# I 疫学からみた脳腫瘍の実態

脳腫瘍の診断・治療を行う際に，対象とする疾患の発生状況や自然経過などはきわめて重要な情報であり，実際の診療はその情報をもとに行われているといっても過言ではない．疫学とは，どの程度その疾患が蔓延しているかを調べ，疾病の原因や要因を見出すとともに，自然経過と予後を調べる学問であり，まさしく診療の基礎となり得る情報をもたらす学問といえる．脳腫瘍の発生原因についてはいろいろな研究がなされているが，現在までのところ，いくつかの遺伝性疾患以外では明確な要因が判明しているものはない．

## A 脳腫瘍の発生要因

### 1 遺伝性疾患

遺伝子の変化が原因となって発症する脳腫瘍があるが，下記の疾患では，原因となる遺伝子がどの染色体上にあるかについても解明されている．

#### 1）神経線維腫症

神経線維腫症（neurofibromatosis）type 1（NF 1）は，常染色体優生遺伝の疾患で，3000～4000人に1人の割合で発生するとされている．末梢の神経線維腫，虹彩小結節，カフェオレ斑（café-au-lait spot），視神経膠腫などを特徴とする．これに対して，数万人に1人の発生といわれる type 2（NF 2）は，両側聴神経鞘腫，髄膜腫，神経膠腫などを発症する．前者の責任遺伝子は17番染色体長腕上に，後者は22番染色体長腕上にあるとされている．

#### 2）リ・フラウメニ症候群

リ・フラウメニ症候群（li-fraumeni syndrome）は多くの悪性腫瘍の発生に関係が深いとされている17番染色体短腕上にある TP 53遺伝子の異常による疾患で，乳がん，骨肉腫，白血病，悪性神経膠腫などの各種腫瘍を発生する．TP 53は星細胞腫の発生の原因の一つとされている．

#### 3）結節性硬化症

結節性硬化症（tuberous sclerosis；TS）は，顔面皮疹，上皮下巨細胞

星細胞腫（subependymal giant cell astrocytoma），知能発育遅延を3主徴とし，責任遺伝子は16番染色体短腕上および9番染色体長腕上にあることが判明している．

### 4）フォン・ヒッペル-リンダウ病

フォン・ヒッペル-リンダウ病（von Hippel-Lindau disease）は常染色体性優生遺伝をし，小脳，脳幹，脊髄，網膜などに血管腫を生じ，腎や膵臓などには嚢胞性腫瘍を形成するほか，副腎の褐色細胞腫などを合併する．責任遺伝子は3番染色体短腕上にあるとされている．

## 2 神経膠腫（グリオーマ）における遺伝子異常

神経膠腫の代表といえる星細胞腫の発生においても遺伝子的な変異が指摘されている．まず，前駆細胞から星細胞腫（grade 2）に変化する時点でTP53および血小板由来成長因子（PDGF）などの変異が必要であるとされ，さらに19番染色体長腕，RB遺伝子の変異により退形成性星細胞腫（grade 3）になり，10番染色体などの異常を経て，膠芽腫（grade 4）へ変化していくものと考えられている．

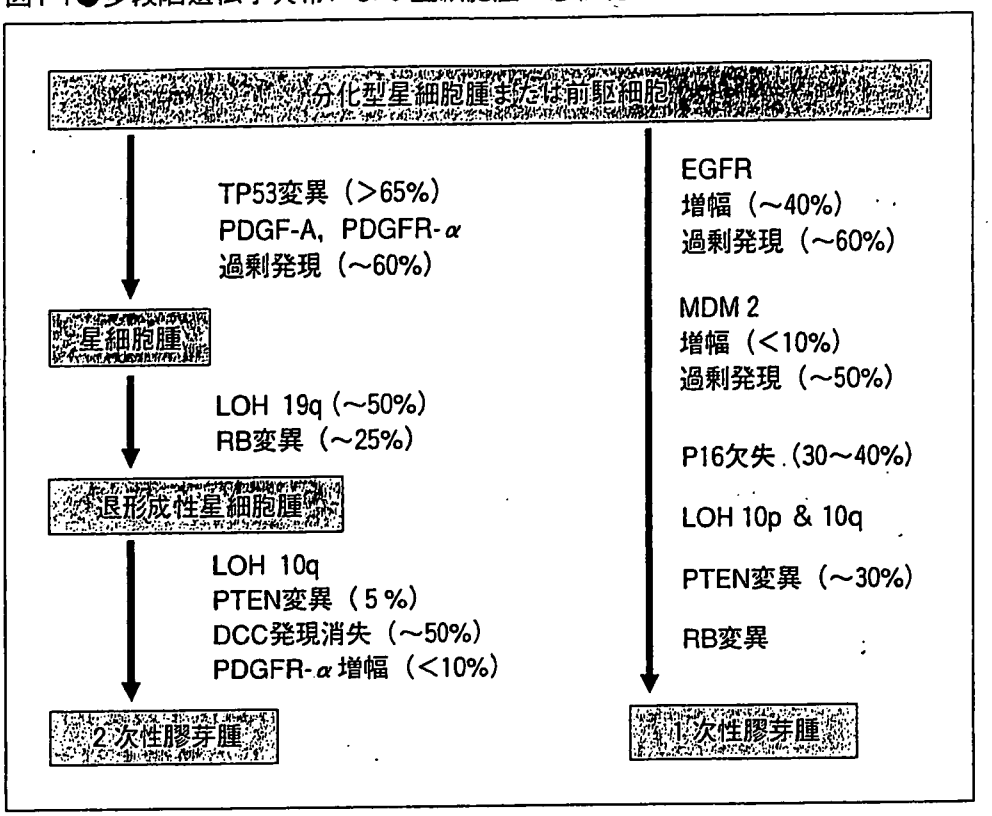
最も悪性度の高い膠芽腫には，このように星細胞腫が順次悪性化してなるもの（2次性膠芽腫）と，このような変化を経ないもの（1次性膠芽腫）が存在するとされ，これらはまったく別の遺伝子異常を伴い，後者のほうがより予後が悪いとされている（図1-1）[1]．しかしながら，星細胞腫でのTP53の異常は30～40%に認められるのみで，この遺伝子異常のみでは星細胞腫の発生について説明は不能であり，環境因子などの他の要因の関与も考えられる．

## 3 環境因子

脳腫瘍の発生と環境因子との明確な関連性が証明されているものはないといえる．職業上で接触あるいは吸入する危険性がある各種有機溶媒，潤滑油，ホルマリン，フェノール，殺虫剤，合成ゴム，塩化ポリビニールおよび母親の喫煙などとの関連性も疑われたが決定的な証拠はない[2]．これに対し，通常の環境とはいえないが，放射線照射は脳腫瘍の発生ときわめて関連性が深い．

かつては，頭皮白癬症や血管腫に対して低線量の放射線照射がなされていたことがあり，数年後あるいは10数年後に神経膠腫，髄膜腫，神経鞘腫などが多発していることが報告されている[3]．また，小児期の白血病患者に対する頭部への予防照射が後の神経膠腫発生の誘因になりうるとも考えられている[4]．

図1-1●多段階遺伝子異常による星細胞腫の悪性化　(kleihues2000)




一方，高圧線の付近に居住する子どもに脳腫瘍や白血病が多発するという報告から，電磁波と脳腫瘍の関係が注目され，これに関連して携帯電話から発生する電磁波が脳腫瘍の発生につながる危険性が危惧されている．現在，国内においても脳腫瘍のなかでも特に聴神経腫瘍との関連性についての疫学調査が実施されている．

現時点では関連性に否定的な結論を出している報告もあるが，完全にその関連性が否定できていないことに加え，携帯電話の使用頻度や使用時間がますます増加する傾向にあることから，今後さらに調査が必要になる可能性もある[5]．

# B 脳腫瘍の疫学調査

## 1 脳腫瘍の発生頻度

国内においては地域がん登録のシステムがまだ十分に整備されておらず，脳腫瘍の発生頻度について正確な数字は得られていない．2006年版の米国中央脳腫瘍登録（central brain tumor registry of the United States；CBTRUS2006）によれば，米国における原発性脳腫瘍の発生率は人口10万人あたり年間14.8人（良性腫瘍7.4人，悪性腫瘍7.4人）であり，男女別では，男性14.5人，女性15.1人とされており，女性に若干多

い[6]．年次別の発生頻度は上昇傾向にあるが，その理由として，1970年代のCTスキャンや1980年代のMRIなどの非侵襲的検査の普及が考えられる．軽度の頭痛や神経症状のみでこれらの検査が実施されることで，偶発的に脳腫瘍が発見されることも珍しくない．近年，特に増加傾向の著しいのは悪性リンパ腫であり，そのほかには，神経鞘腫，下垂体腺腫などの良性腫瘍の増加が指摘されている．また，小児および高齢者の脳腫瘍が増加しているのも近年の傾向の一つである．

## 2 脳腫瘍全国統計

### 1）組織別頻度

脳腫瘍全国統計（brain tumor registry of Japan）[7]は，日本脳神経外科学会のもとで400あまりの脳神経外科施設からのデータを集積し解析したものである．1969（昭和44）年以来，年間おおよそ4000～5000症例，合計9万8000例あまりが登録されており，発生頻度，生存率など各種解析がなされている．

1984（昭和59）～1996（平成8）年の症例5万1818例について，最も頻度の高いものは神経膠腫の27.3%で，髄膜腫26.2%，下垂体腺腫15.2%，神経鞘腫10.4%がそれに続いている（表1-1）．年齢別でみると，15歳未満の小児では，神経膠腫が57.5%と半数以上を占め，続いて胚細胞腫15.4%，頭蓋咽頭腫9.0%，髄膜腫2.0%となっており，成人とはかなり頻度が異なっている．また，70歳以上の高齢者で最も頻度の高いものは髄膜腫42.3%で，続いて神経膠腫26.9%，下垂体腺腫10.1%，神経鞘腫

表1-1●脳腫瘍全国統計による各種脳腫瘍の頻度（1984～1996年登録症例）

| | 全例 | 年齢（歳） | | |
|---|---|---|---|---|
| | | <15 | 15～69 | >70 |
| 神経膠腫 | 27.3% | 57.7% | 24.3% | 26.9% |
| 髄膜腫 | 26.2 | 2.0 | 26.2 | 42.3 |
| 下垂体腺腫 | 15.2 | 1.9 | 17.2 | 10.1 |
| 神経鞘腫 | 10.4 | 1.1 | 11.8 | 7.1 |
| 頭蓋咽頭腫 | 3.5 | 9.0 | 3.3 | 1.6 |
| 悪性リンパ腫 | 2.9 | 0.4 | 2.6 | 6.7 |
| 血管芽腫 | 1.7 | 0.4 | 2.0 | 1.0 |
| 類表皮嚢胞・類皮嚢胞 | 1.6 | 1.6 | 1.7 | 0.5 |
| 胚細胞腫瘍 | 2.8 | 15.4 | 2.0 | 0.0 |
| その他 | 8.4 | 10.5 | 8.9 | 3.8 |
| 合計 | 100.0 (n=51,818) | 100.0 (n=4,070) | 100.0 (n=41,653) | 100.0 (n=6,095) |

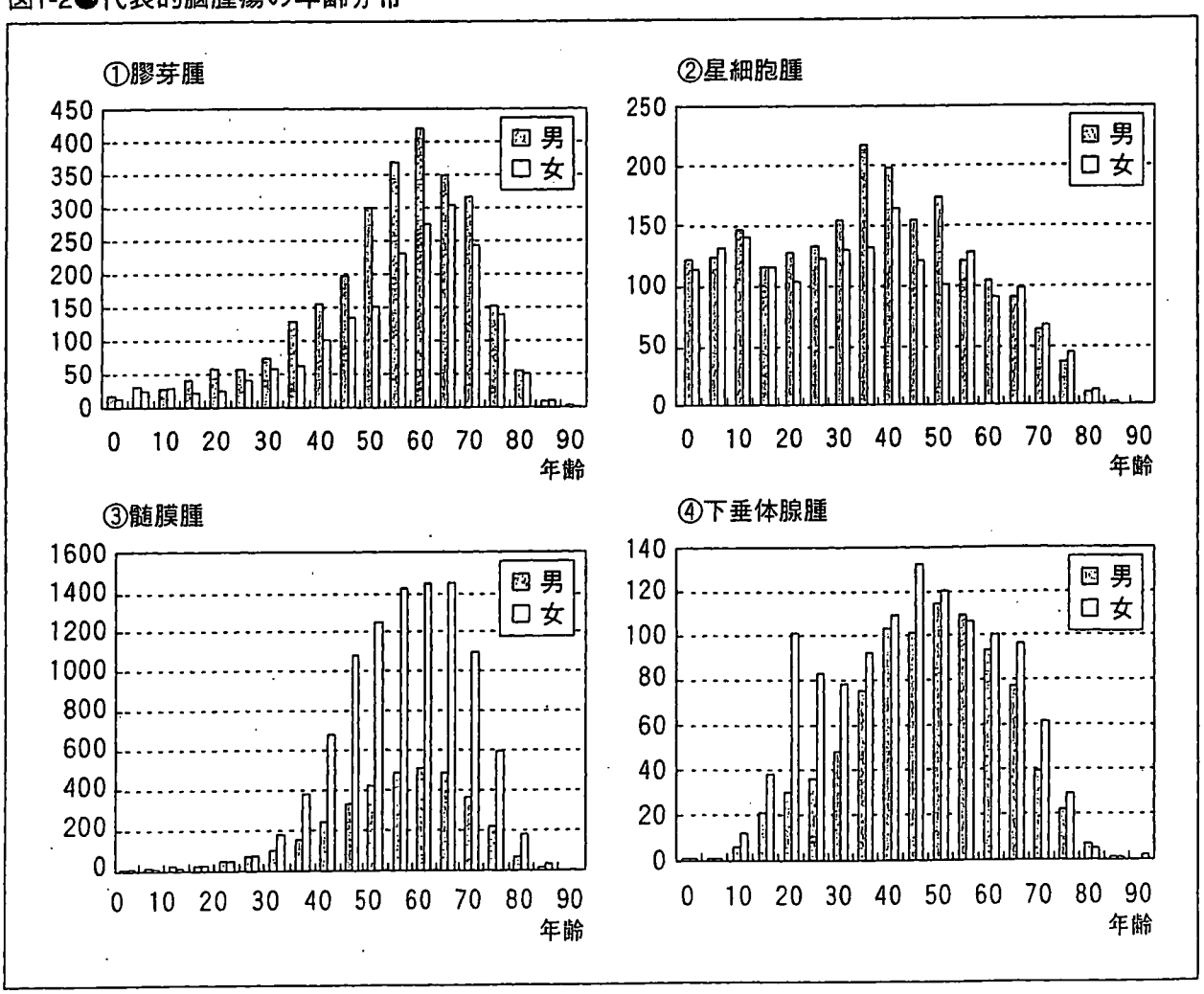
7.1%であり，これに悪性リンパ腫6.7%が続いている．

神経膠腫のなかでの頻度は，膠芽腫（星細胞腫 grade 4）が32.4%と最も多く，続いて星細胞腫（grade 2）27.0%，退形成性星細胞腫（星細胞腫 grade 3）17.2%，乏突起膠腫5.5%となっているが，これも年齢によってその頻度は大きく異なっている．15歳未満の小児では星細胞腫が29.2%，膠芽腫5.4%であるのに対し，70歳以上の高齢者では，59.9%が膠芽腫であり，組織診断上も高齢者の神経膠腫は予後が悪いことが予想される．

## 2）年齢分布

代表的な原発性脳腫瘍の年齢分布を図1-2に示す．膠芽腫は60歳代をピークとする高齢者に多い腫瘍であり，男女比は1.4：1で男性に多い（図1-2①）．これに対し星細胞腫では男女ほぼ同数であり，30歳代後半から40歳代に多く，10歳前後にもう1つのピークがある（図1-2②）．退形成星細胞腫は，この二者の中間的存在である．

図1-2●代表的脳腫瘍の年齢分布



髄膜腫は男女比1：2.8と女性に多い疾患で，好発年齢は50歳代から70歳代まで広く分布する（図1-2③）．神経鞘腫の男女比も1：1.3と女性に若干多く，50歳代から60歳代にピークがある．

下垂体腺腫は，全体では二峰性になっているが，ホルモン非分泌性下垂体腺腫は50歳代から60歳代に1つのピークを示すのみであり，20歳代から30歳代にかけてのピークは女性を主体としたプロラクチン産生性下垂体腺腫である（図1-2④）．最近頻度の増加している悪性リンパ腫は60歳をピークとした高齢者の男性に多く，男女比は1.3：1である．

## 3）予後関連因子

脳腫瘍では，組織診断が決定された時点で予後の推察が可能なことが多い．巨大な腫瘍でも髄膜腫であれば，症状は軽微であることもまれではなく，摘出によって治癒することも多い．それに対し，膠芽腫の場合は，かなり早期に発見されたとしても予後はかなり厳しく，ほかの臓器のようなTNM分類があてはまらない．

表1-2に代表的な脳腫瘍の累積生存率を示す．髄膜腫の5年生存率が93.7%であるのに対し，神経膠腫（グリオーマ）全体では38.1%であり，このうち，星細胞腫は66.5%，退形成星細胞腫は23.4%，膠芽腫は7.0%である．

髄膜腫などの良性腫瘍では，基本的に手術的に摘出することで治癒させることができる．これに対し，神経膠腫の予後に影響を与える因子として，手術，放射線，年齢，術前の活動レベル（performance status）があるといわれている．さらに放射線療法にニトロソウレア（ニトロソ尿素）系の化学療法薬を併用することで，予後の改善がみられることが確認された[8]．脳腫瘍全国統計による生存率でも同様の傾向がみられる．

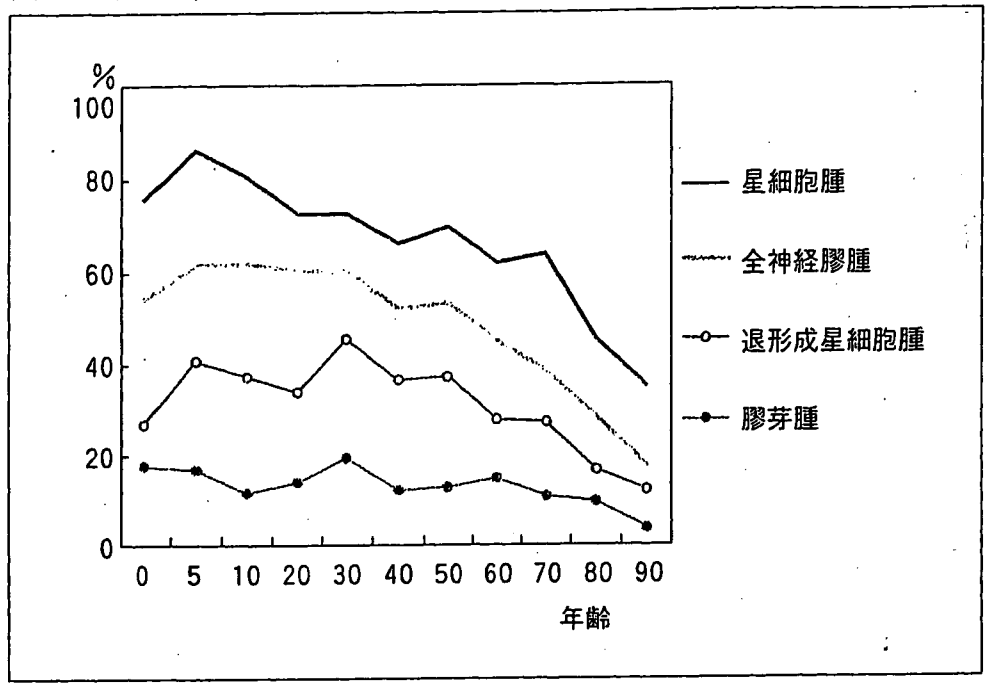
### a）年　齢

神経膠腫の生存率は50歳を過ぎると急激に低下する傾向をもっている．若年者の平均生存率は60%程度であるが，70歳代では40%，80歳代では

表1-2●脳腫瘍全国統計による各種脳腫瘍の5年累積生存率(1991～1996年登録症例)

| | 症例数 | 1年 | 2年 | 3年 | 4年 | 5年 |
|---|---|---|---|---|---|---|
| 星細胞腫 | 1573 | 89.6% | 79.7% | 73.8% | 70.9% | 66.5% |
| 退形成星細胞腫 | 1066 | 67.6 | 43.0 | 32.2 | 26.4 | 23.4 |
| 膠芽腫 | 2125 | 55.2 | 19.6 | 11.4 | 8.8 | 7.0 |
| 乏突起膠腫 | 152 | 95.3 | 91.8 | 87.1 | 83.8 | 82.0 |
| 退形成乏突起膠腫 | 43 | 87.5 | 84.7 | 75.0 | 71.7 | 68.2 |
| 髄膜腫 | 6367 | 97.9 | 96.6 | 95.7 | 94.8 | 93.7 |
| 全グリオーマ | 5757 | 73.3 | 51.9 | 44.4 | 40.9 | 38.1 |

図1-3●疾患別，年齢別5年生存率




30%以下に低下する．高齢者では10歳進むごとに統計的な有意差をもって生存率の低下がみられる（図1-3）．これは，星細胞腫，退形成星細胞腫，膠芽腫のすべてに共通する傾向であり，高齢者のグリオーマは組織診断に関係なく予後不良であるといえる．

**b）術前の活動レベル（performance status）**

脳腫瘍患者の performance status を現す表現法として，karnofsky scale と eastern cooperative oncology group（ECOG）が繁用されているが，脳腫瘍全国統計では臨床悪性度を無症状，自覚症状のみ，巣症状，頭蓋内圧亢進，意識障害，昏睡，呼吸中枢障害の7段階に分けて評価している．

呼吸障害例および昏睡例はいずれも1%以下の症例であるため，これらを除いた5段階での生存曲線を（図1-4）に示す．星細胞腫では，無症状と自覚症状の間に有意差がみられなかったが，膠芽腫では，その差は大きく，悪性腫瘍での早期発見の重要性を示している．

**c）手術摘出度**

悪性神経膠腫をはじめとする悪性脳腫瘍は，浸潤性に発育するため，手術的に全摘出をすることは不可能である．膠芽腫などでは，CT や MRI により造影剤で増強を受ける領域から数 cm 先まで腫瘍の浸潤がみられ，周辺の脳の機能を温存したまま摘出することは困難である．最近では，各種モニタリングやナビゲーションの発達により，安全にしかも最大限に腫瘍を摘出する試みがなされているが，すでに運動や言語の機能をもつ領域に浸潤した腫瘍には外科的侵襲を加えることができない．

図1-5に膠芽腫と星細胞腫の摘出度別の生存曲線を示す．膠芽腫では

図1-4●臨床悪性度別生存率（5段階）

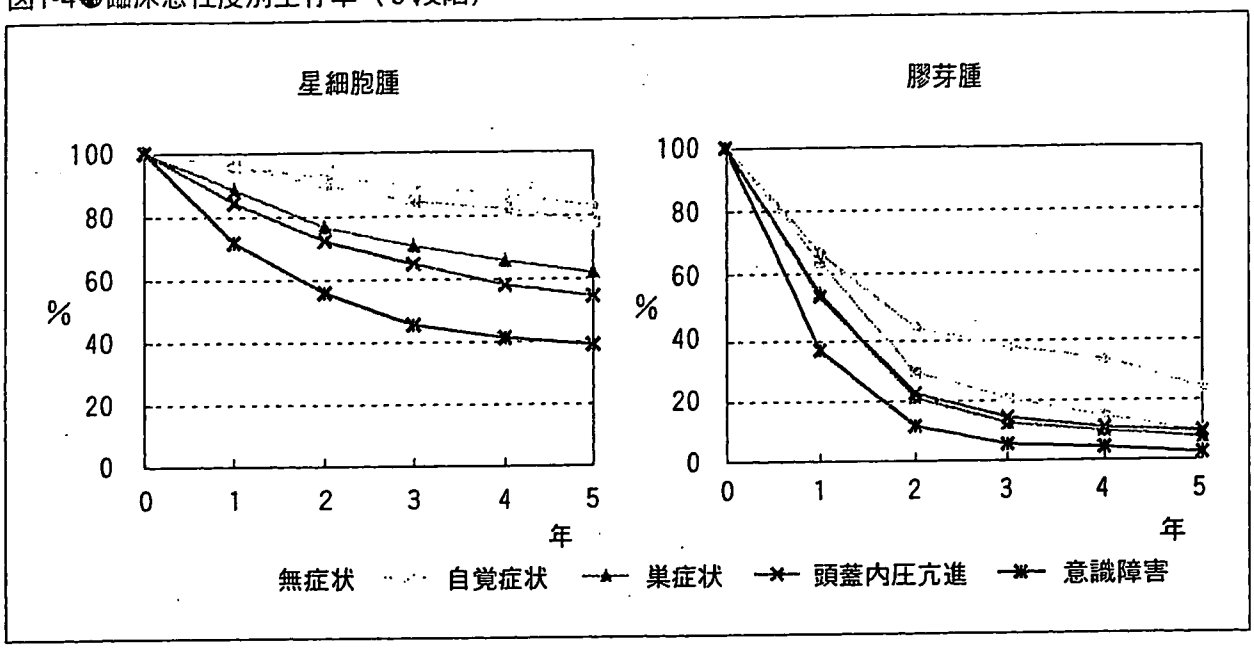
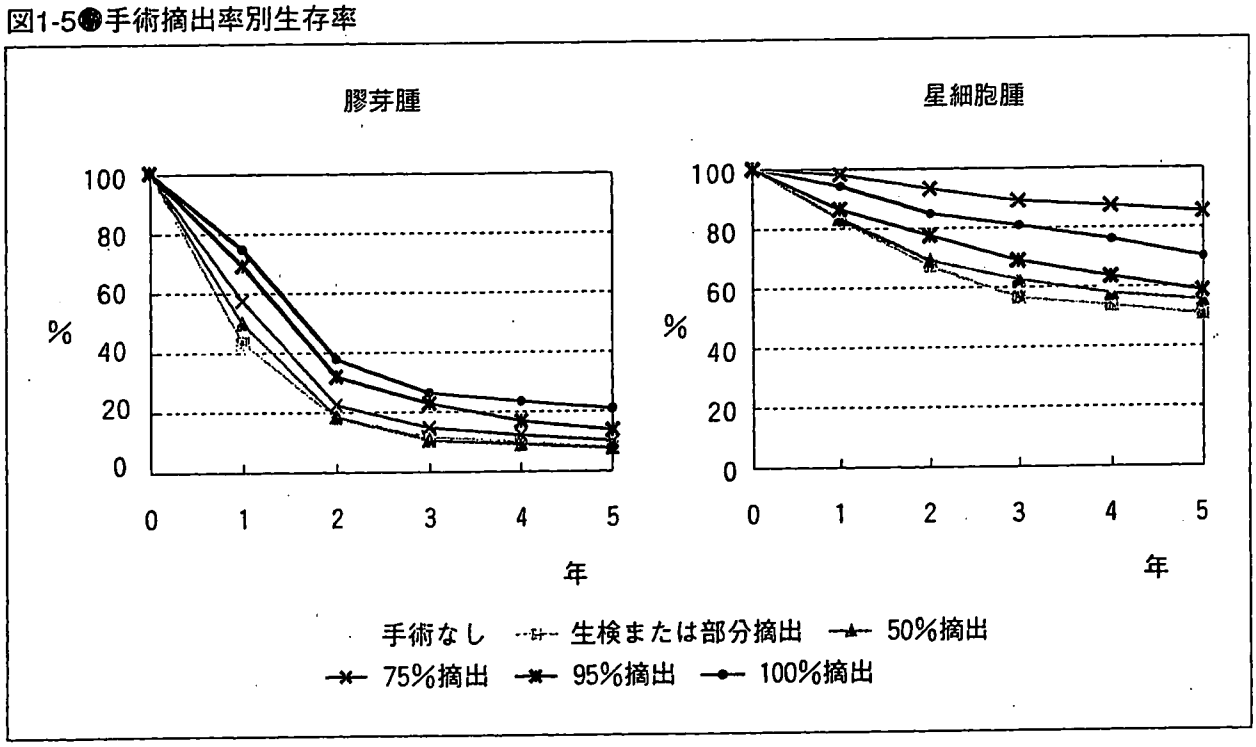



図1-5●手術摘出率別生存率




50%摘出と生検との間に有意差がないが，75%摘出により50%以下の摘出と有意差が出る．さらに摘出度が進むにつれ，それぞれ有意差をもって生存率の上昇がみられる．

そのため最も悪性度の高い膠芽腫でも手術が予後に及ぼす可能性を示すことがわかる．

星細胞腫では，75%までの摘出ではそれ以下と有意差がなく，95%の摘出により75%以下の摘出と有意差をもつようになる．このように本来

全摘出術のできない神経膠腫でも可及的最大限の摘出を図ることが予後の改善につながり，外科手術の重要性を示している．

d）放射線療法と化学療法

悪性神経膠腫に対し，術後の放射線療法は予後を改善する．さらにニトロソウレア系抗がん薬の併用でさらに生存率が改善することが確認された．化学療法薬としては，従来BCNUやACNUなどが用いられていたが，2005年にテモゾロマイド（temozolomide）を用いた大規模臨床試験結果が発表され，統計的な有意差をもって放射線療法単独に比べ，生存期間の延長をみた[9]．最近になって，従来，化学療法にほとんど期待がもてなかった悪性神経膠腫に対し，このほかにも有望な薬剤が出現し始めている．30年来改善されなかった悪性神経膠腫の生存率も徐々に改善することが期待されている．

疫学や統計は，過去の臨床例のデータの解析から，その疾患の特性を知り，有効な治療法をみつけ出し，さらにはその環境を改善することで，疾患の予防にもつながる重要な情報を提供してくれるものである．ある環境条件が腫瘍の発生に影響をもつという事実を証明するには，大規模な調査が必要となり，各臓器の学会と地域がん登録のシステムなどとの密接な連携が必要である．

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# Pharmacokinetic study of temozolomide on a daily-for-5-days schedule in Japanese patients with relapsed malignant gliomas: first study in Asians

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**Abstract**
***Background.*** Temozolomide (TMZ) is widely used in Europe and the United States. For the safe use of TMZ in the Japanese, as representative of Asians, the pharmacokinetics of TMZ was investigated in Japanese patients and compared to that in Caucasians.
***Methods.*** The pharmacokinetics and safety of TMZ following oral administration of 150 and 200 mg/m$^2$ per day for the first 5 days of a 28-day treatment cycle were investigated in six Japanese patients with relapsed gliomas.
***Results.*** The time-to-maximum plasma concentration (tmax) of TMZ was about 1 h and the elimination half-life of terminal excretion phase ($t_{1/2}\lambda z$) was about 2 h. A dose-dependent increase was observed in maximum plasma concentration (Cmax) and AUC, while values for $t_{1/2}\lambda z$, apparent total body clearance (CL/F), and apparent distribution volume (Vz/F) were independent of dose. After administration for 5 days, changes in pharmacokinetics and accumulation were not observed. The plasma 5-(3-methyl)1-triazen-1-yl-imidazole-4-carboxamide (MTIC) concentration changed in parallel with the TMZ plasma concentration, and the Cmax and AUC of MTIC were about 2% of those of TMZ. The pharmacokinetic parameters of TMZ and MTIC in Japanese patients in this study were comparable to those previously determined in Caucasian subjects. Adverse events occurred in all patients, but toxicities were mostly mild or moderate, and continuation of administration was possible by adjusting the dose and by delaying the start of the next treatment cycle.
***Conclusion.*** The pharmacokinetic and safety profile of TMZ in Japanese patients was comparable to that in Caucasians. The treatment regimen used in Europe and the United States will be suitable for Asian patients, including Japanese.

**Key words** Malignant gliomas · Temozolomide · Pharmacokinetics · Japanese

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## Introduction

The treatment of patients with malignant glioma remains the biggest challenge for the neuro-oncologist. Despite maximal safe surgical debulking and radiotherapy, overall survival for the average patient remains poor. In 1999, temozolomide (TMZ) was approved in the United States for refractory anaplastic astrocytoma and in the European Union for recurrent or progressed malignant glioma. In 2005, TMZ was additionally approved in the United States and the European Union for newly diagnosed glioblastoma multiforme, in combination with radiotherapy followed by monotherapy.

TMZ is an oral anticancer drug classified as an alkylating agent. In plasma, under physiological conditions, TMZ undergoes hydrolysis by rapid reaction with an alkaline base, and is transformed into 5-[(1Z)-3-methyltriaz-1-en-1-yl]-1H-imidazole-4-carboxamide (MTIC).[1–5] MTIC rapidly undergoes degeneration to the active form, methyldiazonium ion (DNA alkylating molecule)[3,5] and the inactive compound 5-aminoimidazole-4 carboxamide (AIC). TMZ has relatively high permeability through the blood-brain barrier as an unchanged drug.[6] These features contribute to its efficacy in patients with malignant gliomas.

Biological factors, including individual and ethnic differences, are considered to have little effect on the pharmacokinetics of TMZ. This consideration is based on the following findings: the bioavailability of TMZ with oral administration is nearly 100%,[7] linearity in pharmacokinetics is observed over a wide dose range,[8,9] the bioavailability of TMZ is not substantially affected by physiological conditions such as meals and gastric pH,[8,10] and the biotransformation from TMZ to MTIC and the formation of

methyldiazonium ion from MTIC are both nonenzymatic decomposition reactions.[1-5] Although TMZ is already being used in Taiwan and South Korea, no results have been reported of a pharmacokinetic study of TMZ in Asians.

We therefore investigated the pharmacokinetics of TMZ in Japanese patients, as representing Asians, to confirm its safety in Asian patients.

## Patients and methods

### Patient eligibility

Male and female patients with histologically proven relapsed gliomas with evidence of recurrence confirmed by magnetic resonance imaging (MRI) and whose Karnofsky performance status (KPS) was 50 or more were eligible for participation in this study. All pathology slides were reviewed by an independent central neuropathologist (Professor Yoichi Nakazato, Department of Human Pathology, Gunma University Graduate School of Medicine) based on WHO classification.[11] Patients also had to be 18 to less than 75 years in age, with male patients weighing at least 50 kg and female patients weighing at least 45 kg.

As prior treatment, patients must have undergone radiotherapy and chemotherapy. If the tumor was surgically resected at the time of relapse, MRI should have been conducted within 72 h after surgery and at least 8 days must have elapsed between the day of the surgery and the start of TMZ administration in the first cycle.

Patients also had to have an assessable tumor site confirmed by MRI, and the results of hematology and biochemistry tests had to meet defined criteria. Clinical laboratory values (performed within 14 days prior to TMZ [Temozolomide; Schering-Plough, Tokyo, Japan] administration, including the day of initial administration) had to be as follows: neutrophil count, $\geq 1500/mm^3$; platelet count, $\geq 100000/mm^3$; hemoglobin, $\geq 10.0$ g/dl; blood urea nitrogen, <1.5 times the upper limit of laboratory standard value; serum creatinine, <1.5 times the upper limit of laboratory standard value; serum total bilirubin, ≤upper limit of laboratory standard value; transaminase, <3 times the upper limit of laboratory standard value; alkaline phosphatase, <2 times the upper limit of laboratory standard value.

Patients also had to have a life expectancy of at least 12 weeks.

This study was conducted after obtaining approval from the institutional review board at each study site. Written informed consent, according to the principles of the Declaration of Helsinki and the rules of Good Clinical Practice was obtained from all patients.

### Clinical endpoints

*Pharmacokinetics*

To examine the pharmacokinetics of TMZ in Japanese patients, pharmacokinetic parameters were calculated based on TMZ plasma concentrations, MTIC plasma concentrations, and TMZ urinary concentrations. The pharmacokinetic parameters of TMZ and MTIC plasma concentrations were then compared with those obtained in Caucasian patients.

*Safety*

Laboratory values (hematology, blood biochemistry, and urinalysis), body weight, body temperature, blood pressure, and pulse rate were measured, and adverse events and adverse reactions were investigated, according to the National Cancer Institute (NCI) common toxicity criteria (Version 2.0). The appropriateness of the safety evaluation made by the investigator was evaluated by an Efficacy and Safety Evaluation Committee (Yukitaka Ushio, Director of Otemae Hospital; Kazuo Tabuchi, Director of Koyanagi Memorial Hospital; and Professor Yuta Shibamoto, Department of Quantum Radiotherapy, Nagoya City University Graduate School of Medical Sciences).

### Treatment

One treatment cycle consisted of once-daily oral administration of TMZ on an empty stomach (2 h before breakfast) for 5 consecutive days, followed by 23 days without treatment, in a 28-day treatment cycle. The dose was 150 mg/m$^2$ per day in the first cycle, and the dose in subsequent cycles was 100, 150, or 200 mg/m$^2$ per day, based on the criteria for dose adjustment (Table 1). If adequate recovery had not occurred, the start of the next cycle was delayed until the criteria were met.

### Plasma and urine sampling

*Collection of plasma samples*

Immediately before TMZ administration (0 h) and at 15, 30, and 45 min, and 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after administration on days 1 and 5 in the first cycle (150 mg/m$^2$) and second cycle (200 mg/m$^2$), 5 ml of venous blood was collected using a pre-chilled heparinized vacuum tube, and blood samples were cooled in an ice-water bath from immediately after sampling and centrifuged (4°C, 3000 rpm, 10 min) to separate plasma within 5 min of blood collection. For the determination of TMZ plasma concentrations, 1.0 ml of plasma sample immediately after centrifugation was placed in a polypropylene tube to which 50 µl of 8.5% phosphoric acid solution (stabilizer) had been pre-added. The acidified plasma was vortexed, and the tube was then sealed and stored frozen at −20°C or below until analysis. For determination of MTIC plasma concentrations, about 1 ml of plasma sample was dispensed immediately after centrifugation to a pre-cooled polypropylene tube. The tube was then sealed and frozen immediately on dry ice-methanol. The sample was stored frozen at −80°C or below until analysis.

**Table 1.** Dose adjustment criteria based on neutrophil count, platelet count, and onset of adverse events

| | Grade | Dose adjustment criteria based on neutrophil count and platelet count | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| | | Nadir neutrophil count >1500/mm$^3$ Nadir platelet count >100000/mm$^3$ | Nadir neutrophil count 1000–1500/mm$^3$ Nadir platelet count 50000–100000/mm$^3$ | Nadir neutrophil count <1000/mm$^3$ Nadir platelet count <50000/mm$^3$ |
| Dose adjustment criteria based on onset of adverse events | CTC Grade 0, 1 | Increase by 50mg/m$^2$ per day | No change | Reduce by 50mg/m$^2$ per day |
| | CTC Grade 2 | No change | No change | Reduce by 50mg/m$^2$ per day |
| | CTC Grade 3, 4 | Reduce by 50mg/m$^2$per day | Reduce by 50mg/m$^2$ per day | Reduce by 50mg/m$^2$ per day |

*Collection of urine samples*

Urine was collected before TMZ administration and in 0–4, 4–8, and 8- to 24-h blocks after administration on days 1 and 5 in both the first and second cycles. The total volume of urine accumulated up to each prescribed time point was collected in plastic urine collection containers to which 2 ml of 8.5% phosphoric acid solution (stabilizer) had been pre-added. These plastic containers were refrigerated throughout the urine accumulation time period. The pH of the urine in the plastic container was determined after each voiding. If the pH was 4 or more, 8.5% phosphoric acid solution was added again. Each urine sample was collected in a polypropylene tube (total, 20ml) and the tubes were sealed and stored frozen at –20°C or below until analysis.

Assay method

TMZ and MTIC plasma concentrations were both determined by validated high-performance liquid chromatography-tandem mass spectrometry. The lower limits of quantitation of temozolomide and MTIC were 0.020μg/ml and 5.00ng/ml, respectively. Urinary temozolomide concentration was determined by validated high-performance liquid chromatography. The lower limit of quantitation was 1.00μg/ml.

Pharmacokinetic analysis

The pharmacokinetic analysis of TMZ and MTIC plasma concentrations was performed by noncompartmental analysis[12] and pharmacokinetic parameters, including the maximum plasma concentration (Cmax), time-to-maximum plasma concentration (tmax), the area under the plasma concentration-time curve (AUC) up to the final observation point ($AUC_{0-t}$), AUC up to 24h after administration ($AUC_{0-24}$), AUC up to infinite time ($AUC_{0-\infty}$), elimination half-life of terminal excretion phase ($t_{1/2}\lambda z$), apparent total body clearance (CL/F), apparent distribution volume (Vz/F), and accumulation index (R) were calculated by patient. With TMZ urinary concentrations, the amount of urinary excretion (Ae), the urinary excretion rate (Ae%), and renal clearance (CLr) were calculated for each patient.

Role of the funding source

The supporter of this study was responsible for the study design, quality assurance, and quality control systems to ensure that the study was done and data were generated, documented, analyzed, and reported in compliance with the protocol. The supporter had no role in the interpretation of the data. The corresponding author had full access to all data in the study, including those for safety, and had the final responsibility to submit the paper for publication.

## Results

Patient characteristics

Table 2 shows the major background factors of all six patients. The mean age was 43.3 years, with three patients under 40 and the remaining three between 40 and 64 years. The six patients consisted of five men and one woman. The mean body weight was 63.85kg, and the mean body mass index was 22.92kg/m$^2$. KPS was assessed to be 50, 60, 70, and 80 in one patient each and 90 in two patients.

According to the results of the central pathology review, one patient had anaplastic astrocytoma (AA), three had glioblastoma multiforme (GBM), one had anaplastic oligodendroglioma (AO), and one had malignant glioma. Four patients had received surgical treatment once and two had received surgical treatment twice. All patients had experienced one recurrence, and the time to recurrence was less than 6 months in one patient and 6 months or more in five patients.

Pharmacokinetics

Pharmacokinetics was examined in six patients who completed administration at 150mg/m$^2$ per day in the first cycle and in three patients whose dose was increased to 200mg/m$^2$ per day in the second cycle.

**Table 2.** Demographics

| Item | Classification, etc. | All subjects |
|---|---|---|
| Age (years) $n = 6$ | Mean ± standard deviation | 43.3 ± 12.4 |
| | Median value | 39.5 |
| | Minimum value–maximum value | 29–62 |
| Age classification (years) $n = 6$ | <40 | 3 (50%) |
| | ≥40 to <65 | 3 (50%) |
| | ≥65 | 0 |
| Sex $n = 6$ | Male | 5 (83%) |
| | Female | 1 (17%) |
| Body weight (kg) $n = 6$ | Mean ± standard deviation | 63.85 ± 9.14 |
| | Median value | 62.1 |
| | Minimum value–maximum value | 52.6–78.0 |
| BMI (kg/m$^2$) $n = 6$ | Mean ± standard deviation | 22.92 ± 3.71 |
| | Median value | 22.15 |
| | Minimum value–maximum value | 19.6–28.7 |
| KPS before start of administration $n = 6$ | Mean ± standard deviation | 73.3 ± 16.3 |
| | Median value | 75 |
| | Minimum value–maximum value | 50–90 |
| Central pathology judgment of lesion tissue $n = 6$ | AA | 1 (17%) |
| | Other than AA | 5 (83%) |
| Number of operations $n = 6$ | 0 | 0 |
| | 1 | 4 (67%) |
| | 2 | 2 (33%) |
| | 3 or more | 0 |
| Recurrence $n = 6$ | Once | 6 (100%) |
| | 2 Times or more | 0 |
| Duration from initial diagnosis to initial recurrence (months) $n = 6$ | <6 | 1 (17%) |
| | ≥6 | 5 (83%) |
| Steroid use $n = 6$ | No | 3 (50%) |
| | Yes | 3 (50%) |
| Most recent steroid dose[a] (mg/day) $n = 3$ | Mean ± standard deviation | 16.13 ± 0.98 |
| | Median value | 16.7 |
| | Minimum value–maximum value | 15.0–16.7 |
| Classification of most recent steroid dose[a] $n = 3$ | <10 mg/day | 0 |
| | ≥10 mg/day <20 mg/day | 3 (100%) |
| | ≥20 mg/day | 0 |

[a] Calculated as dose of prednisolone (excluding topical steroid)

*TMZ and MTIC plasma concentration-time profiles and pharmacokinetic parameters*

Table 3 shows the pharmacokinetic parameters of TMZ and MTIC plasma concentrations on days 1 and 5 of TMZ administration in the first cycle (150 mg/m$^2$ per day) and second cycle (200 mg/m$^2$ per day). Figure 1 shows the mean TMZ and MTIC concentration-time profiles on days 1 and 5 of the first and second cycles.

TMZ in the six patients in the first cycle reached tmax at about 1 h after administration, with a monophasic decrease up to 12 h after administration. TMZ plasma concentrations were below the lower limit of quantitation (0.020 μg/ml) in five of six patients after 24 h. The Cmax values on days 1 and 5 were 7.87 and 8.38 μg/ml, respectively; $AUC_{0-t}$ values were 25.7 and 25.2 μg·h/ml; $AUC_{0-24}$ values were26.5 and 25.9 μg·h/ml; and $AUC_{0-\infty}$ values were 26.1 and 25.6 μg·h/ml. The accumulation index, based on Cmax and $AUC_{0-24}$, was 1.11 and 0.986, respectively, indicating no accumulation due to repeated administration. The $t_{1/2}\lambda z$ values on days 1 and 5 of administration were 2.14 and 2.29 h, respectively; CL/F values were 2.57 and 2.56 ml/min per kg; and the Vz/F values were 0.468 and 0.492 l/kg, indicating no change due to repeated administration. These coefficients of variation for AUC, $t_{1/2}\lambda z$, CL/F, and Vz/F ranged from 9% to 35%.

As with the first cycle, TMZ in the three patients in the second cycle who received 200 mg/m$^2$ per day reached tmax at about 1 h after administration, with a monophasic decrease up to 12 h after administration. TMZ plasma concentrations were below the lower limit of quantitation in all patients after 24 h. The Cmax values on days 1 and 5 were 15.3 and 14.0 μg/ml, respectively; $AUC_{0-t}$ values were 35.1 and 36.0 μg·h/ml, $AUC_{0-24}$ values were 36.4 and 37.3 μg·h/ml; and $AUC_{0-\infty}$ values were 35.7 and 36.7 μg·h/ml. The accumulation index, based on Cmax and $AUC_{0-24}$, was 0.868 and 1.03, respectively, indicating no accumulation due to repeated administration. The $t_{1/2}\lambda z$ values on days 1 and 5 of administration were 2.03 and 2.02 h, respectively; CL/F values were 2.37 and 2.27 ml/min per kg; and Vz/F values were 0.415 and 0.395 l/kg, indicating no change due to repeated administration, and the values were nearly the same as those observed after the administration of 150 mg/m$^2$ per day. The coefficients of variation for AUC, $t_{1/2}\lambda z$, CL/F, and Vz/F ranged from 4% to 9%.

The concentration-time profile of MTIC plasma concentrations in both the first cycle (150 mg/m$^2$ per day) and the second cycle (200 mg/m$^2$ per day) was nearly parallel to that

**Table 3.** Pharmacokinetic parameters of temozolomide and MTIC plasma concentrations in cycle 1 (150 mg/m$^2$ per day) and cycle 2 (200 mg/m$^2$ per day)

| Analyte | Dose (mg/m$^2$) | Dosing day | Tmax (h) | Cmax (μg/ml) | $t_{1/2}\lambda z$ (h) | AUC (μg·h/ml) | | | CL/F (ml/min per kg) | Vz/F (l/kg) | R | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 0–t | 0–24 | 0–∞ | | | Cmax | $AUC_{0-24}$ |
| Temozolomide | 150 ($n$ = 6) | Day 1 | 1.42 (52) | 7.87 (38) | 2.14 (25) | 25.7 (15) | 26.5 (14) | 26.1 (14) | 2.57 (18) | 0.468 (23) | – | – |
| | | Day 5 | 0.958 (53) | 8.38 (36) | 2.29 (35) | 25.2 (10) | 25.9 (9) | 25.6 (10) | 2.56 (14) | 0.492 (21) | 1.11 (24) | 0.986 (8) |
| | 200 ($n$ = 3) | Day 1 | 0.583 (25) | 15.3 (5) | 2.03 (4) | 35.1 (6) | 36.4 (6) | 35.7 (6) | 2.37 (5) | 0.415 (7) | – | – |
| | | Day 5 | 0.917 (57) | 14.0 (30) | 2.02 (5) | 36.0 (4) | 37.3 (5) | 36.7 (4) | 2.27 (9) | 0.395 (5) | 0.868 (39) | 1.03 (7) |
| MTIC | 150 ($n$ = 6) | Day 1 | 1.42 (52) | 0.145 (38) | 1.98 (24) | 0.426 (15) | 0.451 (14) | 0.463 (14) | – | – | – | – |
| | | Day 5 | 1.08 (43) | 0.154 (28) | 1.83 (12) | 0.425 (12) | 0.445 (13) | 0.454 (13) | – | – | 1.14 (29) | 1.00 (16) |
| | 200 ($n$ = 3) | Day 1 | 0.750 (33) | 0.272 (15) | 1.93 (6) | 0.594 (7) | 0.622 (8) | 0.632 (8) | – | – | – | – |
| | | Day 5 | 0.917 (57) | 0.284 (33) | 1.87 (3) | 0.636 (7) | 0.665 (7) | 0.676 (7) | – | – | 1.03 (17) | 1.07 (1) |

Values are means, with coefficient of variation % in parentheses
Tmax, time of each plasma concentration; Cmax, maximun plasma concentration; $t_{1/2}\lambda z$, elimination half-life terminal excretion phase; AUC, area under the plasma concentration time curve; CL/F, apparent total body clearance; Vz/F, apparent distribution volume; R, accumulation index

of the TMZ plasma concentrations on day 1 as well as on day 5. The tmax and $t_{1/2}\lambda z$ values of MTIC plasma concentrations were 0.750 to 1.42 h and 1.83 to 1.98 h, respectively, which closely matched the tmax and $t_{1/2}\lambda z$ values of the TMZ plasma concentrations. After the administration of 150 and 200 mg/m$^2$ per day, the Cmax values were 0.145 to 0.154 and 0.272 to 0.284 μg/ml, respectively; $AUC_{0-t}$ values were 0.425 to 0.426 and 0.594 to 0.636 μg·h/ml, respectively; $AUC_{0-24}$ values were 0.445 to 0.451 and 0.622 to 0.665 μg·h/ml, respectively; and $AUC_{0-\infty}$ values were 0.454 to 0.463 and 0.632 to 0.676 μg·h/ml, respectively. Cmax and AUC exhibited a dose-dependent increase in relation to the administration of 150 mg/m$^2$ per day and 200 mg/m$^2$ per day. The ratios of MTIC to TMZ, based on Cmax and AUC, were 1.78% to 2.03% and 1.66% to 1.84%, respectively. The accumulation index, based on Cmax and $AUC_{0-24}$, was 1.03 to 1.14 and 1.00 to 1.07, indicating no accumulation due to repeated administration, as in the case of TMZ plasma concentrations. The coefficients of variation for AUC and $t_{1/2}\lambda z$ ranged from 3% to 24%.

### *TMZ urinary excretion rate*

Table 4 shows the amount of urinary excretion, excretion rate, and renal clearance by urine accumulation intervals to 24 h after the administration of TMZ on days 1 and 5 in the first cycle (150 mg/m$^2$ per day) and second cycle (200 mg/m$^2$ per day). One of the three patients in the second cycle mistakenly discarded the 0- to 4-h urine after administration on day 5, and the cumulative urinary excretion data for this patient on day 5 of the administration of 200 mg/m$^2$ per day was considered missing.

The cumulative urinary excretion rates of TMZ (up to 24 h after administration) were 7.42% and 5.93% on days 1 and 5, respectively, at 150 mg/m$^2$ per day, and 4.81% and 5.21% on days 1 and 5, respectively, at 200 mg/m$^2$ per day. The renal clearance of TMZ was 0.193 and 0.155 ml/min per kg on days 1 and 5, respectively, at 150 mg/m$^2$ per day, and 0.114 and 0.119 ml/min per kg on days 1 and 5, respectively, at 200 mg/m$^2$ per day. No change due to the difference in dose or to repeated administration was observed in the urinary excretion rate or renal clearance of TMZ. Calculation of the proportion of renal clearance to total body clearance (2.27–2.57 ml/min per kg) indicated a value of 4.81% to 7.51%.

### *Safety*

Adverse events occurred in all patients; most of these events were either mild or moderate.

The adverse events observed at an incidence of 50% or more were: constipation in 67% (four patients), nausea in 67% (four patients), increased alanine aminotransferase in 67% (four patients), increased aspartate aminotransferase in 67% (four patients), and increased blood alkaline phosphatase in 50% (three patients). These adverse events also corresponded to the adverse events for which a causal rela-

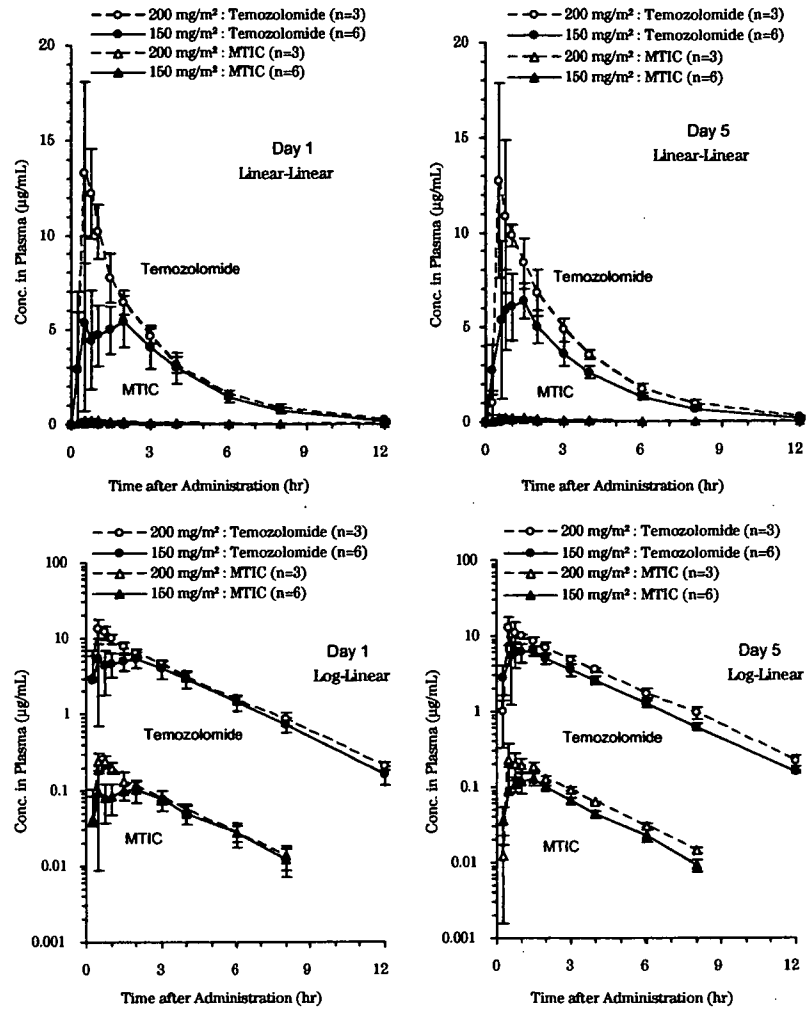



**Fig. 1.** Time-course change in mean plasma temozolomide and 5-(3-methyl)1-triazen-1-yl-imidazole-4-carboxamide (*MTIC*) concentrations on days 1 and 5 in cycle 1 (150 mg/m$^2$ per day) and cycle 2 (200 mg/m$^2$ per day)

**Table 4.** Amount of urinary temozolomide excretion (Ae), excretion rate (Ae%), and renal clearance (CLr) by urine accumulation intervals in cycle 1 (150 mg/m$^2$ per day) and cycle 2 (200 mg/m$^2$ per day)

| Parameter | Dose (mg/m$^2$) | Administration day | Time after administration (Urine accumulation interval) (h) | | | |
|---|---|---|---|---|---|---|
| | | | 0–4 | 4–8 | 8–24 | 0–24 |
| Ae (mg) | 150 | Day 1 | 11.1 (25) | 5.64 (59) | 1.49 (82) | 18.2 (22) |
| | $n = 6$ | Day 5 | 10.3 (43) | 3.37 (46) | 0.915 (55) | 14.6 (26) |
| | 200 | Day 1 | 12.8 (53) | 3.42 (62) | 0.178 (173) | 16.4 (53) |
| | $n = 3$ | Day 5 | 12.8[a] | 3.93 (32) | 1.00 (89) | 17.7[a] |
| Ae% (%) | 150 | Day 1 | 4.51 (29) | 2.32 (65) | 0.593 (80) | 7.42 (28) |
| | $n = 6$ | Day 5 | 4.20 (48) | 1.36 (47) | 0.366 (55) | 5.93 (33) |
| | 200 | Day 1 | 3.75 (57) | 1.00 (66) | 0.0524 (173) | 4.81 (57) |
| | $n = 3$ | Day 5 | 3.75[a] | 1.15 (36) | 0.300 (90) | 5.21[a] |
| CLr (ml/min per kg) | 150 | Day 1 | – | – | – | 0.193 (33) |
| | $n = 6$ | Day 5 | – | – | – | 0.155 (42) |
| | 200 | Day 1 | – | – | – | 0.114 (60) |
| | $n = 3$ | Day 5 | – | – | – | 0.119[a] |

Values are means, with coefficient of variation % in parentheses
[a] $n = 2$

tion to TMZ could not be ruled out (adverse reactions) that were observed at an incidence of 50% or more.

As myelosuppression-related adverse events, a decrease in neutrophil count (grade 2), platelet count (grade 1), and leukocyte count (grade 2) occurred in one patient each (17%). Grade 3 toxicity observed in hematology tests was a decreased lymphocyte count in one patient, and no other grade 3 or 4 toxicity was observed. Leukocyte count, platelet count, and neutrophil count were within normal ranges. No grade 3 or 4 toxicities were observed in biochemistry tests or urinalysis, except for a grade 3 increase in alanine aminotransferase in two patients.

Two adverse events resulted in death. The first was brain damage in one patient, resulting in death 23 days after the final administration in the first cycle. The second was a decreased level of consciousness in one patient who discontinued participation in the study 23 days after the final administration in the first cycle and who died about 3 months after discontinuation. The study was also discontinued in another patient 24 days after the final administration in the sixth cycle due to progression of the primary disease, and this patient died about 3.5 months after discontinuation due to aggravation of the primary disease. Three deaths occurred in this study, but the cause of death in all three patients was attributed to the primary disease.

## Discussion

We investigated the pharmacokinetics of TMZ in Japanese patients to determine whether or not the treatment regimen used in the United States and Europe could be used in Japan.

After the oral administration of 150 and 200 mg/m$^2$ per day, TMZ plasma concentration reached tmax about 1 h after administration, followed by a monophasic decrease. Although a dose-dependent increase in Cmax and AUC was observed, these values did not increase after 5 days of repeated administration (accumulation index was about 1), indicating no accumulation of this drug. The elimination of TMZ from plasma was rapid, and no change due to difference in the dose or to repeated administration was observed in CL/F or Vz/F. The coefficients of variation for AUC, $t_{1/2}\lambda z$, CL/F, and Vz/F were small, at 4% to 35%, suggesting that the interpatient difference in pharmacokinetics was small. Plasma MTIC concentrations were observed to change in parallel with TMZ plasma concentrations at both 150 and 200 mg/m$^2$ per day, and tmax and $t_{1/2}\lambda z$ values generally corresponded to those of TMZ plasma concentrations. The Cmax and AUC of MTIC plasma concentration were 1.8% to 2.0% and 1.7% to 1.8% of those of TMZ plasma concentrations. With TMZ, no accumulation was observed with repeated administration. These results suggested that the plasma MTIC concentration is dependent on the plasma TMZ concentration and that the reaction rate from MTIC to AIC is clearly more rapid than that from TMZ to MTIC. Based on the results obtained by the administration of 150 and 200 mg/m$^2$ per day, no marked change in pharmacokinetics due to the difference in dose or to repeated administration was noted. The cumulative urinary excretion rate of TMZ was 4.8% to 7.4% (up to 24 h after administration). The renal clearance of TMZ was 0.114 to 0.193 ml/min per kg, accounting for 4.8% to 7.5% of total body clearance. It is possible, however, that actual renal clearance was underestimated because of the possible effect of decomposition during the retention of urine in the bladder. The above plasma and urinary pharmacokinetic profile of TMZ in Japanese was essentially the same as that already observed in Caucasians.[8-10]

The pharmacokinetic parameters of TMZ and MTIC plasma concentrations in Japanese patients obtained in this study were compared with those obtained in pharmacokinetic studies (Schering-Plough data on file)[8,10,12] conducted

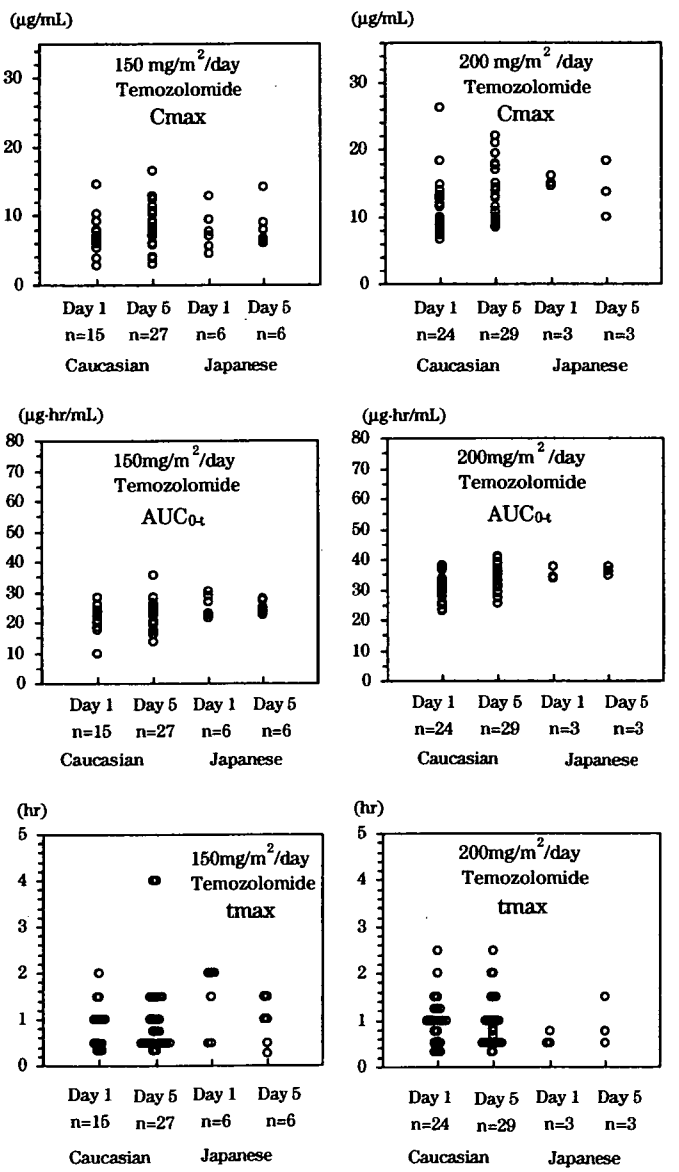
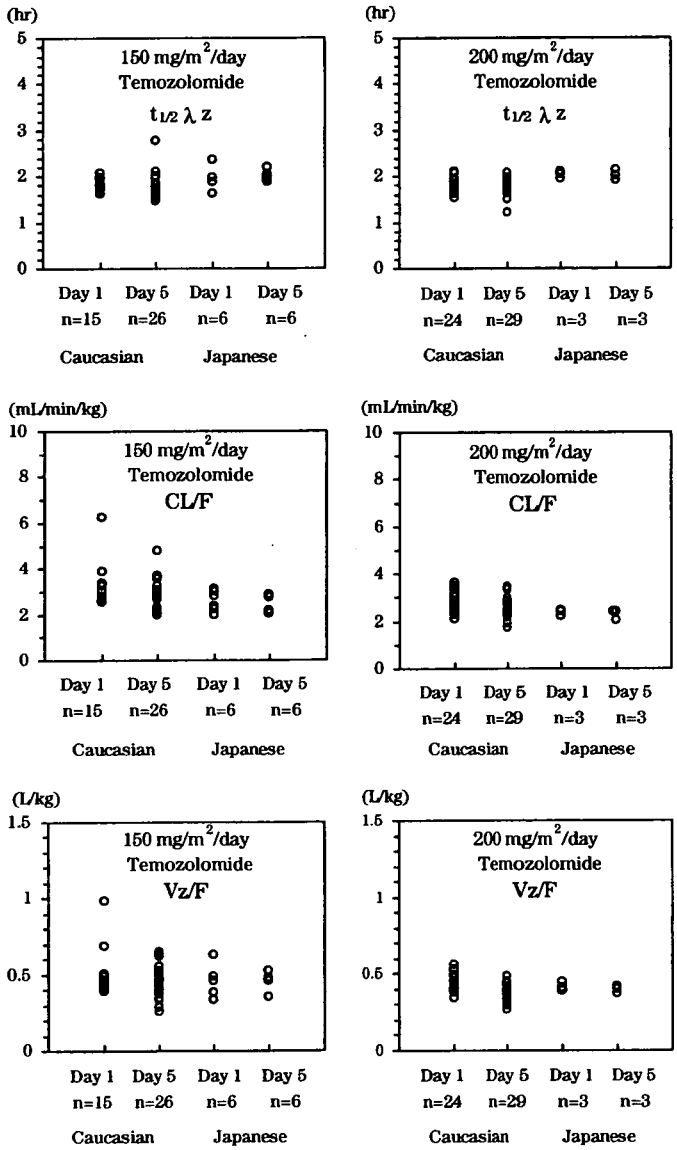



**Fig. 2.** Maximum plasma concentration (*Cmax*), area under the plasma concentration-time curve up to the final observation point ($AUC_{0\text{-}t}$), and time-to-maximum plasma concentration (*tmax*) of temozolomide: comparison between Japanese and Caucasians. Data of Caucasians are cited from Schering-Plough data on file[8,10,12]



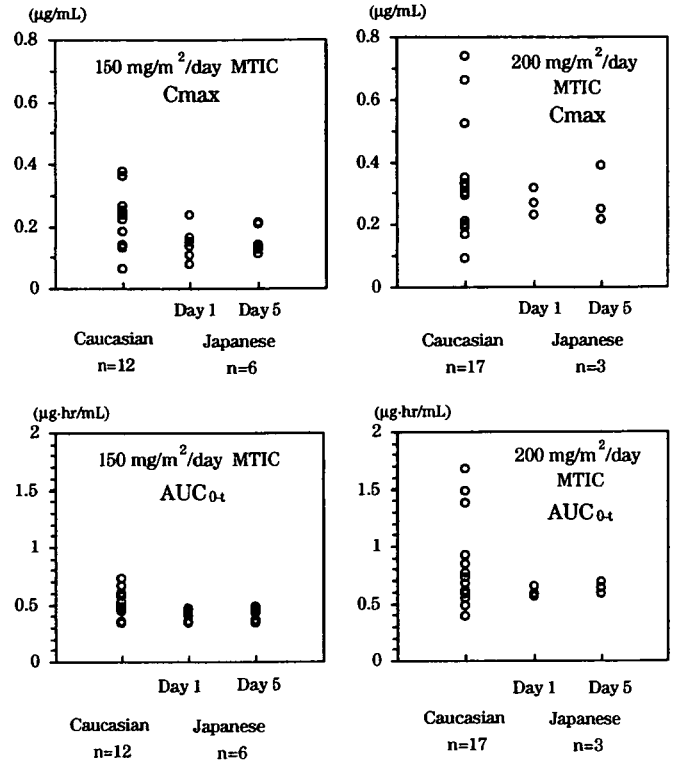




**Fig. 3.** Elimination half-life of terminal excretion phase ($t_{1/2}\lambda z$), apparent total body clearance (*CL/F*), and apparent distribution volume (*Vz/F*) of plasma temozolomide: comparison between Japanese and Caucasians. Data of Caucasians are cited from Schering-Plough data on file[8,10,12]

**Fig. 4.** Maximum plasma concentration (Cmax) and the area under the plasma concentration-time curve ($AUC_{0-t}$) of 5-(3-methyl)-1-triazen-1-yl-imidazole-4-carboxamide (MTIC) concentration: comparison between Japanese and Caucasians. Data of Caucasians are cited from Schering-Plough data on file[8,10,12]

in Caucasians in the United States. As shown in Figs. 2 to 5, the pharmacokinetic parameters (Cmax, tmax, $AUC_{0-t}$, $t_{1/2}\lambda z$, CL/F, and Vz/F) of plasma TMZ concentration and the pharmacokinetic parameters (Cmax, tmax, $AUC_{0-t}$, and $t_{1/2}\lambda z$) of plasma MTIC concentration obtained from Japanese patients all fell in the range of data obtained from Caucasian patients. These results confirm the assumption that there is little possibility that the pharmacokinetics of TMZ would be affected by biological factors including ethnic differences.

Adverse events occurred in all patients, but most were judged to be mild or moderate in severity. Continued administration was therefore possible with dose adjustment and delay in the start of administration of the next cycle. The incidence of nausea and constipation was high, but with prophylactic antiemetic administration during the administration period, no patient discontinued or interrupted treatment due to nausea during the 5 days of administration in each cycle. Constipation was managed with laxatives. Delay in the start of administration and dose modification due to myelosuppression was required in one of the four patients who continued to receive treatment with TMZ in the second cycle. The safe continuation of treatment was considered possible by monitoring for adverse reactions and adjusting the dose. No increase of myelosuppression with increased dose was observed.

The treatment regimen in this study was generally well tolerated in Japanese patients with relapsed gliomas.

The confirmation of the safety of TMZ in Japanese patients in this study contributes greatly to the assurance of safety in Asians, including patients in Taiwan and South Korea, where TMZ is already being used. The possibility is very high that the treatment regimen in the United States and Europe is applicable to all ethnic groups.

## Conflict of interest

All authors declare no conflict of interest.

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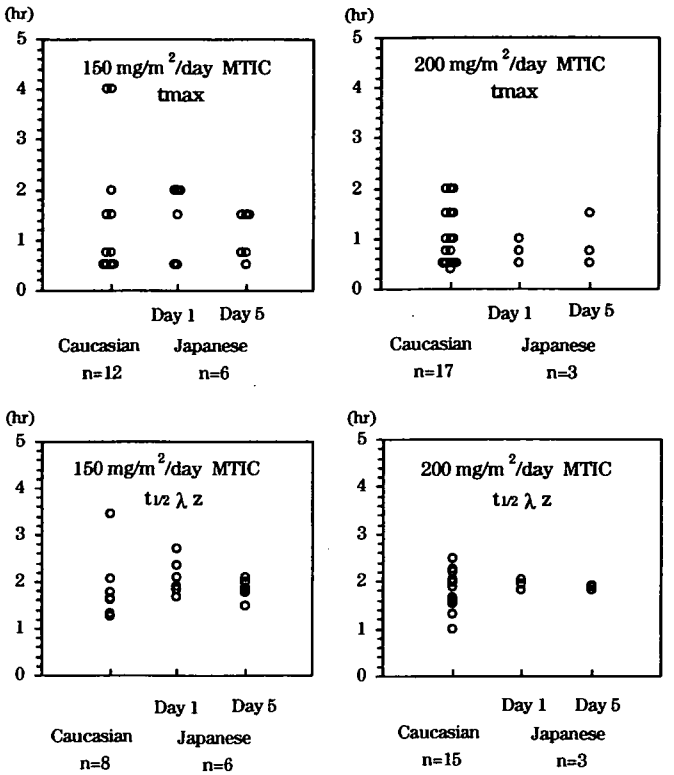


**Fig. 5.** Values for time of each plasma concentration (tmax) and terminal excretion phase ($t_{1/2}\lambda z$) of plasma 5-(3-methyl)-1-triazen-1-yl-imidazole-4-carboxamide (MTIC) concentration: comparison between Japanese and Caucasians. Data of Caucasians are cited from Schering-Plough data on file[8,10,12]

ticipate in this study, and to the study nurse and data managers for their collaboration.

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ORIGINAL ARTICLE

# Neuropilin-1 promotes human glioma progression through potentiating the activity of the HGF/SF autocrine pathway

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**Neuropilin-1 (NRP1) functions as a coreceptor through interaction with plexin A1 or vascular endothelial growth factor (VEGF) receptor during neuronal development and angiogenesis. NRP1 potentiates the signaling pathways stimulated by semaphorin 3A and VEGF-A in neuronal and endothelial cells, respectively. In this study, we investigate the role of tumor cell-expressed NRP1 in glioma progression. Analyses of human glioma specimens (WHO grade I–IV tumors) revealed a significant correlation of NRP1 expression with glioma progression. In tumor xenografts, overexpression of NRP1 by U87MG gliomas strongly promoted tumor growth and angiogenesis. Overexpression of NRP1 by U87MG cells stimulated cell survival through the enhancement of autocrine hepatocyte growth factor/scatter factor (HGF/SF)/c-Met signaling. NRP1 not only potentiated the activity of endogenous HGF/SF on glioma cell survival but also enhanced HGF/SF-promoted cell proliferation. Inhibition of HGF/SF, c-Met and NRP1 abrogated NRP1-potentiated autocrine HGF/SF stimulation. Furthermore, increased phosphorylation of c-Met correlated with glioma progression in human glioma biopsies in which NRP1 is upregulated and in U87MG NRP1-overexpressing tumors. Together, these data suggest that tumor cell-expressed NRP1 promotes glioma progression through potentiating the activity of the HGF/SF autocrine c-Met signaling pathway, in addition to enhancing angiogenesis, suggesting a novel mechanism of NRP1 in promoting human glioma progression.**
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**Keywords:** neuropilin-1; HGF/SF; c-Met; glioma

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## Introduction

Neuropilin-1 (NRP1) is a type I cell surface co-receptor that plays important roles in the development of the nervous system and angiogenesis (Bagri and Tessier-Lavigne, 2002). During neuronal development, NRP1-mediated signal transduction requires the formation of a functional semaphorin (Sema) 3A-NRP1-plexin A1 complex, which inhibits axonal guidance signals to the projecting neurons (Bagri and Tessier-Lavigne, 2002). In endothelial cells, NRP1 enhances the interaction of heparin-binding vascular endothelial growth factor $(VEGF)_{165}$ with its receptors (VEGFRs) and modulates VEGF-stimulated angiogenesis. Elevated expression of NRP1 was also found in tumor cells in various types of human cancers (Klagsbrun *et al.*, 2002). Overexpression of NRP1 in prostate and colon cancer cells enhances angiogenesis and tumor growth in animals (Miao *et al.*, 2000; Parikh *et al.*, 2004), whereas expression of an antagonist of NRP1 inhibited vessel growth and tumor expansion (Gagnon *et al.*, 2000).

Hepatocyte growth factor/scatter factor (HGF/SF) modulates various cellular functions such as proliferation, migration and morphogenesis through its cognate surface receptor c-Met (Gao and Vande Woude, 2005). Activation of the HGF/SF/c-Met signaling pathway correlates with the malignancy of human gliomas (Abounader and Laterra, 2005). Overexpression of HGF/SF in glioma cells resulted in enhanced tumorigenicity and growth *in vivo* (Laterra *et al.*, 1997). Inhibition of endogenous HGF/SF and c-Met in human cancer cells, including gliomas, reversed their malignant phenotype (Abounader *et al.*, 2002). Additionally, the activation of signaling molecules such as extracellular signal-regulated kinase (ERK) and Bcl-2 antagonist of cell death (Bad) is involved in the HGF/SF/c-Met pathway in cancer cells (Abounader and Laterra, 2005). NRP1 was recently demonstrated to interact directly with a subset of heparin-binding growth factors, such as fibroblast growth factor-2 (FGF-2), FGF-4 and HGF/SF and potentiates FGF-2 stimulation of endothelial cells (West *et al.*, 2005), suggesting that NRP1 expression in glioma cells may augment

HGF/SF/c-Met stimulation of tumor progression through an autocrine loop.

In this study, we investigated the roles of tumor cell-expressed NRP1 in human glioma progression. We show that upregulated NRP1 is primarily expressed in tumor cells, and NRP1 expression correlates with tumor progression in clinical glioma specimens. We demonstrate that NRP1 expression promotes glioma growth and survival *in vitro* and *in vivo* through an autocrine HGF/SF/c-Met signaling pathway involving activation of c-Met, ERK and Bad, thus suggesting a novel mechanism of NRP1 expression in promoting cancer cell survival and proliferation.

## Results

*Upregulation of NRP1 is correlated with the malignancy of human astrocytic tumors*

To determine the association of NRP1 expression with glioma progression, we performed immunohistochemistry (IHC) analyses on a total of 92 human glioma specimens and four normal human brain biopsies using three well-characterized anti-NRP1 antibodies (Ding *et al*., 2000) and isotype matched IgGs as negative controls that all showed no staining (see the insets in Figure 1A). As shown in Figure 1Aa, in all four normal brain tissues analysed, weak immunoactivity for the anti-NRP1 antibody was detected in neurons (red arrow) or cells within a blood vessel (arrowhead). In four pilocytic astrocytoma specimens (P.A., WHO grade I), NRP1 was weakly stained in a few tumor cells (Figure 1Ab, arrows) and vessels (panel b, arrowhead). In 24 WHO grade II gliomas, NRP1 protein was detected in tumor cells (panel c, arrows) and vessels (panel c, arrowhead). In 21 WHO grade III glioma biopsies, a greater intensity of NRP1 staining was detected in tumor cells (Figure 1Ad, arrows). In 43 WHO grade IV glioblastoma multiforme (GBM) specimens, high expression of NRP1 was found in tumor cells (Figure 1Ae, arrows). In general, no increase in staining for NRP1 protein was found in hypoxic/pseudopalisading regions, but heterogeneous staining for NRP1 expression was seen within the gliomas. As summarized in Figure 1B and Supplementary Table S1 (Supplementary Material), statistical analyses of our IHC data revealed a significant correlation between NRP1 expression and human glioma progression. There was a significant difference in IHC staining for NRP1 among the three groups as well as a correlation between NRP1 expression and the malignancy of human glioma (Figure 1B).

*Overexpression of NRP1 in U87MG xenografts promotes tumor growth and angiogenesis* in vivo

To further investigate whether upregulation of NRP1 by glioma cells promotes tumor progression, we first examined expression of NRP1 in human glioma cell lines by immunoblot (IB) analyses. As shown in Figure 2a, NRP1 protein was detected at various levels in all glioma cell lines examined. As U87MG cells express NRP1 at a relatively low level and are highly tumorigenic in mice (Hu *et al*., 2003), we utilized this cell line to stably overexpress NRP1. Among various U87MG cell clones that stably express NRP1, we chose two cell clones, U87MG/NRP1-no. 1 and U87MG/NRP1-no. 8, that expressed exogenous NRP1 at medium (NRP1-no. 1) or high levels (NRP1-no. 8) compared with U87MG (Figure 2b) or LacZ (see below) cells for further studies. Next, we separately implanted U87MG and NRP1 cells into the flank or the brain of nude mice. On the 26th day post-implantation, inoculation of NRP1 cells into the flank resulted in formation of tumors with an average volume of $1205 \pm 307\,mm^3$, whereas mice that received U87MG or LacZ cells (Guo *et al*., 2001) developed tumors with similar volumes in 45 days (Figure 3A). In the brain, mice receiving NRP1 cells developed tumors with a volume of $34 \pm 6.8\,mm^3$ in $25 \pm 3$ days ($n = 17$) (Figure 3Bc, d and 3C), whereas mice inoculated with U87MG or LacZ cells developed tumors of $15 \pm 4.5\,mm^3$ in the same period of time ($n = 15$) (Figure 3Ba, b and 3C). Afterwards, we stained the brain tumor tissue using anti-CD31 (Figure 3Bb and d) and anti-bromodeoxyuridine (BrdUrd) plus anti-von Willebrand factor (vWF) antibodies (Figure 3E). We found that NRP1 intracranial tumors had a 2.5-fold increase in vessel density (Figure 3D) and a 2.6-fold increase in BrdUrd incorporation in the tumor cells when compared with U87MG tumors (Figure 3F).

*NRP1 promotes U87MG cell growth through enhancing autocrine HGF/SF/c-Met signaling*

Our results show that overexpression of NRP1 by glioma cells enhances tumor cell proliferation *in vivo*, which could possibly be due to NPR1-modulated autocrine intracellular signaling stimulation in glioma cells or caused indirectly by an increase in angiogenesis. To distinguish these possibilities, we performed a trypan blue vital dye exclusion assay. NRP1 overexpression did not significantly affect NRP1 cell growth compared with U87MG or LacZ cells when cultured in medium containing 10% fetal bovine serum (FBS) (data not shown). However, as shown in Figure 4A, in the absence of serum, NRP1 cells showed a 1.8-fold increase in cell survival, whereas U87MG or LacZ cells demonstrated a slight decrease of cell survival in a 4-day culture, suggesting that autocrine signaling through NRP1 promotes cell survival in these glioma cells.

A recent study demonstrated NRP1 also interacts with several heparin-binding growth factors, such as FGF-2, FGF-4 and HGF/SF, and potentiates the growth stimulatory activity of FGF-2 on endothelial cells (West *et al*., 2005). As HGF/SF and FGF-2 were shown to stimulate cell growth through receptor-mediated autocrine signaling in glioma cells (Abounader and Laterra, 2005), we performed enzyme-linked immunosorbent assay (ELISA) and determined whether the autocrine signaling activities of these growth factors were involved in the NRP1-stimulated U87MG cell survival and growth. In a 48-h cell culture,