. Glioblastoma is differentiated from low-grade astrocytomas based on the histological presence of tumor necrosis and associated microvascular proliferation [11]. Large necroses are attributable to insufficient blood supply and thrombosed tumor vessels are often observed. We speculate that the local platelet aggregation and thrombus formation might be increased by podoplanin-expressing malignant astrocytic tumor cells, resulting in tumor vessel obstruction and subsequent necrosis. Indeed, our unpublished results suggest that podoplanin expressed by glioblastoma cells induces platelet aggregation in vitro (data not shown).

In conclusion, podoplanin expression was markedly higher in glioblastomas than in anaplastic astrocytomas. Furthermore, podoplanin expression was not observed in diffuse astrocytoma. It will be intriguing to investigate the functional basis of the association between podoplanin expression and malignant progression of astrocytomas.

Acknowledgments This study was supported in part by Kanae Foundation for Life and Socio-medical Science (to Y.K.) and by Osaka Cancer Research Foundation (to Y.K.). We thank Drs Fujita and Tsuruo (University of Tokyo) for their great help, and Ms Kunita, Mr Nakazawa (University of Tokyo), Ms Totake, and Ms Kobo (Saitama Medical School) for their kind assistance. We thank Dr Sugiyama (Hiroshima University) for providing us clinical samples.

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Int. J. Cancer: **119**, 1491–1494 (2006)

SHORT REPORT

Frequent *EGFR* mutations in brain metastases of lung adenocarcinoma

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Lung adenocarcinomas often metastasize to the brain, and the prognosis of patients with brain metastases is still very poor. The *epidermal growth factor receptor* (*EGFR*) gene is mutated in a considerable fraction of primary lung adenocarcinomas, in particular those with drastic response to EGFR tyrosine kinase inhibitors. The present study was designed to elucidate the prevalence of *EGFR* mutations in brain metastases and the timing of their occurrence during cancer progression. *EGFR* mutations were detected in 12 of 19 metastatic lung adenocarcinomas to the brain (63%). This frequency was higher than those in previous studies for *EGFR* mutations at various stages of lung adenocarcinoma in East Asia, including Japan (*i.e.*, 20–55%). In 6 cases with *EGFR* mutations, the corresponding primary lung tumors were also examined for the mutations, and in all of them, the same types of *EGFR* mutations were detected also in the primary tumors. In 2 of them, second metastatic brain tumors in addition to the first ones were also available for analysis, and the same types of *EGFR* mutations were detected in both the first and second ones in both cases. These results indicate that *EGFR* mutations are present frequently in brain metastases and occur preceding brain metastasis. These findings will be highly informative for treatment of metastatic lung adenocarcinoma to the brain.

Key words: *EGFR* mutation; metastatic brain tumor; lung adenocarcinoma

Epidermal growth factor receptor (EGFR) is a member of a family comprised of 4 homologous receptors, EGFR (ERBB1), HER-2/neu (ERBB2), HER-3 (ERBB3) and HER-4 (ERBB4). Ligand binding to EGFR leads to receptor tyrosine kinase (TK) activation and a series of downstream signaling activation that mediates proliferation, migration, invasion and suppression of apoptosis.[1] Recently, it was revealed that most lung adenocarcinoma patients who were responsive to gefitinib, an EGFR TK inhibitor, had somatic mutations in the kinase domain of the *EGFR* gene in their tumor cells.[2,3] Subsequently, it was reported that *EGFR* mutations are present in a considerable fraction of lung adenocarcinoma and occur more frequently in East Asian patients, including Japanese, than in Caucasian patients.[4–10] Furthermore, the incidence of *EGFR* mutations was significantly high in female patients and patients without smoking histories. In our previous study, *EGFR* mutations were detected frequently in noninvasive bronchioloalveolar carcinomas, suggesting that *EGFR* mutations occur early in the development of adenocarcinoma, and those with the mutations further progress to invasive and metastatic carcinomas.[11] However, to our knowledge, there is no report showing the prevalence of *EGFR* mutations in metastatic lung adenocarcinomas, and thus, it remains unclear whether *EGFR* mutations are indeed retained in metastatic lung adenocarcinoma or not. Elucidation of this issue will be implicative for treatment with EGFR TK inhibitors against advanced lung adenocarcinomas, which often metastasize systemically to diverse sites, such as brain, bone, adrenal glands and liver.[12] Therefore, we examined metastatic lung adenocarcinomas to the brain for *EGFR* mutations and compared the mutation status in the metastatic brain tumors with the corresponding primary tumors, if they were available for the analysis. We also examined these tumors for *KRAS* mutations, which have been reported as being mutually exclusive for *EGFR* mutations.[4,7]

A total of 21 metastatic brain tumor tissues were obtained from 19 patients who were treated during the period from 1986 to 2001 at the National Cancer Center Hospital, Tokyo, Japan. These tumor tissues were obtained at surgery or at autopsy. In 2 of the 19 cases, the second brain surgery was performed against the second recurrence in the brain 15 months and 24 months after the first brain surgery, respectively, and thus brain tumor tissues were obtained twice during their clinical courses. In 8 of the 19 cases, the corresponding primary lung tumors were obtained at lung surgery preceded by brain surgery. None of the patients were treated with gefitinib through all clinical courses. In 16 of the 19 cases, primary and metastatic tumors were macrodissected and were subjected to genomic DNA extraction by the method described previously.[13] In the remaining 3 cases, from which 3 primary tumors and 5 metastatic tumors were obtained, cancer cells were microdissected using the Pixcell Laser Capture Microdissection system (Arcturus Engineering, Mountain View, CA). Their genomic DNAs were isolated by SDS/proteinase K digestion and phenol/chloroform extraction as described previously.[14] Exons 18–21 of the *EGFR* gene and exons 1 and 2 of the *KRAS* gene were examined for mutations by genomic PCR amplification and direct sequencing of PCR products. PCR primer sequences and PCR conditions are described previously.[11]

Table I shows the result of all cases examined. In 12 of the 19 cases (63%), *EGFR* mutations were detected in their metastatic brain tumors. In 2 cases (cases 2 and 3), for which both the first and second metastatic brain tumors were available for the analysis, *EGFR* mutations were detected in both the metastatic tumors, and the type of mutation in the second metastatic tumor was the same as that in the first one in both cases. In 8 cases, for which primary lung tumors were also available for the analysis, 6 cases had *EGFR* mutations in their primary tumors. All metastatic tumors from the 6 cases with *EGFR* mutations in the primary tumors had the same types of mutations as those in the primary tumors, respectively. Figure 1 shows representative sequence chromatograms of case 1, for which cancer cells of primary and metastatic tumors were collected by microdissection. There was no case that *EGFR* mutations were not detected in primary tumors and were detected in the corresponding metastatic brain tumors. Additionally, there was also no case that *EGFR* mutations were detected only in primary tumors and were not detected in the corresponding metastatic brain tumors. Thus, it was shown that *EGFR* mutations in primary adenocarcinomas are retained in their metastatic brain tumors.

The first two authors contributed equally to this work.

Grant sponsors: Ministry of Health, Labor and Welfare; National Institute of Biomedical Innovation (NiBio).

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Received 21 October 2005; Accepted 7 February 2006
DOI 10.1002/ijc.21940
Published online 26 April 2006 in Wiley InterScience (www.interscience.wiley.com).

TABLE I – *EGFR* AND *KRAS* MUTATIONS IN LUNG ADENOCARCINOMA WITH BRAN METASTASIS

Case		Clinical characteristics					Mutation		
No.	Tumor	Age	Gender	Smoking	Stage[1]	Interval time (months)[2]	Gene	Exon	Amino acid change
1	P, M	48	F	–	IIIA	26	*EGFR*	19	E746-A750 del
2	P, M1, M2	43	F	–	IIA	M1, 18; M2, 33	*EGFR*	19	E746-E749 del, AT750-751VA
3	P, M1, M2	59	M	+	IIB	M1, 40; M2, 64	*EGFR*	19	L747-T751 del
4	P, M	49	F	–	IIIB	0.5	*EGFR*	19	E746-A750 del
5	P, M	54	M	+	IIIB	6	*EGFR*	19	E746-A750 del
6	P, M	70	M	+	IIIA	14	*EGFR*	21	L858R
7	P, M	64	M	+	IB	16			
8	P, M	58	M	+	IIIA	22			
9	M	56	M	+			*EGFR*	19	E746-A750 del
10	M	51	F	–			*EGFR*	19	E746-A750 del
11	M	56	F	–			*EGFR*	19	E746-A750 del
12	M	49	M	+			*EGFR*	19	E746-A750 del, T751A
13	M	59	M	+			*EGFR*	19	E746-T751del, SP752-753IS
14	M	48	F	–			*EGFR*	21	L858R
15	M	53	M	+			*KRAS*	1	G12C
16	M	59	M	+			*KRAS*	1	G12C
17	M	62	M	+					
18	M	74	M	+					
19	M	67	M	+					

P, primary tumor; M, metastatic brain tumor; M1, first metastatic brain tumor; M2, second metastatic brain tumor.
[1]Pathological stage according to the TNM classification at the time of lung surgery for the primary tumor.–[2]Interval time from lung surgery to brain surgery.

Types of *EGFR* mutations detected in the present study were 10 in-frame deletions (83%) in exon 19 and 2 point mutations (17%) in exon 21. The most frequent mutation was a simple deletion of 5 amino acid residues from codon 746 to 750 (6/12, 50%). Both of the 2 point mutations were the leucine to arginine mutation at codon 858 (L858R). These 2 types of mutation are known to be the most common ones in lung cancer, especially in lung adenocarcioma. The remaining types of *EGFR* mutations detected were a simple deletion of 5 amino acid residues from codon 747 to 751 and 3 deletions coupled with 1 or 2 amino acid substitutions. No mutation was detected in exons 18 and 20 in the present study, although several point mutations and in-frame insertions have been identified in those exons in primary lung adenocarcinomas in previous studies.[4,7]

KRAS mutations were detected in 2 of 7 metastatic brain tumors without *EGFR* mutations. Both of the 2 mutations were the glycine to cysteine mutation at codon 12 (G12C). We also analyzed the association of *EGFR* mutations with clinicopathological characteristics, such as age, gender and smoking history (Table I). All female patients, who were never-smokers, had *EGFR* mutations in their tumors and the mutations were significantly more frequent in female patients (6/6, 100%) than in male patients (6/13, 46%) (Fisher's exact test, $p = 0.0436$). Therefore, the mutual exclusiveness of *EGFR* and *KRAS* mutations as well as frequent mutations in female nonsmokers was consistent with previous findings.[4,6–8,10]

We demonstrated here that *EGFR* mutations were frequently present in metastatic brain tumors of lung adenocarcinoma. In previous studies for *EGFR* mutations in various stages of lung adenocarcinomas in East Asia, the frequency of the mutations were 20–55%.[4,5,8,15] The higher incidence of *EGFR* mutations in our study raises a possibility that the mutations may be associated with metastasis of lung adenocarcinoma. In recent studies, it was suggested that *EGFR* mutations occur early in the development of lung adenocarcinoma.[11,16–18] Yoshida *et al.*[17] showed that *EGFR* mutations were present in 3% of atypical adenomatous hyperplasia (AAH), which is considered to be a precursor lesion of lung adenocarcinoma, and the presence of the mutations was increasingly frequent during sequential progression from AAH to invasive adenocarcinoma through bronchioloalveolar carcinoma (BAC). Our previous study also demonstrated that a majority of BACs had *EGFR* mutations.[11] Moreover, Tang *et al.*[18] reported that *EGFR* mutations identical to those in tumors were present in the histologically normal respiratory epithelium in 9 of 21 patients with lung adenocarcinoma carrying *EGFR* mutations in the tumors. In the present study, we also showed that all 6 cases with *EGFR* mutations in their metastatic brain tumors had the identical mutations in the corresponding primary tumors. Thus, *EGFR* mutations are likely to be an early genetic alteration in multistage carcinogenic processes of lung adenocarcinoma, and additional genetic alterations responsible for brain metastasis may occur in cancer cells with *EGFR* mutations. Indeed, our previous study, using the same samples as those from 16 of the 19 cases, showed the sequential accumulation of allelic losses during tumor progression. In particular, in case 6, which has *EGFR* mutations in both the primary and metastatic tumors, loss of heterozygosity (LOH) on chromosomes 2q, 13q and 18q was shown to accumulate during tumor progression.[19] In the present study, we showed that *EGFR* mutations, which had been present in the primary tumors at stages IIB-IIIB, were all retained in their brain metastases. Although *EGFR* mutations may be associated with the genesis and/or early progression of lung adenocarcinoma, our results showing the retention and frequent presence of the mutations in metastatic brain tumors indicate that lung adenocarcinomas with *EGFR* mutations may also have a higher potential of metastasizing to the brain. For this reason, it should be noted that *EGFR* gene alterations occur frequently in gliomas, a common brain tumor.[20,21] However, since there is no information on the prevalence of *EGFR* mutations in metastatic lung adenocarcinomas to sites other than the brain at present, further studies will be needed to clarify the association of *EGFR* mutations with metastatic sites of lung adenocarcinoma.

The brain is one of the most frequent metastatic sites of lung adenocarcinoma. Since traditional chemotherapy is not so effective against metastatic brain tumors of lung adenocarcinoma, radiotherapy is, to date, the main treatment for patients with them. Nevertheless, the prognosis of those patients is poor and median survival is only 3–6 months.[22] Interestingly, recent reports demonstrated that metastatic brain tumors of lung adenocarcinoma frequently and drastically responded to gefitinib, an EGFR TK inhibitor,[23–25] although *EGFR* mutations were not examined in these cases. Those reports could have an impact on treatment for metastatic brain tumors of lung adenocarcinoma; however, the role of gefitinib in therapeutic strategies against metastatic lung adenocarcinoma has not yet been established. This is the first report, to our knowledge, to analyze a considerable number of metastatic brain tumors of lung adenocarcinoma for *EGFR* mutations and to demonstrate the frequent presence of them. These results will provide us with a rationale for the use of this drug for treatment against metastatic brain tumors of lung adenocarcinoma.

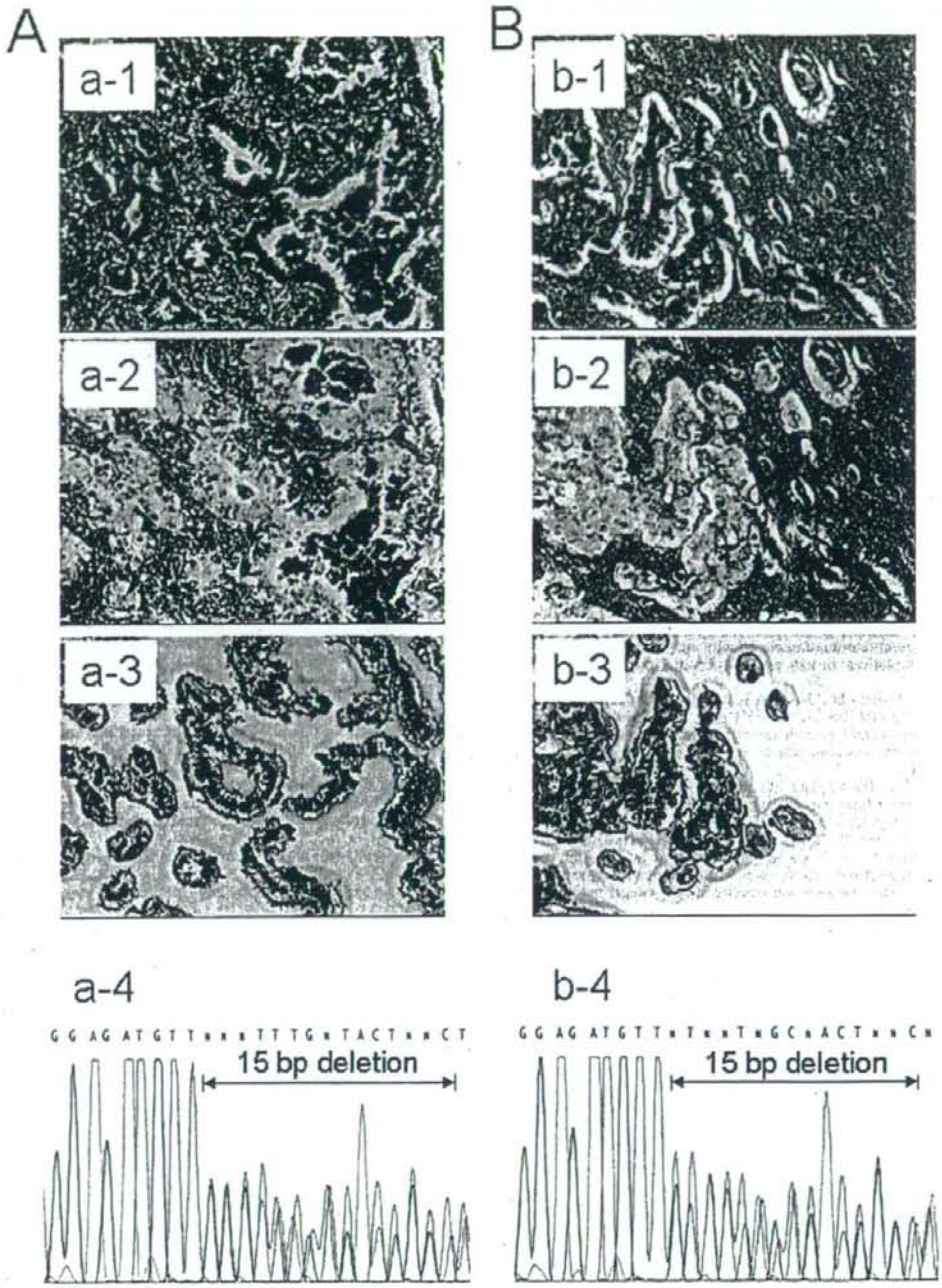

FIGURE 1 – Representative photographs of laser capture microdissection and sequence data obtained from a primary lung adenocarcinoma (*a*) and a corresponding metastatic brain tumor (*b*) in the same patient (case 1 in Table I; magnification of photographs, ×100). (*a-1*) and (*b-1*) shows the tumors in hematoxylin-stained tissue sections before microdissection. (*a-2*) and (*b-2*) shows the same sections after microdissection. (*a-3*) and (*b-3*) shows the cells captured on the transfer films. The sequence chromatogram from the primary tumor is shown in (*a-4*), and that from the metastatic brain tumor is shown in (*b-4*). Both show sequence chromatograms in exon 19 using antisense sequencing primer. A heterozygous in-frame 15 bp deletion was detected from both the samples, demonstrating a deletion of 5 amino acid residues from codon 746 to 750.

Acknowledgements

T.K. was the recipient of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan during the study. This work was supported by the Ministry of Health, Labor and Welfare for the 3rd-term Comprehensive 10-year Strategy for Cancer Control, for Cancer Research (16S-1), and the program for promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio).

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JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 102, No. 1, 46–52. 2006
DOI: 10.1263/jbb.102.46

Classification of Intramural Metastases and Lymph Node Metastases of Esophageal Cancer from Gene Expression Based on Boosting and Projective Adaptive Resonance Theory

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Received 22 February 2006/Accepted 15 April 2006

Esophageal cancer is a well-known cancer with poorer prognosis than other cancers. An optimal and individualized treatment protocol based on accurate diagnosis is urgently needed to improve the treatment of cancer patients. For this purpose, it is important to develop a sophisticated algorithm that can manage a large amount of data, such as gene expression data from DNA microarrays, for optimal and individualized diagnosis. Marker gene selection is essential in the analysis of gene expression data. We have already developed a combination method of the use of the projective adaptive resonance theory and that of a boosted fuzzy classifier with the SWEEP operator denoted PART-BFCS. This method is superior to other methods, and has four features, namely fast calculation, accurate prediction, reliable prediction, and rule extraction. In this study, we applied this method to analyze microarray data obtained from esophageal cancer patients. A combination method of PART-BFCS and the U-test was also investigated. It was necessary to use a specific type of BFCS, namely, BFCS-1,2, because the esophageal cancer data were very complexity. PART-BFCS and PART-BFCS with the U-test models showed higher performances than two conventional methods, namely, *k*-nearest neighbor (kNN) and weighted voting (WV). The genes including *CDK6* could be found by our methods and excellent IF-THEN rules could be extracted. The genes selected in this study have a high potential as new diagnosis markers for esophageal cancer. These results indicate that the new methods can be used in marker gene selection for the diagnosis of cancer patients.

[**Key words:** cancer classification, boosting, projective adaptive resonance theory, esophageal cancer, intramural metastases]

Cancer is a major cause of human deaths in the many countries. Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer-related mortality in the world (1). This cancer is a well-known cancer with poorer prognosis than other cancers. Lymph node metastasis is one of the reasons for its poor prognosis in potentially resectable solid epithelial tumors. Furthermore, intramural metastasis (skip metastasis) has poorer prognosis than lymph node metastasis (2). From such situations, the prognosis of cancer patients with the same clinical diagnosis can differ, frequently. Therefore, it is important that the prognosis of cancer patients is made accurately and that an adequate treatment is proposed. However, the diagnosis of cancer patients is determined by a complex causality involving multiple factors because the mechanisms of cancer development (or malignancy) are extremely complex. Gene expression data from DNA microarrays are individualized and useful in the diagnosis and prognosis of diseases (3). To conduct this analysis, it is necessary to select genes significantly expressing mRNA and strongly related to the diagnosis or prognosis of disease, because the performance of classification analysis can decline owing to such large quantities of data.

Feature selection has been performed to screen candidate genes for modeling. There are two types of approach: the wrapper and filter approaches. In the former, features (genes) are selected as a part of mining algorithms, such as support vector machines (SVMs) (4), a fuzzy neural networks (FNNs) combined with the SWEEP operator method (FNN-SWEEP) (3), and a boosted fuzzy classifier with the SWEEP operator method (BFCS) (5, 6). On the other hand, in the filter approach, features are selected by filtering methods, such as the U-test, the t-test, signal-to-noise statistic (S2N)

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