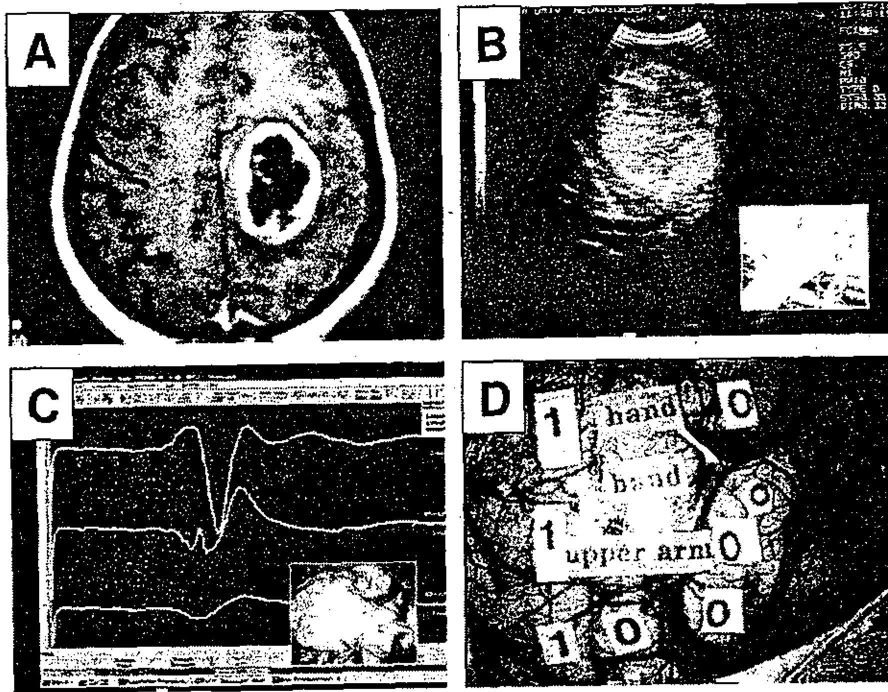

Fig.1
Intraoperative monitoring of motor-evoked potentials (MEPs). A. Contrast-enhanced T1-weighted MR image demonstrating a ringed enhanced tumor in the left frontal lobe. B. Intraoperative ultrasound detection of the tumor. C. Computer monitor showing positive responses for MEP upon electrical cortical stimulation. D. Cortical mapping for motor function. 0: no response, 1: positive response.

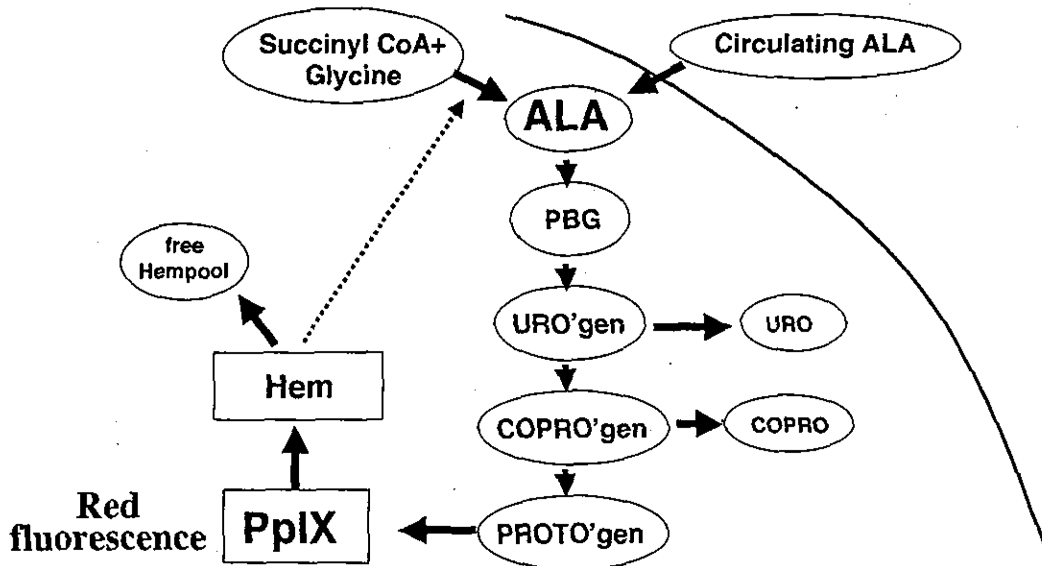

Fig.2
Molecular pathways of hem biosynthesis in cells. A hem precursor 5-aminolevulinic acid (ALA) is catalyzed by multiple enzymes to generate fluorescent protoporphyrin (Pp) IX in malignant tumor cells.

ロパックΣ、日本光電)。腫瘍摘出中は最も検出のよかった脳表皮質上にて数秒間隔の刺激を継続し、手術操作によるMEP減弱の有無をモニター、減弱が生じた場合は手術操作休止の処置をとるようにしている[3]。

2000年3月から2002年10月までに、テント上の悪性脳腫瘍計20例に術中MEPを施行した。その内訳は、astrocytoma 1例、anaplastic astrocytoma 2例、GBM 11例、*anaplastic oligodendroglioma* 1例、metastatic tumor 3例、PCNSL 1例、sarcoma 1例である。Eloquent areaとしては、motor cortexおよびsubcortical tract 15例、深部motor neuron tract 3例、perforator(*lenticulostriate arteries, anterior choroidal arteries*)領域2例であった。結果としては、14例(70%)でMEP 測定が可能であった。術前の患側上下肢MMTが3/5以上の症例が11例と、比較的術前の運動機能が維持されている症例が大部分を占めた。しかし1例で術前MMT 0/5と完全麻痺を呈していたが、術中MEP測定が可能で、術後MMT 2/5までの回復がみられた。術前MMT 3/5で、術中MEPは終始維持されていた症例では、術後完全麻痺を呈したが、約2週間で3/5までの回復が認められ、術中MEP測定結果の有用性が示された。一方、6例(30%)で術中MEP測定が不可能であった。このうち3例は術前の患側上下肢麻痺がMMT 2/5と比較的強度であった。1例はMMT4/5であったが、病変部がface motor 領域で、導出筋電図を上下肢のみでモニターしていたため術野内での刺激では、不十分であった可能性がある。残る2例は術前麻痺を認めていなかった症例であるが、1例は初期の症例で、条件設定などが不完全であった可能性がある。他の1例は再発腫瘍で、先行治療の影響と考えられる脳表面と硬膜との強固な癒着があり、MEP測定以前の皮質損傷が一因となっている可能性も考えられる。

現在、主に単電極による刺激を行っているが、より限局した部位同定のために双極刺激電極の使用を予定している。また脳表のみならず、腫瘍摘出中の摘出壁における深部motor tractの検出が術中の機能損傷の回避には重要であり、その検出率の向上を図っている。このような神経モニタリングが、eloquent regionにある悪性脳腫瘍患者の生命および機能予後の改善に如何に寄与できるかが今後の検討課題であろう。

3) 5-Aminolevulinic acid(5ALA)を用いた術中蛍光診断:

Gliomaの手術においては、腫瘍を摘出する際に周囲正常脳との境界が不明瞭のことが少なくなく、摘出範囲の決定や残存腫瘍の有無を肉眼的に判断することは一般に容易ではない。われわれは2001年より5ALAを術前投与し、術中腫瘍蛍光診断を施行している。

(1)5ALAによる術中蛍光診断の特徴:

5ALAは生体内に存在する分子で、hemの生合成に必要な前駆物質である。従って5ALA自体の生体への毒性は少ないと考えられる。5ALA自体は蛍光物質ではなく、図に示すように幾つかの酵素反応を介して、hemの前駆体であり青色光により赤色の蛍光を発するprotoporphyrin(Pp)IXが細胞内で生合成される(Fig.2)。また、このPpIX生合成に必要な酵素活性は正常脳細胞に比べ悪性腫瘍、特にGBMなどで高いことが知られており、腫瘍細胞に特異的な反応とされている。即ち、理論的には5ALAが含まれている血液の混入や脳組織内への浸透によっては蛍光は誘発されず、低いfalse positivityで、PpIXが合成された腫瘍細胞を特異的に検出することが可能であろうと考えられる[4]。

(2)方法・対象:

5ALA(Nakalai)は手術室入室前に、30 mlの5% glucose溶液に溶解し、約~20mg/kg(1g/body)の容量で経口投与した。手術中、手術用顕微鏡に搭載されているXenon光源を用いて405nm(±10%)のexcitation filterを通して腫瘍表面に照射し、赤色のPpIXの発光を455 nm~の観察用cut filterを介して肉眼的に観察した。2001年3月より2003年1月までに計24例の脳腫瘍症例に施行したが、本法については、杏林大学医学部の倫理委員会の承認を受けて、各症例で文書による同意を得て施行している。

(3)結果・考察:

当初の7症例では、明らかなPpIXの赤色の蛍光発光は検出が出来なかった。逆に最近では12例中11例で、明瞭な赤色発光を確認することが可能であった。これは光源や励起用並びに観察用のfilterなどhardwareの改良が原因と考えられる。蛍光発光が認められたこれら11症例の内訳は、anaplastic astrocytoma(recurrent)2例、GBM(再発例1例を含む)6例、転移性脳腫瘍3例であった。この全例で、術前のGd造影MRIにて造影増強効果が認められていた。発色が確認できなかった最近の1例は、深部小脳のpilocytic astrocytomaで、腫瘍全摘後に施行したためと、狭く深い術野のため、励起光が到達しなかったことが原因と考えられる。悪性リンパ腫はこれまでに脳原発3例、転移性1例で施行したが、いずれも陰性であった。

術中にPpIX発光の陽性所見が得られた症例(2002年10月まで)において、肉眼的発光度の程度と、腫瘍細胞の密度につき、病理組織学的に検討した。術中、強い蛍光強度が得られた部位の病理所見は、6例全てで比較的高い細胞密度を示す腫瘍本体であった。一方、腫瘍辺縁で蛍光がnegativeであった部位は、明らかな腫瘍細胞を認めなかった。術中、淡い蛍光を発していた部位では、脳組織の中に腫瘍細胞が巣状にあるいは浸潤しながら存在しており(2症例)、Stummerの報告と同様に[5]、術中のPpIX蛍光強度と病理所見とは比較的良好な相関関係があると考えられた(Fig.3,4)。従って、5ALAによる術中蛍光診断の腫瘍摘出への応用法としては、強度の蛍光を認める部位は積極的に摘出を図ることが望ましく、淡い蛍光が認められる部位には腫瘍

Fig.3

A case with recurrent anaplastic astrocytoma in the right frontal lobe. The fluorescent intensity of PpIX of the tumor tissues correlates well with cellularity of tumor cells examined histologically.

Fig.4

A case with glioblastoma multiforme in the left frontal lobe. A resected tumor specimen showing high intensity of PpIX (upper panel) consists of hypercellular tumor, whereas a specimen with low fluorescence contains infiltrative tumor cells in the normal brain parenchyma.

以外に正常脳組織も存在していたことから、non-eloquent areaであれば積極的に、eloquent areaであれば慎重に他のモニタリング等も併用しながら摘出範囲を決定すべきであろうと考えられる。

全例において、5ALA経口摂取後の嘔気などの消化器症状は認めなかった。また光過敏症状もみられていない。術後2週間までの手術回復期に、5ALAを投与した19例中、血清creatinineのgrade 1レベル(JCOG基準)の上昇が2例、GOT上昇grade 1が5例、GOT上昇はgrade 1が7例、grade 2が2例の計9例、白血球の減少がgrade 2が1例、grade 3が1例に認められたが、何れも速やかに正常域に回復し、異常値が遷延したり特別な治療を要したものは認められなかった。また、これらの異常値が5ALA投与による直接の副作用であるかは不明であり、5ALAの投与は比較的安全に行いえると考えている。

我々のこれまでの経験から、術前の造影MRIで良好な造影増強効果がみられる腫瘍では、高率でPpIX発光が陽性となることが期待され、励起光の照射が困難な深部腫瘍を除いて、本法の良い適応になるものと考えられる。一方、造影剤にて造影されないようなlow grade astrocytomaにおける蛍光診断については、今後更に症例を重ねて検討して行く必要があろう。また、現行のシステムでは、PpIX蛍光を観察するために、手術用の顕微鏡の光源を落とし、手作業でPpIX励起用光の照射を行う必要が有り、操作がやや煩雑であるという欠点がある。更にPpIXの赤色発光を通常の手術顕微鏡のモニターカメラで記録することは、波長・信号強度などの影響で十分ではなく、高感度CCDカメラが必要である。PpIXの蛍光強度を定量的に計測するためには特殊な計測機器が必要であり、より客観的な評価を進めるうえで、built-in microscopeをはじめとして更にhardwareの面での改良が望まれる。本法を発展させた形として、蛍光発光を利用したphotodynamic therapyによる残存腫瘍の治療が現在検討されており、本アプローチはますます注目されていくことと予想される。

③術後補助療法：

1）放射線療法：

術後の残存腫瘍・浸潤部に対して、LINACによる局所外照射を通常60 Gy(2 Gy/fr x 30 fr, 5 fr/week, 6 weeks)を原則施行している。

2）化学療法：MGMT発現度による個別化化学療法

(1)背景：

最近、オーダーメイド治療と呼ばれる悪性腫瘍に対する個別化治療(individual adjuvant therapy, IAT)が各治療側面から検討されるようになってきた。悪性gliomaにおいては、腫瘍細胞の抗癌剤耐性と薬剤耐性関連遺伝子の発現の間に相関性あるいは関連性があることが報告されてきており[6]、個々の腫瘍においてそのような薬剤耐性関連遺伝子の発現状態により使用すべき抗癌剤の選択を行なう個別化化学療法は、合理的な治療法になりえる可能性をもっている。特にアルキル化剤であるACNUへの耐性は、特殊なDNA修復酵素であるO6-methylguanine-DNA methyltransferase (MGMT)の発現が関与していることが示されており[7]、MGMT mRNAの発現量を基にした個別化化学療法が試みられている[8-10]。

(2)方法・対象：

2000年3月以降、手術摘出腫瘍標本からtotal lysateを抽出し、Western blot法によるMGMT蛋白発現の解析を行っている(Fig.5)[11]。この間、悪性gliomaの、anaplastic astrocytoma 8例、GBM 16例についてMGMT蛋白の発現量を定量し、対照として用いるACNU耐性でMGMTを高発現しているヒトglioma細胞株T98GのMGMT発現量を100%として、相対的MGMT蛋白発現量を算定した。標本間での蛋白量の比較のためβ-actinの発現量を用いて標準化した。原則として、MGMTが低発現(T98GのMGMT発現量の20%以下)の腫瘍にはACNUとVP16の併用療法を、高発現(T98GのMGMT発現量の20%以上)の腫瘍にはCBDCA、VP16の併用療法を放射線治療と併用して施行した。PD以外の症例では、放射線治療後も維持療法として同じregimenで1～2ヶ月間隔で繰り返し施行した。

(3)結果：

Anaplastic astrocytoma 8例のうち、相対MGMT発現量が20未満の症例が4例、20以上の症例4例であった。MGMT値20未満の2症例でACNUが用いられ、Time to tumor progression(TTP)(日)は、448+, 1047+で、共に未再発であったのに対し、MGMT値20以上で、ACNU治療がなされた2症例では、TTPが185, 191と早期に再発が認められた。MGMT値20以上で、platinum系のCDDP或いはCBDCAが使用された2症例では、TTPがそれぞれ207+, 359+と再発が認められていない。

GBM16例のうち、MGMT値が20未満であった症例は8例(50%)であった。このうち6例でACNUを含む化学療法が施行され、TTPは58+, 140, 177, 238+(gross total removal : GTR), 863+, 879+(GTR)であり、長期にdisease freeで生存する患者がみられた。MGMT値が20以上であった症例も8例(50%)あり、内4例がACNU+VP16で治療された。評価可能な3症例のTTPは、107 (GTR), 112+, 289+(extended lobectomy)であった。残る4例ではCBDCA+VP16(3例)あるいはCDDP+VP16(1例)が施行され、TTPはCBDCAの症例で102+(GTR), 114, 126, CDDPの症例で62と早期に再発する傾向が認められた。

これまでのところ、GBMでは中間追跡期間が289日で、1年progression-free survival(PFS)は37.5%(Fig.6)、1年生存率は53.5%(Fig.7)、anaplastic astrocytomaでは中間追跡期間は404日で、1年PFSは58.3%、1年生存率は72.9%であった。

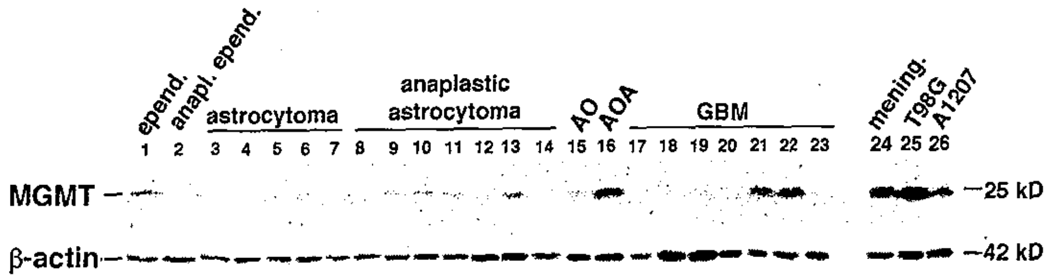

Fig.5

Western blot analyses of MGMT expression in human glioma tissues. Human glioma cell lines T98G and A1207 are used as positive controls. β-actin expression is shown as an internal control. Epend, ependymoma; Anapl, anaplastic; AO, anaplastic oligodendroglioma; AOA, anaplastic oligoastrocytoma.

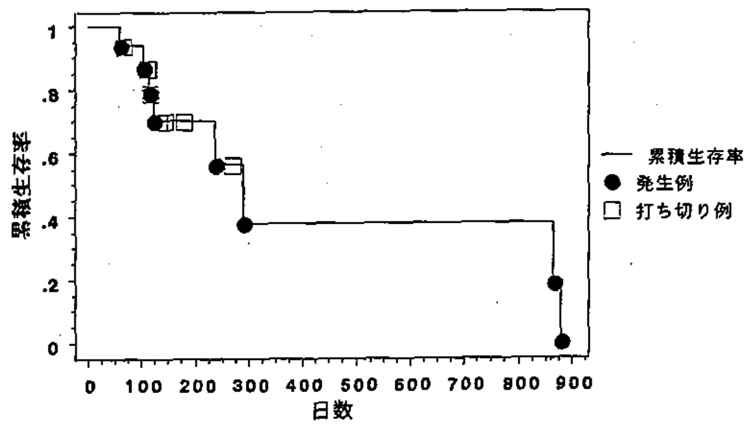

Fig.6

Kaplan-Meier progression-free survival curve of patients with glioblastoma treated in the Kyorin University Hospital since 2000

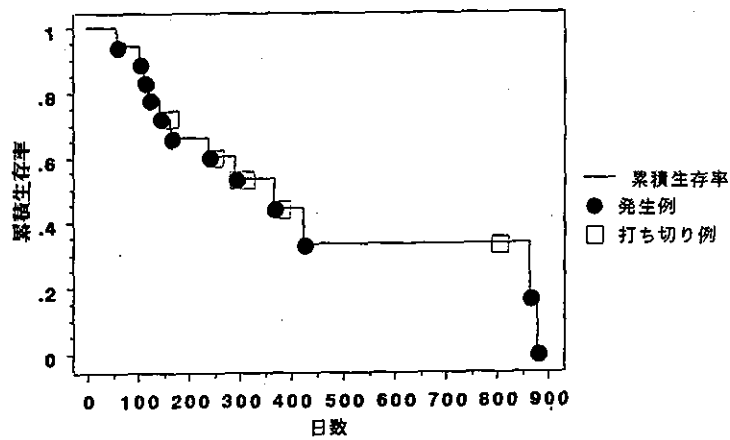

Fig.7

Kaplan-Meier overall survival curve of patients with glioblastoma treated in the Kyorin University Hospital since 2000.

(4)考 察:

悪性gliomaに対して一律に同様の化学療法を行うのではなく、個々の腫瘍の性質に応じて化学療法剤を選択する個別化化学療法の概念は、これまで以上に化学療法の有効性を高め、また不必要な副作用を回避することが理論的には可能であり、21世紀における癌医療の一つの方向性を示していると考えられる。こと悪性gliomaに関しては、手術・放射線治療のみでは治癒を期待することが未だ困難であり、化学療法の担う役割は大きいといえる。悪性脳腫瘍の化学療法で最も標準的に使用されているnitrosourea系抗癌剤(本邦では主としてACNU)に関しては、これまでその殺細胞効果の最初のステップで形成されるO6-alkylguanineに対する特異的修復酵素であるMGMTの活性あるいは発現量が、ACNUの耐性度と良好な相関関係があることを報告されてきた[7]。従って、摘出した腫瘍においてMGMTが高発現している場合はACNUによる治療効果が期待できない可能性が予想される。

我々は比較的簡便に施行できる蛋白レベルでのMGMT発現の検出を行っている。遺伝子発現の検出にはmRNAレベルでも可能であるが、実際に耐性の機序を担うのは遺伝子産物の蛋白・酵素であり、またmRNA発現量と蛋白発現量は必ずしも相関しないことも知られているため、蛋白レベルでの定量がより有用と考えている。実際、現在使用している抗MGMTモノクローナル抗体により、特異的且つ高感度にMGMTの発現がほぼ全てのglioma標本に検出された。更にβ-actin等のhouse keeping geneの産物をinternal controlとすることで発現量を標準化し、標本間での発現量差を比較することが可能であった。

MGMT発現量によるIATを行う際に問題となるのは、ACNUに抵抗性のMGMT高発現の判定基準である。MGMTの発現の有無で選別する方法では、ほぼ全てのgliomaでMGMTの発現が検出されたことからACNUを使用できる症例が殆どないということになり、非現実的と考えられた。そこで現在はACNU耐性のT98G細胞のMGMT発現量に対し、20%の発現量を目安とした選別を行っているが、in vitroのMGMT overexpressionの実験から、このMGMT発現量はACNUのIC50値として60μM程度の耐性度に相当することが示唆された[11]。しかし臨床上、ACNUを100~150mg/bodyで静注した後の最高血中濃度は10μM程度であることから、この選別レベルが適切であるか否か、今後更なる検討が必要と考えられる。これまでの症例では、相対MGMT発現量が20%未満の症例が約半数であった。その中でACNUを使用した症例では、anaplastic astrocytoma, GBMともに治療に反応を示した腫瘍や、再発せずに生存している症例が認められている。一方で、相対MGMT発現量が20以上でplatinum系の抗癌剤を使用した症例では、早期に再発をきたした症例が多い。当初は30%の発現量をcut-off lineとしていたため、MGMT発現量が20-30の3症例でACNUが使用されたが、1例が早期再発、1例は3ヶ月でNC、1例ではlobec-tomyが施行され10ヶ月間再発を認めていない。これらの結果から、MGMT発現量が20%未満の場合にACNUを第一選択とすることは妥当と考えられるが、MGMTが高発現の場合に使用する薬剤の選択は未だ解決されていない重要な問題である。Platinum系の薬剤の有効性は、投与量にもよると考えられるが、これまでのところ明らかではない。Platinum系薬剤の耐性機序は多因子性と考えられており、現時点ではACNUに対するMGMTと同様なアプローチでの個別化は困難である。また全例でetoposideを併用しており、etopo-side耐性の一因とされているTopoisomerase IIの蛋白発現レベルの検量を試みているが、使用したモノクローナル抗体は細胞株では陽性であったが腫瘍標本での感受性が低く、IAT化には至っていない。更にP-glycoprotein(MDR1遺伝子産物)やMRP family蛋白の発現とetoposide感受性との関連性など解明されるべき事項が残されている。

MGMTの高発現が認められる場合、ACNU以外の他剤を選択する方法の他に、MGMTを一時的に失活させることでACNU耐性の克服を計る方法も検討されている。我々はACNU耐性rat glioma細胞株C6ARにおいて、antisense MGMT RNAによるACNU感受性化を報告したが[12]、欧米ではO6-benzylguanineを用いたclinical trialが現在実施されており、今後の成果が期待されている[13]。

薬剤耐性関連遺伝子の他にも、anaplastic oligodendro-gliomaにおける染色体1番短腕および19番長腕の欠失のように化学療法感受性と強い相関を示すgenetic markerの存在が明らかにされてきている[14,15]。また化学療法剤の解毒機構とも関与の深い薬剤代謝酵素系には個体差がみられ、その違いをsingle nucleotide polymorphism(SNP)sの解析により判定し、化学療法施行上の指標としていこうとする検討も精力的に進められており[16]、多方面からのIAT化が今後推進されていくものと考えられる。

【結 語】

現在、杏林大学病院にて行っている悪性gliomaに対する治療法につき概説した。まだ症例数が少なく、また治療期間も短いこともあり、有意な予後改善が得られるまでには至っていないが、今後更に症例を重ね、本法の有効性について検討していく予定である。

【文 献】

1) The Commettee of Brain Tumor Registry of Japan: Report of Brain Tumor Registry of Japan (1969-1993), 19th ed. Neurl Med chir (Tokyo) 40 (Suppl): 2000.

2) Lacroix M, Abi-Said D, Fourney DR, Gokaslan ZL, Shi W, DeMonte F, Lang FF, McCutcheon IE, Hassenbusch SJ, Holland E, Hess K, Michael C, Miller D, Sawaya R : A multivariate analysis of 416 patients with glioblastoma multiforme : prognosis, extent of resection, and survival.
J Neurosurg 95: 190-198., 2001.

3) 飯野奈央子，塩川芳昭，斎藤勇．運動野近傍の脳腫瘍摘出時の運動誘発電位 Motor evoked potential (MEP)によるマッピングとモニタリングの有用性．医学検査 51: 1294-1298, 2002.

4) 金子貞男．脳腫瘍に対する光モニタリング--ALA induced Pp IXによる術中脳腫瘍蛍光診断--．脳外29: 1019-1031, 2001.

5) Stummer W, Novotny A, Stepp H, Goetz C, Bise K, Reulen HJ : Fluorescence-guided resection of glioblastoma multiforme by using 5-aminolevulinic acid-induced porphyrins: a prospective study in 52 consecutive patients.
J Neurosurg 93 : 1003-1013., 2000.

6) Nagane M, Asai A, Shibui S, Oyama H, Nomura K, Kuchino Y: Expression pattern of chemoresistance-related genes in human malignant brain tumors : a working knowledge for proper selection of anticancer drugs. Jpn J Clin Oncol 29: 527-534, 1999.

7) Nagane M, Asai A, Shibui S, Nomura K, Matsutani M, Kuchino Y : Expression of O6-methylguanine-DNA methyltransferase and chloroethylnitrosourea resistance of human brain tumors.
Jpn J Clin Oncol 22: 143-149, 1992.

8) Nagane M, Shibui S, Oyama H, Asai A, Kuchino Y, Nomura K : Investigation of chemoresistance-related genes mRNA expression for selecting anticancer agents in successful adjuvant chemotherapy for a case of recurrent glioblastoma.
Surg Neurol 44: 462-468 ; discussion 468-470, 1995.

9) Tanaka S, Kobayashi I, Utsuki S, Oka H, Fujii K, Watanabe T, Nagashima T, Hori T: O6-methylguanine-DNA methyltranspherase gene expression in gliomas by means of real-time quantitative RT-PCR and clinical response to nitrosoureas. Int J Cancer 103 : 67-72., 2003.

10) Tanaka S, Kamitani H, Amin MR, Watanabe T, Oka H, Fujii K, Nagashima T, Hori T : Preliminary individual adjuvant therapy for gliomas based on the results of molecular biological analyses for drug-resistance genes.
J Neurooncol 46: 157-171, 2000.

11) 永根基雄，山口竜一，水谷徹，三木啓全，伊藤宣行，伊東聡行，塩川芳昭，齋藤勇．薬剤耐性関連遺伝子の蛋白発現量によるグリオーマの個別化化学療法の試み．ポストシークエンス時代における脳腫瘍の研究と治療，田渕和雄・白石哲也編，九州大学出版，pp375-381, 2002.

12) Nagane M, Asai A, Shibui S, Nomura K, Kuchino Y : Application of antisense ribonucleic acid complementary to O6-methylguanine -deoxyribonucleic acid methyltransferase messenger ribonucleic acid for therapy of malignant gliomas.
Neurosurgery 41: 434-440; discussion 440-431, 1997.

13) Friedman HS, Pluda J, Quinn JA, Ewesuedo RB, Long L, Friedman AH, Cokgor I, Colvin OM, Haglund MM, Ashley DM, Rich JN, Sampson J, Pegg AE, Moschel RC, McLendon RE, Provenzale JM, Stewart ES, Tourt-Uhlig S, Garcia-Turner AM, Herndon JE, 2nd, Bigner DD, Dolan ME : Phase I trial of carmustine plus O6-benzylguanine for patients with recurrent or progressive malignant glioma.
J Clin Oncol 18: 3522-3528., 2000.

14) Smith JS, Perry A, Borell TJ, Lee HK, O'Fallon J, Hosek SM, Kimmel D, Yates A, Burger PC, Scheithauer BW, Jenkins RB : Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. J Clin Oncol 18: 636-645., 2000.

15) Cairncross JG, Ueki K, Zlatescu MC, Lisle DK, Finkelstein DM, Hammond RR, Silver JS, Stark PC, Macdonald DR, Ino Y, Ramsay DA, Louis DN: Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas.
J Natl Cancer Inst 90: 1473-1479., 1998.

16) Pirmohamed M, Park BK : Genetic susceptibility to adverse drug reactions.
Trends Pharmacol Sci 22: 298-305., 2001.

Correlation of Histology and Molecular Genetic Analysis of 1p, 19q, 10q, *TP53*, *EGFR*, *CDK4*, and *CDKN2A* in 91 Astrocytic and Oligodendroglial Tumors[1]

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ABSTRACT

Purpose: **The histological diagnosis of human gliomas is of great importance for estimating patient prognosis and guiding therapy but suffers from being subjective and, therefore, variable. We hypothesized that molecular genetic analysis could provide a more objective means to classify tumors and, thus, reduce diagnostic variability.**

Experimental Design: **We performed molecular genetic analysis on 91 nonselected gliomas for 1p, 19q, 10q, *TP53*, epidermal growth factor receptor, and cyclin-dependent kinase 4 abnormalities and compared with the consensus diagnoses established among four independent neuropathologists.**

Results: **There were six astrocytomas, seven anaplastic astrocytomas, 45 glioblastomas, 21 oligodendrogliomas, eight anaplastic oligodendrogliomas, three oligoastrocytomas, and one anaplastic oligoastrocytoma. Twenty-nine cases had either 1p or 19qloss of heterozygosity (LOH) while retaining both copies of 10q, of which 25 (86%) were histologically oligodendroglioma, anaplastic oligodendroglioma, oligoastrocytoma, or anaplastic oligoastrocytoma. As for the oligodendroglial tumors, unanimous agreement of the initial diagnoses was almost restricted to those cases with combined 1p/19qLOH, whereas all nine tumors without 1p loss initially received variable diagnoses. Interestingly, *TP53* mutation was inversely related to 1pLOH in all gliomas ($P = 0.0003$) but not 19qLOH ($P = 0.15$).**

Conclusions: **These data demonstrate that molecular genetic analysis of 1p/19q/10q/*TP53* has significant diagnostic value, especially in detecting oligodendroglial tumors. In addition, 1pLOH and *TP53* mutations in gliomas may be markers of oligodendroglial and astrocytic pathways, respectively, which may separate gliomas with the same histological diagnosis, especially oligodendroglial tumors and glioblastomas. Testing for those molecular genetic alterations would be essential to obtain more homogeneous sets of gliomas for the future clinical studies.**

INTRODUCTION

Treatment of diffuse gliomas still remains one of the toughest challenges in oncology. Surgical cure of these tumors infiltrating into the brain is practically impossible, and their clinical course is primarily determined by the biological behavior of the tumor cells, including growth rate and their responsiveness to radiation therapy and chemotherapy. For the prediction of such biological behavior, the most reliable, proven method has been the histological diagnosis based on the microscopic morphology of the tumor since the era of Bailey and Cushing. However, histological diagnosis suffers from being subjective and, therefore, variable in some cases, which occasionally hampers the clinical studies on gliomas. On the other hand, recent progress in the molecular biology and molecular genetics is showing a potential to provide new means to dissect the biological features of gliomas.

Various genetic alterations have been identified in the tumorigenesis and progression of diffuse gliomas (1–4). Amplification of oncogenes has been observed for *EGFR*,[3] *CDK4*, and *MDM2* genes (5, 6) and inactivating mutation or deletion of tumor suppressor genes has been discovered for *TP53*(17p), *RB*(13q), *CDKN2A*(9p), *PTEN*(10q), and *DMBT1*(10q) (7–11). Moreover, several chromosomal loci presumed to contain tumor

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; CDK, cyclin-dependent kinase; LOH, loss of heterozygosity; AA, anaplastic astrocytoma; AC, astrocytoma; GBM, glioblastoma; OG, oligodendroglioma; AOG, anaplastic oligodendroglioma; OA, oligoastrocytoma; AOA, anaplastic oligoastrocytoma.

suppressor genes, such as *1p, 19q, 11p,* and *22q,* show frequent allelic losses in gliomas, which are easily detected as LOH (2). Among those, LOH at short arm of chromosome 1 (1p) and long arm of chromosome 19 (19q) is found in 60–70% of OGs (12–15), and recent studies demonstrated that 1pLOH in AOGs, frequently accompanied by 19qLOH, predicted sensitivity to specific chemotherapy and better overall survival (16, 17). Although the underlying molecular biological mechanism for this observation is yet unknown, 1pLOH seems to represent a specific biological feature of tumor cells, at least in OGs. On the other hand, 1pLOH is also found in 50–70% in OAs and 10–20% of astrocytic tumors, including GBMs (4, 18), and its significance within these histological types is not yet clear. Therefore, we studied a series of diffuse gliomas of various grades and histologies for a range of genetic markers known to be important in glioma tumorigenesis. Importantly, we carefully classified these 91 gliomas using independent histological review by four neuropathologists, enabling us to compare genotype with both individual and consensus diagnosis. In this manner, we sought to determine whether a cumulative analysis of those genetic markers could provide insight into the significance of molecular genetic examination in glioma diagnosis.

MATERIALS AND METHODS

Tumor samples were obtained at surgery performed at the Saitama Medical School Hospital, Teikyo University Hospital, and University of Tokyo Hospital and its affiliated hospitals. In total, 91 cases of diffuse gliomas operated during the period of 1996–1999 were studied. Tumor samples were collected on an availability basis and were not selected for specific histological types. Portions of resected tumors were snap frozen in liquid nitrogen and stored at −80°C until use. DNA from the tumor tissue was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) after the manufacturer's protocol. Constitutional DNA used as a control for the microsatellite analysis was extracted as described previously from peripheral blood obtained after written informed consent (19). All experiments using the human samples were approved by the ethical committee at the University of Tokyo Hospital.

Histology slides were reviewed by four independent neuropathologists (T. H., N. F., J. H., and Y. N.), and consensus diagnoses were made following the latest WHO classification (20). In cases with divergent opinions, the senior neuropathologist (Y. N.) made the final diagnosis. For GBM cases, the neuropathologists were also asked to describe if there were regions showing distinct oligodendroglial morphological features. Histological evaluations were blinded to the molecular genetic data.

Molecular Genetic Analysis. LOH and single-strand conformational polymorphism assays were performed using the Genetic Analyzer 310 (PE Biosystems, Norwalk, CT) capillary electrophoresis system, following the manufacturer's protocols. For the LOH assay, the following microsatellite markers located at the most frequently deleted sites in gliomas were used: *D1S244, D1S2734,* and *D1S402* for 1p (1p36); *D19S112, D19S596, D19S412,* and *D19S219* for 19q (19q13); and *D10S1744, D10S1680,* and *D10S583* for 10q (10q22–23; Refs. 10, 17, and 21). Primer sequences for these markers are available from the Genome Database.[4]

The single-strand conformational polymorphism assay for exons 5–8 of *TP53* was performed using the previously published primer pairs (7). For each primer set, the sense and antisense primers were labeled with two different fluorescent dyes to allow specific detection of each strand. PCR products were separated by capillary electrophoresis and were analyzed with the GeneScan program (PE Biosystems) to detect tumor-specific migration shifts. Exons showing migration shifts were reamplified from the tumor DNA with nonlabeled primers, gel purified, and directly sequenced using the BigDye Terminator Sequencing Kit (PE Biosystems). The sequencing reaction products were separated and analyzed by the Genetic Analyzer 310 following the manufacturer's protocol.

Previously described, established comparative multiplex PCR assays were used to detect gene amplification for *EGFR* and *CDK4* and homozygous deletion of *CDKN2A.* (9, 22)

RESULTS

Of the 91 cases, the consensus histological diagnosis was AC (WHO grade 2) in 6, AA (grade 3) in 7, GBM (grade 4) in 45, OG (grade 2) in 21, AOG (grade 3) in 8, mixed OA (grade 2) in 3, and mixed AOA (grade 3) in 1 (Table 1). Complete agreement of the initial diagnoses among all four neuropathologists was seen in 49 of 91 cases (54%): 4 AC (including 2 cases on which one neuropathlogist did not made a diagnosis), 0 AA, 36 GBM, 6 OG, 3 AOG, 1 OA, and 1 AOA. Disagreement was much more common in AA (seven of seven, 100%), OA (two of three, 67%), OG (15 of 21, 71%), and AOG (five of eight, 63%), compared with AC (two of eight, 25%) and GBM (9 of 45, 20%; Fig. 1, *A–D*).

The results of the molecular genetic analysis are also summarized in Table 1. At least one of the three examined 1p markers was informative in 88 cases, of which 30 cases (33%) showed LOH: one of six AC (17%), zero of seven AA (0%), 10 of 42 GBM (24%), 12 of 21 OG (57%), five of eight AOG (63%), two of three OA (67%), and none of one AOA (0%). Similarly, at least one of the four 19q markers was informative in 90 cases, of which 38 cases (42%) showed LOH: one of six AC (17%), two of seven AA (29%), 13 of 44 (31%) GBM, 12 of 21 OG (57%), seven of nine AOG (78%), two of three OA (67%), and one of one AOA.

Among the 30 cases with 1pLOH, 25 cases (86%) also had 19qLOH: one of one AC (100%), 6 of 10 GBM (60%), 11 of 12 OG (92%), five of five AOG (100%), and two of two OA (100%). The positive correlation between 1pLOH and 19qLOH was statistically significant not only in the 34 tumors with oligodendroglial features (OG, AOG, OA, and AOA, $P < 0.0001$; Fisher's exact test) but also within the 58 astrocytic tumors (AC, AA, and GBM); of 11 astrocytic tumors with 1pLOH in total, 7 (64%) had 19qLOH ($P = 0.0055$).

At least one of the three examined 10q markers was informative in 88 cases, and LOH was detected in 29 cases (33%):

[4] Internet address: http://www.gdb.org.

Table 1 Histology and molecular genetic data on 91 gliomas[a]

No.	Cons.	o-comp.	A	B	C	D	EGFR	TP53	CDKN2A del.	CDK4 amp.
1	AC		AC	AC	OA	AC	nl	wt	(-)	(-)
2	AC		AC	AC	AC	AC	nl	mut: exon 5, codon 161 GCC->GGC (Ala->Gly)	(-)	(-)
3	AC		AC	AC	AC	AA	nl	mut: exon 8, codon 286 GAA->GGA (Glu->Gly)	(-)	(-)
4	AC		AC	AC	AC	AC	nl	mut: exon 7, codon 248 CGG-> CAG (Arg->Gln)	HD	(-)
5	AC		AC	AC	ND	AC	nl	wt	(-)	(-)
6	AC		AC	AC	ND	AC	nl	wt	(-)	(-)
7	AA		AA	AA	AOA	AA	nl	mut: exon 8, codon 273 CGT->CAT (Arg->His)	(-)	(-)
8	AA		AA	GBM	AA	AOA	nl	mut: exon 8, codon 306 CGA->TGA (Arg->Stop)	(-)	(-)
9	AA		AOA	OA	AA	AA	nl	mut: exon 7, codon 245 GGC->AGC (Gly->Ser)	(-)	(-)
10	AA		AA	GBM	AA	AC	nl	mut: exon 7, codon 248 CGG->CAG (Arg->Gln)	(-)	(-)
11	AA		AA	AA	GBM	GBM	nl	wt	(-)	(-)
12	AA		AA	OG	AA	AA	nl	wt	HD	(-)
13	AA		AA	GBM	AA	AA	nl	wt	(-)	(-)
14	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
15	GBM	(+)	AOG	GBM	GBM	GBM	amp	wt	(-)	(-)
16	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
17	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 7, codon 245 GGC->AGC (Gly->Ser)	(-)	(-)
18	GBM	(-)	GBM	AOG	GBM	GBM	amp	wt	(-)	(-)
19	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
20	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
21	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
22	GBM	(-)	AOG	GBM	A.Ep	GBM	nl	wt	(-)	(-)
23	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	HD	(-)
24	GBM	(-)	AOG	GBM	GBM	GBM	nl	wt	HD	(-)
25	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
26	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
27	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
28	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 7, codon 245 GGC->AGC (Gly->Ser)	HD	(-)
29	GBM	(+)	GBM	GBM	GBM	GBM	amp	mut: exon 6, agGT->aaGT (splice donor site)	HD	(-)
30	GBM	(-)	GBM	GBM	AC	GBM	nl	wt	HD	(-)
31	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	HD	(-)
32	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 5, codon 175 CGC->CAC (Arg->His)	(-)	(-)
33	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 8, codon 273 CGT->CAT (Arg->His)	(-)	(-)
34	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
35	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	HD	(-)
36	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	HD	(-)
37	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
38	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
39	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	(-)	amp
40	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
41	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
42	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	(-)	amp
43	GBM	(-)	AA	AA	GBM	GBM	nl	wt	HD	(-)
44	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
45	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	amp
46	GBM	(+)	GBM	GBM	GBM	GBM	nl	mut: exon 7, codon 238 TGT->TGA (Cys->stop)	HD	(-)
47	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 6, codon 220 TAT->TGT (Tyr->Cys)	(-)	(-)
48	GBM	(-)	GBM	GBM	AA	GBM	nl	mut: exon 5, codon 175 CGC->CAC (Arg->His)	(-)	amp
49	GBM	(-)	GBM	GBM	GBM	GBM	nl	mut: exon 5, codon 135 TGC->TAC (Cys->Tyr)	(-)	(-)
50	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
51	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
52	GBM	(+)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
53	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
54	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	(-)	(-)
55	GBM	(-)	GBM	GBM	GBM	GBM	nl	wt	HD	(-)
56	GBM	(+)	GBM	AOG	AOA	GBM	nl	wt	(-)	(-)
57	GBM	(+)	GBM	AOG	AOA	GBM	nl	wt	HD	(-)
58	GBM	(-)	GBM	GBM	GBM	GBM	amp	wt	(-)	(-)
59	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
60	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
61	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
62	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
63	OG		OG	OG	OG	AOA	nl	wt	(-)	(-)
64	OG		AOG	OG	OG	OG	nl	wt	(-)	(-)
65	OG		OA	AOG	OG	OG	nl	wt	HD	(-)
66	OG		OA	OG	OG	OG	nl	wt	HD	(-)
67	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
68	OG		OG	OG	OG	OG	nl	wt	(-)	(-)
69	OG		OA	OG	OG	OG	nl	wt	(-)	(-)
70	OG		DNT	OG	AC	OG	nl	wt	(-)	(-)
71	OG		OA	OG	AA	OA	amp	mut: exon 8, codon 286 GAA->AAA (Glu->Lys)	(-)	(-)
72	OG		OA	OG	ND	AA	nl	mut: exon 8, codon 273 CGT->TGT (Arg->Cys)	(-)	(-)
73	OG		OG	OG	AC	AC	nl	mut: exon 5, agTA->ggTA	(-)	(-)
74	OG		AA	OG	AOG	ND	nl	mut: exon 7, codon 248 CGG->TGG (Arg->Cys)	(-)	(-)
75	OG		OG	OG	OG	AOG	nl	mut: exon 8, codon 273 CGT->TGT (Arg->Cys)	(-)	(-)
76	OG		AC	OG	OA	ND	nl	mut: exon 5, codon 157 GTC->GGC (Val-> Gly)	(-)	(-)
77	OG		OA	OG	OG	OG	nl	wt	(-)	(-)
78	OG		OG	AC	OA	AA	nl	wt	(-)	(-)
79	OG		OG	OG	OG	AA	nl	wt	(-)	(-)
80	AOG		AOG	AOG	OA	AOG	nl	wt	(-)	(-)
81	AOG		AOG	AOG	OA	AOG	nl	wt	(-)	(-)
82	AOG		AOG	AOG	OG	AOG	nl	wt	(-)	(-)
83	AOG		AOG	AOG	AOG	AOG	nl	wt	HD	(-)
84	AOG		AOG	AOG	AOG	AOG	nl	wt	(-)	(-)
85	AOG		AOG	AOG	AOG	AOG	nl	wt	HD	(-)
86	AOG		AOG	AOG	OG	OG	nl	wt	HD	(-)
87	AOG		AOG	AOG	AOG	GBM	nl	wt	(-)	(-)
88	OA		OA	OA	OA	OA	nl	wt	(-)	(-)
89	OA		OA	OA	OA	AC	nl	wt	(-)	(-)
90	OA		OG	OA	OA	OA	nl	mut: exon 8, codon 273 CGT->TGT (Arg->Cys)	(-)	(-)
91	AOA		AOA	AOA	AOA	AOA	nl	mut: exon 8, codon 273 CGT->TGT (Arg->Cys)	(-)	(-)

Cons: consensus diagnosis, A-D: diagnosis by neuropathologists A-D
AC: astrocytoma, AA: anaplastic astrocytoma, GBM: glioblastoma, OG: oligodendroglioma, AOG: anaplastic oligodendroglioma,
OA: oligoastrocytoma, AOA:anaplastic oligoastrocytoma, wt: wild type, mut: mutated, amp: gene amplification
ND: not determined, A.Ep: anaplastic ependymoma, DNT: dysembryoplastic neuroepithelial tumor, o-comp: oligo-component
□: heterozygous, ▨: non-informative, ■: loss of heterozygosity (LOH)

[a] Cons, consensus diagnosis; A–D, diagnosis by neuropathologists A–D; wt, wild type; mut, mutated; amp, gene amplification; ND, not determined; A.Ep, anaplastic ependymoma; DNT, dysembryoplastic neuroepithelial tumor; o-comp, oligo-component; □, heterozygous; ▨, noninformative; ■, LOH.

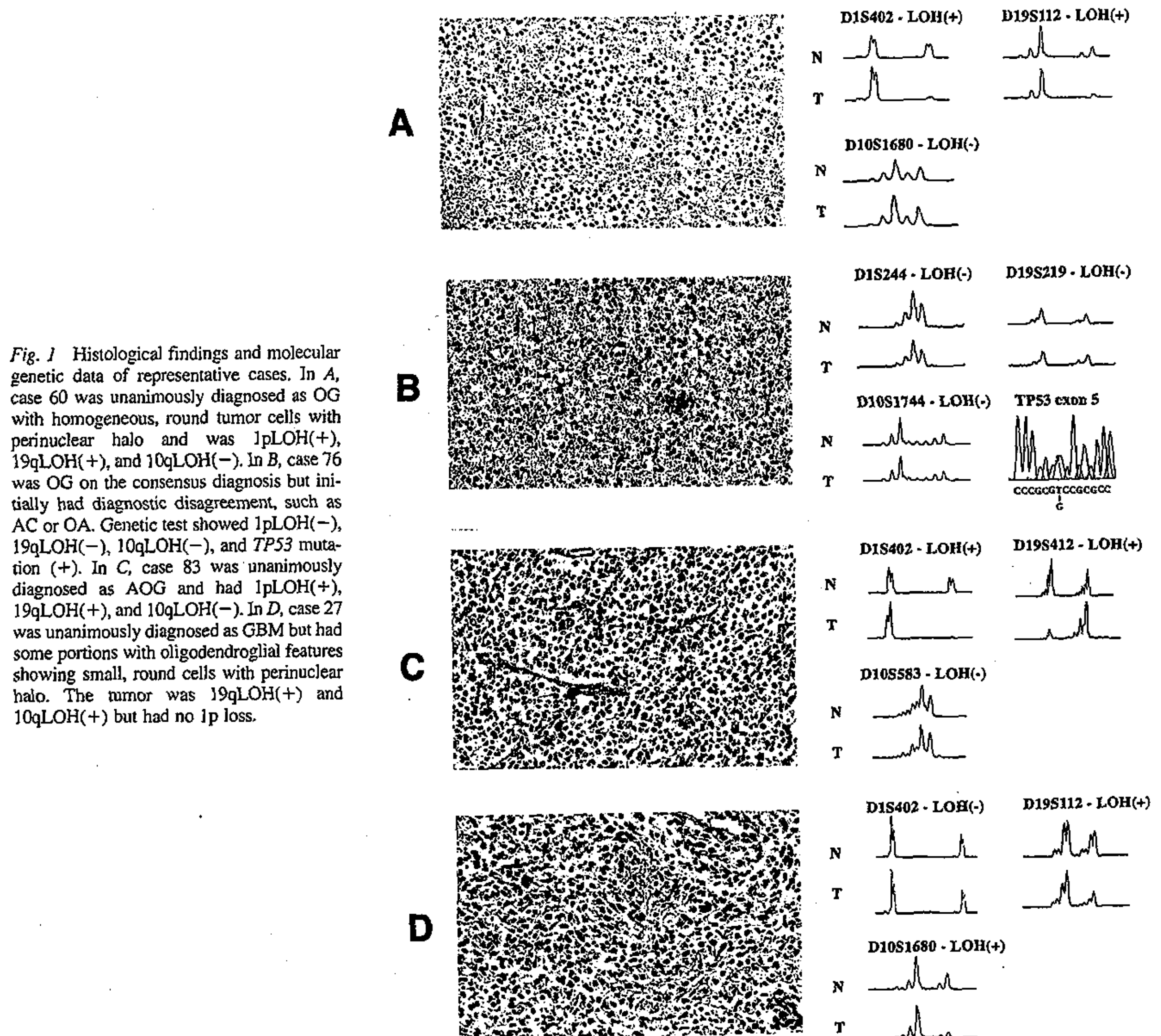

Fig. 1 Histological findings and molecular genetic data of representative cases. In *A*, case 60 was unanimously diagnosed as OG with homogeneous, round tumor cells with perinuclear halo and was 1pLOH(+), 19qLOH(+), and 10qLOH(−). In *B*, case 76 was OG on the consensus diagnosis but initially had diagnostic disagreement, such as AC or OA. Genetic test showed 1pLOH(−), 19qLOH(−), 10qLOH(−), and *TP53* mutation (+). In *C*, case 83 was unanimously diagnosed as AOG and had 1pLOH(+), 19qLOH(+), and 10qLOH(−). In *D*, case 27 was unanimously diagnosed as GBM but had some portions with oligodendroglial features showing small, round cells with perinuclear halo. The tumor was 19qLOH(+) and 10qLOH(+) but had no 1p loss.

1 AC, 1 AA, and 27 GBM. Among the 43 cases with either 1p or 19qLOH, 14 cases had 10qLOH, and 29 cases retained both copies of 10q. All of the 14 cases (100%) with 10qLOH were GBMs, whereas 25 of 29 cases (86%) retaining both copies of 10q were OG, AOG, OA, or AOA.

Mutation of *TP53* was detected in 24 cases: three of six AC (50%), four of seven AA (57%), 9 of 45 GBM (20%), 6 of 21 OG (29%), zero of nine AOG (0%), one of three OA (33%), and one of one AOA (100%). When correlation with 1p status was examined, only one GBM (case 17) had both 1pLOH and *TP53* mutation, and none of the remaining 29 cases with 1pLOH had *TP53* mutation. This relative exclusiveness between 1pLOH and *TP53* mutation was statistically significant when examined in all cases (P = 0.0003) but did not reach statistical significance when the 58 astrocytic tumors (AC, AA, and GBM) were separately analyzed, probably because a considerable number of cases had neither of the two genetic alterations (P = 0.055). On the other hand, *TP53* mutation and 19qLOH were not inversely correlated in all gliomas (P = 0.29), with seven cases showing both 19qLOH and *TP53* mutation: two AA, three GBM, one OG, and one AOA.

Homozygous deletion of *CDKN2A* was observed in one AC, one AA, 17 GBM, two OG, three AOG, and in none of OA or AOA. There was no significant association of *CDKN2A* status with 1pLOH, 19qLOH, or *TP53* mutation in any tumor types. However, *CDKN2A* deletion positively correlated with 10qLOH (P = 0.04).

Gene amplification of *EGFR* was detected in 11 cases: 10 GBMs and 1 OG. In agreement with previous studies, most GBM cases with *EGFR* amplification (9 of 10) had 10qLOH, but only one had *TP53* mutation (23–25). However, this inverse correlation did not reach statistical significance in this series (P = 0.15). There was no apparent correlation between *EGFR*

amplification and 1p/19q status: two had both 1pLOH and 19qLOH, one had 1pLOH alone, two had 19qLOH alone, and four had neither 1p nor 19qLOH. Of the 45 GBMs, 12 cases were described to contain portions with oligodendroglial morphological features. Five of the 12 cases (42%) had 19qLOH, and only 1 (8%) had 1pLOH (Table 1; Fig. 1*D*) .

DISCUSSION

The data presented here showed that molecular genetic evaluation of chromosomes 1p, 19q, and 10q in diffuse gliomas has significant diagnostic value, especially in identifying OGs and OAs. When 1p or 19qLOH was detected without 10qLOH in diffuse gliomas, the tumor usually demonstrated consensus oligodendroglial histological features, compatible with the diagnosis of grade II or III OG or OA. A recent study using real-time quantitative PCR to detect allelic loss also showed this concordance between 1p/19q/10q status and the histological diagnosis of OGs (26). When 1p or 19qLOH was accompanied by additional 10qLOH, on the other hand, such tumors were most likely (86%) GBMs on consensus diagnosis. Interestingly, tumors unanimously agreed as OGs on their initial diagnoses almost always carried combined loss of 1p and 19q; all six OGs and two of three AOGs with unanimous diagnosis had 1p and 19q losses, and the remaining one AOG had 19q loss with noninformative 1p markers (Fig. 1, *A* and *C*). Therefore, it appears that tumors showing typical histological features of OGs are likely to carry 1p and 19q losses both in grade II and grade III tumors. On the contrary, all nine consensus OGs without 1p loss had disagreement on the diagnosis initially, indicating that these might be the cases potentially leading to the diagnostic variability (Fig. 1*B*).

The distinction between OG, OA, and AC can be subjective and sometimes difficult, especially in high-grade, undifferentiated tumors (20, 27). Our study suggested that simple LOH analysis of 1p, 19q, and 10q may provide important supportive information in making diagnoses for such difficult cases, which may affect the clinical management. For instance, a trend in postsurgical treatment for OGs is to start with chemotherapy alone and defer radiation therapy until the tumors show progression (28–30). Whether a similar approach is appropriate in treating patients with OAs or AOAs remains to be investigated, and to do so requires homogeneous defined sets of tumors. Genetic profiles on 1p/19q/10q and *TP53* should be one of the important objective factors to be considered in such studies.

There was a small number of outlying cases in our series: one AC, one AA, and one GBM showed 1p and 19qLOH while maintaining allelic balance at 10q, the typical genetic profile for OGs. Therefore, molecular genetic analysis cannot replace histological diagnosis. On the other hand, it is still unknown which of the two measures, histological diagnosis or genetic profiling, would better represent the biological characteristics of a tumor when the two evaluations disagree.

As reported previously, 1p LOH was highly associated with 19qLOH in oligodendroglial tumors, but our study further showed that this positive correlation seems to extend to astrocytic tumors, including GBMs ($P = 0.0055$; Refs. 13 31, and 32). Although they showed such a strong positive correlation, 1pLOH and 19qLOH did have slight differences in their patterns of appearance. 1pLOH demonstrated a tight inverse correlation with *TP53* mutation ($P = 0.0003$), a presumed key genetic event in AC tumorigenesis (2, 33–35). On the other hand, 19qLOH accompanied *TP53* mutation more frequently both in oligodendroglial tumors and GBMs. On the basis of such findings, it is tempting to hypothesize that diffuse gliomas with 1pLOH may constitute a genetic subset that is associated with oligodendroglial lineage, whereas *TP53* mutation represents another subset associated with astrocytic lineage. Such proposition has been made previously on OA, in which 1p/19qLOH was associated to be more OG predominant, and *TP53* mutation was associated with more AC predominant morphology (36). Curiously, however, partial oligodendroglial morphological features in GBMs were more frequently detected in tumors with 19q loss in our study, not in 1p loss, indicating that morphological features do not necessary follow the genetic profile (Fig. 1*D*).

Genetic subsets in gliomas were first noticed in GBMs, in which *TP53* mutation and *EGFR* gene amplification occur in a mutually exclusive fashion, thereby defining two genetic subsets (23, 25). Clinical significance of these genetic subsets, one with *TP53* mutation and the other with *EGFR* amplification, was shown by the fact that secondary GBMs mostly belonged to the former, and primary GBMs mostly belonged to the latter (20, 25). Whether the putative subsets by 1p loss and *TP53* mutation suggested in our study also would have any clinical relevance remains to be investigated. However, it is noteworthy that AOGs with 1pLOH had already been shown to have better treatment response and prognosis (16, 17), and another recently published study on seven high-grade gliomas with unusual long survival demonstrated 1pLOH in all seven cases (37). Although such association was not proven in OA and astrocytic tumors thus far (17), it is therefore possible that 1pLOH may be a marker indicative of gliomas associated with better treatment response and survival. Unfortunately, we currently do not have sufficient follow-up data on our series to look for correlation between the genetic data and clinical outcomes, such as the difference between GBMs with or without 1pLOH. Additional studies in a larger series with sufficient follow-up should address this clinically important question. Nonetheless, use of molecular genetic markers will allow objective evaluation of diffuse gliomas and, therefore, enable such studies to be done, perhaps without the time-consuming and difficult requirement of independent histological review by multiple neuropathologists.

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REFERENCES

1. Louis, D. N., and Gusella, J. F. A tiger behind many doors: multiple genetic pathways to malignant glioma. Trends Genet., *11:* 412–415, 1995.

2. Louis, D. N. A molecular genetic model of astrocytoma histopathology. Brain Pathol., *7:* 755–764, 1997.

3. Collins, V. P. Progression as exemplified by human astrocytic tumors. Semin. Cancer Biol., *9:* 267–276, 1999.

4. Smith, J. S., and Jenkins, R. B. Genetic alterations in adult diffuse glioma: occurrence, significance, and prognostic implications. Front. Biosci., *5:* D213–D231, 2000.

5. Bigner, S. H., Burger, P. C., Wong, A. J., Werner, M. H., Hamilton, S. R., Muhlbaier, L. H., Vogelstein, B., and Bigner, D. D. Gene

amplification in malignant human gliomas: clinical and histopathologic aspects. J. Neuropathol. Exp. Neurol., *47:* 191-205, 1988.

6. Reifenberger, G., Reifenberger, J., Ichimura, K., Meltzer, P. S., and Collins, V. P. Amplification of multiple genes from chromosomal region 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. Cancer Res., *54:* 4299-4303, 1994.

7. Fults, D., Brockmeyer, D., Tullous, M. W., Pedone, C. A., and Cawthon, R. M. p53 mutation and loss of heterozygosity on chromosomes 17 and 10 during human astrocytoma progression. Cancer Res., *52:* 674-679, 1992.

8. Henson, J. W., Schnitker, B. L., Correa, K. M., von Deimling, A., Fassbender, F., Xu, H. J., Benedict, W. F., Yandell, D. W., and Louis, D. N. The retinoblastoma gene is involved in malignant progression of astrocytomas. Ann. Neurol., *36:* 714-721, 1994.

9. Ueki, K., Ono, Y., Henson, J. W., Efird, J. T., von Deimling, A., and Louis, D. N. CDKN2/p16 or RB alterations occur in the majority of glioblastomas and are inversely correlated. Cancer Res., *56:* 150-153, 1996.

10. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. Identification of a candidate tumour suppressor gene, *MMAC1,* at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet., *15:* 356-362, 1997.

11. Mollenhauer, J., Wiemann, S., Scheurlen, W., Korn, B., Hayashi, Y., Wilgenbus, K. K., von Deimling, A., and Poustka, A. DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. Nat. Genet., *17:* 32-39, 1997.

12. von Deimling, A., Louis, D. N., von Ammon, K., Petersen, I., Wiestler, O. D., and Seizinger, B. R. Evidence for a tumor suppressor gene on chromosome 19q associated with human astrocytomas, oligodendrogliomas, and mixed gliomas. Cancer Res., *52:* 4277-4279, 1992.

13. Reifenberger, J., Reifenberger, G., Liu, L., James, C. D., Wechsler, W., and Collins, V. P. Molecular genetic analysis of oligodendroglial tumors shows preferential allelic deletions on 19q and 1p. Am. J. Pathol., *145:* 1175-1190, 1994.

14. Smith, J. S., Alderete, B., Minn, Y., Borell, T. J., Perry, A., Mohapatra, G., Hosek, S. M., Kimmel, D., O'Fallon, J., Yates, A., Feuerstein, B. G., Burger, P. C., Scheithauer, B. W., and Jenkins, R. B. Localization of common deletion regions on 1p and 19q in human gliomas and their association with histological subtype. Oncogene, *18:* 4144-4152, 1999.

15. Bigner, S. H., Matthews, M. R., Rasheed, B. K., Wiltshire, R. N., Friedman, H. S., Friedman, A. H., Stenzel, T. T., Dawes, D. M., McLendon, R. E., and Bigner, D. D. Molecular genetic aspects of oligodendrogliomas including analysis by comparative genomic hybridization. Am. J. Pathol., *155:* 375-386, 1999.

16. Cairncross, J. G., Ueki, K., Zlatescu, M. C., Lisle, D. K., Finkelstein, D. M., Hammond, R. R., Silver, J. S., Stark, P. C., Macdonald, D. R., Ino, Y., Ramsay, D. A., and Louis, D. N. Specific genetic predictors of chemotherapeutic response and survival in patients with anaplastic oligodendrogliomas. J. Natl. Cancer Inst. (Bethesda), *90:* 1473-1479, 1998.

17. Smith, J. S., Perry, A., Borell, T. J., Lee, H. K., O'Fallon, J., Hosek, S. M., Kimmel, D., Yates, A., Burger, P. C., Scheithauer, B. W., and Jenkins, R. B. Alterations of chromosome arms 1p and 19q as predictors of survival in oligodendrogliomas, astrocytomas, and mixed oligoastrocytomas. J. Clin. Oncol., *18:* 636-645, 2000.

18. Bigner, S. H., Mark, J., Burger, P. C., Mahaley, M. S., Bullard, D. E., Muhlbaier, L. H., and Bigner, D. D. Specific chromosomal abnormalities in malignant human gliomas. Cancer Res., *48:* 405-411, 1988.

19. Ueki, K., Rubio, M. P., Ramesh, V., Correa, K. M., Rutter, J. L., von Deimling, A., Buckler, A. J., Gusella, J. F., and Louis, D. N. *MTS1/CDKN2* gene mutations are rare in primary human astrocytomas with allelic loss of chromosome 9p. Hum. Mol. Genet., *3:* 1841-1845, 1994.

20. Kleihues, P., and Cavenee, W. K. WHO classification, Tumors of the Central Nervous System. Lyon, France: IARC, 2000.

21. Rosenberg, J. E., Lisle, D. K., Burwick, J. A., Ueki, K., von Deimling, A., Mohrenweiser, H. W., and Louis, D. N. Refined deletion mapping of the chromosome 19q glioma tumor suppressor gene to the D19S412-STD interval. Oncogene, *13:* 2483-2485, 1996.

22. Louis, D. N., Rubio, M. P., Correa, K. M., Gusella, J. F., and von Deimling, A. Molecular genetics of pediatric brain stem gliomas. Application of PCR techniques to small and archival brain tumor specimens. J. Neuropathol. Exp. Neurol., *52:* 507-515, 1993.

23. von Deimling, A., von Ammon, K., Schoenfeld, D., Wiestler, O. D., Seizinger, B. R., and Louis, D. N. Subsets of glioblastoma multiforme defined by molecular genetic analysis. Brain Pathol., *3:* 19-26, 1993.

24. von Deimling, A., Louis, D. N., von Ammon, K., Petersen, I., Hoell, T., Chung, R. Y., Martuza, R. L., Schoenfeld, D. A., Yasargil, M. G., Wiestler, O. D., *et al.* Association of epidermal growth factor receptor gene amplification with loss of chromosome 10 in human glioblastoma multiforme. J. Neurosurg., *77:* 295-301, 1992.

25. Watanabe, K., Tachibana, O., Sata, K., Yonekawa, Y., Kleihues, P., and Ohgaki, H. Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas. Brain Pathol., *6:* 217-223; discussion 23-24, 1996.

26. Nigro, J. M., Takahashi, M. A., Ginzinger, D. G., Law, M., Passe, S., Jenkins, R. B., and Aldape, K. Detection of 1p and 19q loss in oligodendroglioma by quantitative microsatellite analysis, a real-time quantitative polymerase chain reaction assay. Am. J. Pathol., *158:* 1253-1262, 2001.

27. Coons, S. W., Johnson, P. C., Scheithauer, B. W., Yates, A. J., and Pearl, D. K. Improving diagnostic accuracy and interobserver concordance in the classification and grading of primary gliomas. Cancer (Phila.), *79:* 1381-1393, 1997.

28. Streffer, J., Schabet, M., Bamberg, M., Grote, E. H., Meyermann, R., Voigt, K., Dichgans, J., and Weller, M. A role for preirradiation PCV chemotherapy for oligodendroglial brain tumors. J. Neurol., *247:* 297-302, 2000.

29. Fortin, D., Cairncross, G. J., and Hammond, R. R. Oligodendroglioma: an appraisal of recent data pertaining to diagnosis and treatment. Neurosurgery, *45:* 1279-1291; discussion 191, 1999.

30. Burton, E., and Prados, M. New chemotherapy options for the treatment of malignant gliomas. Curr. Opin. Oncol., *11:* 157-161, 1999.

31. Kraus, J. A., Koopmann, J., Kaskel, P., Maintz, D., Brandner, S., Schramm, J., Louis, D. N., Wiestler, O. D., and von Deimling, A. Shared allelic losses on chromosomes 1p and 19q suggest a common origin of oligodendroglioma and oligoastrocytoma. J. Neuropathol. Exp. Neurol., *54:* 91-95, 1995.

32. Bello, M. J., Leone, P. E., Vaquero, J., de Campos, J. M., Kusak, M. E., Sarasa, J. L., Pestana, A., and Rey, J. A. Allelic loss at 1p and 19q frequently occurs in association and may represent early oncogenic events in oligodendroglial tumors. Int. J. Cancer, *64:* 207-210, 1995.

33. Sidransky, D., Mikkelsen, T., Schwechheimer, K., Rosenblum, M. L., Cavanee, W., and Vogelstein, B. Clonal expansion of p53 mutant cells is associated with brain tumour progression. Nature (Lond.), *355:* 846-847, 1992.

34. Bogler, O., Huang, H. J., and Cavenee, W. K. Loss of wild-type p53 bestows a growth advantage on primary cortical astrocytes and facilitates their *in vitro* transformation. Cancer Res., *55:* 2746-2751, 1995.

35. Ohgaki, H., Schauble, B., zur Hausen, A., von Ammon, K., and Kleihues, P. Genetic alterations associated with the evolution and progression of astrocytic brain tumours. Virchows Arch., *427:* 113-118, 1995.

36. Maintz, D., Fiedler, K., Koopmann, J., Rollbrocker, B., Nechev, S., Lenartz, D., Stangl, A. P., Louis, D. N., Schramm, J., Wiestler, O. D., and von Deimling, A. Molecular genetic evidence for subtypes of oligoastrocytomas. J. Neuropathol. Exp. Neurol., *56:* 1098-1104, 1997.

37. Ino, Y., Zlatescu, M. C., Sasaki, H., Macdonald, D. R., Stemmer-Rachamimov, A. O., Jhung, S., Ramsay, D. A., von Deimling, A., Louis, D. N., and Cairncross, J. G. Long survival and therapeutic responses in patients with histologically disparate high-grade gliomas demonstrating chromosome 1p loss. J. Neurosurg., *92:* 983-990, 2000.

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Distinction in gene expression profiles of oligodendrogliomas with and without allelic loss of 1p

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Oligodendrogliomas frequently, but not always show sensitivity to chemotherapy and recent studies demonstrated that allelic loss of chromosome 1p is highly associated with this chemosensitivity. To gain insight into the molecular mechanism of such difference, we examined comprehensive gene expression profiles of 11 oligodendroglial tumors, six with and five without 1pLOH (loss of heterozygosity), and two normal brain tissues using the oligonucleotide microarray (GeneChip). Statistically significant numbers of genes were expressed differentially between the two genetic subsets. Clustering analysis separated the tumor subsets well. The tumors with 1pLOH had similar expression profiles to the normal brain for those differentially expressed genes. Many genes showing higher expression in tumors with 1pLOH were presumed to have functions in nervous tissues. Notably, the majority of the 123 genes showing significant expression reduction in tumors with 1pLOH were either on chromosome 1 (50%) or on 19 (10%), and the average expression reduction ratio was about 50% (0.54 ± 0.13) possibly reflecting the chromosomal deletion. Thus, the biological difference between the genetic subsets of oligodendroglioma was indeed reflected to gene expression profile, which provided baseline information for further studies to elucidate the mechanism of chemosensitivity in gliomas.
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Keywords: oligodendroglioma; oligonucleotide microarray; loss of heterozygosity

Introduction

Oligodendrogliomas are a major type of gliomas which constitute approximately 5% of all primary brain tumors or 10 to 25% of all intracranial gliomas (Kleihues and Cavenee, 2000). One of the important recent findings in neuro-oncology was that those oligodendrogliomas frequently showed remarkable sensitivity to chemotherapy, especially to a regimen using procarbazine, CCNU and vincristine (PCV therapy) (Cairncross and Macdonald, 1988). However, the response rate to PCV therapy remains 60–80%, and 20–30% of tumors are resistant to chemotherapy and have worse prognosis. Therefore, within this histologically indistinguishable entity, there apparently exist subgroups showing different biological behavior. Recent molecular genetic studies on oligodendrogliomas revealed that allelic loss of chromosome 1p, which is found in 60–80% of oligodendrogliomas and often accompanied with allelic loss of 19q (Smith *et al.*, 1999), was highly associated with the treatment responsiveness and also with a better prognosis (Cairncross *et al.*, 1998; Ino *et al.*, 2001). Thus, it is now being recognized that loss of chromosome 1p is a marker separating oligodendrogliomas into subgroups showing different biological behavior. In addition to its important clinical implications, understanding of the underlying molecular mechanisms of such a difference may lead to a new treatment strategy for all gliomas. Unfortunately, the putative tumor suppressor genes at chromosomes 1p and 19q, obvious keys to investigate the molecular biologic features of the tumor cells, are yet to be identified despite vigorous investigations. Several attractive candidates on chromosome 1p include *TP73* (1p36.3) and *CDKN2C* (1p32), but neither has been shown to be altered in the majority of oligodendrogliomas (Husemann *et al.*, 1999; Mai *et al.*, 1998). Although 1p loss is also found in many other neoplasms including neuroblastomas, the search for the suppressor gene in such neoplasms has not been successful either (Ohira *et al.*, 2000; Schwab *et al.*, 1996). To gain insight into the molecular basis of the biological difference among oligodendrogliomas, we turned to recently developed oligonucleotide microarray technology. By analysing comprehensive gene expressions, several studies have now shown that the expression profiles correlated well with the histology

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and clinical grades in human neoplasms including gliomas (Golub et al., 1999; Huang et al., 2000; Watson et al., 2001). Therefore, we performed a comparative study of the gene expression profiles between the genetic subgroups of oligodendrogliomas based on the 1p status.

Results

Genetic alterations in oligodendroglial tumor samples

Of 40 oligodendroglial tumors we could collect, we selected six cases with 1pLOH (loss of heterozygosity) and five cases without 1pLOH from which we could obtain good quality RNA evaluable with the GeneChip system (Affymetrix, Santa Clara, CA, USA). Histological diagnoses and the results of molecular genetic analysis are summarized in Table 1. There were seven oligodendrogliomas, one oligoastrocytoma and three anaplastic oligodendrogliomas. In all six tumors with 1pLOH, all of the informative 1p markers showed LOH, indicating that deletion involved the whole arm of chromosome 1p (data not shown). Of the six cases with 1pLOH, five cases also had 19qLOH and one case was non-informative on examined 19q markers. None of the six tumors with 1pLOH had *TP53* mutation, and three of the five tumors without 1pLOH had *TP53* mutation. No case had 10qLOH.

The statistical analysis of genes differentially expressed by 1p status

To select genes that were expressed differentially by 1p status, we used prediction value (*P*-value) in neighborhood analysis, which was recently described as useful for extracting genes expressed uniformly high in one group and low in the other (Golub et al., 1999). We listed a total of 209 genes that had an absolute *P*-value of more than one, of which 86 genes showed higher expression and 123 genes showed lower expression in tumors with 1pLOH. These numbers of the genes were significantly higher than expected in random grouping tested by 1000 times permutation test ($P<0.01$), indicating that these two subgroups indeed have significantly different gene expression profiles. When Mann–Whitney test with cut-off *P*-values of 0.05 and 0.01 were used, 288 and 123 genes were detected as differentially expressed by the 1p status, and those numbers were higher than the expected numbers in permutation test which were 115 and 33 in median, respectively. Of the 209 genes selected by prediction value, more than 90% (192 genes) were also included in the 288 genes selected by a *P*-value of 0.05 by Mann–Whitney test, indicating the consistency of those two methods in selecting differentially expressed genes. We used the 209 genes for further analysis.

Clustering analysis was performed to classify all 13 samples using Pearson correlation with these extracted 209 genes (Figure 1). The tumor subsets were separated well and the normal brain samples were clustered into the same group with the tumors with 1pLOH. Among the five tumors without 1pLOH, expression profiles were not markedly different between the tumors with and without *TP53* mutation in this clustering analysis.

Genes showing higher expression in tumors with 1pLOH

Of the 86 genes selected by *P*-value, 24 genes whose mean average difference had more than threefold difference between the two groups were listed in Table 2. The average differences of those genes in normal brain RNA were close to those in tumors with 1pLOH as expected by the clustering analysis. Based on the UniGene on National Center for Biotechnology Information (NCBI), 14 of the 24 genes were predominantly expressed in brain or neural tissues (*KIAA0985*, *RGS7*, human clone 23695, *INA*, *KIAA0750*, *MYT1L*, human clone 23560, *PTPRN*, *SLC1A2*, *HAPIP*, *SNCB*, *SNAP25*, *L1CAM* and *OLFM1*), and were likely to have some function in the nervous system. In the normal brain samples, genes that are predominantly expressed in glial cells such as glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP) were also well expressed, indicating that these samples contained many glial cells.

Table 1 Summary of oligodendroglial tumors used in GeneChip experiments

Tumor no.	*Gender*	*Age*	*Histology*	*1pLOH*	*19qLOH*	*10qLOH*	*TP53*	*CDKN2A*
1	F	47	AOG	(+)	(+)	(−)	Wt	HD
2	F	23	OG	(+)	(+)	(−)	Wt	Normal
3	M	44	OG	(+)	(+)	(−)	Wt	Normal
4	M	49	OG	(+)	(+)	(−)	Wt	HD
5	F	22	OA	(+)	NI	(−)	Wt	Normal
6	F	48	AOG	(+)	(+)	(−)	Wt	Normal
7	M	44	OG	(−)	(−)	(−)	Mutation	Normal
8	F	60	OG	(−)	(−)	(−)	Mutation	Normal
9	F	25	OG	(−)	(−)	(−)	Mutation	Normal
10	M	67	AOG	(−)	(−)	(−)	Wt	HD
11	F	27	OG	(−)	(−)	(−)	Wt	Normal

OG: oligodendroglioma, OA: oligoastrocytoma, AOG: anaplastic oligodendroglioma, LOH: loss of heterozygosity, NI: non-informative, Wt: wild type, HD: homozygous deletion

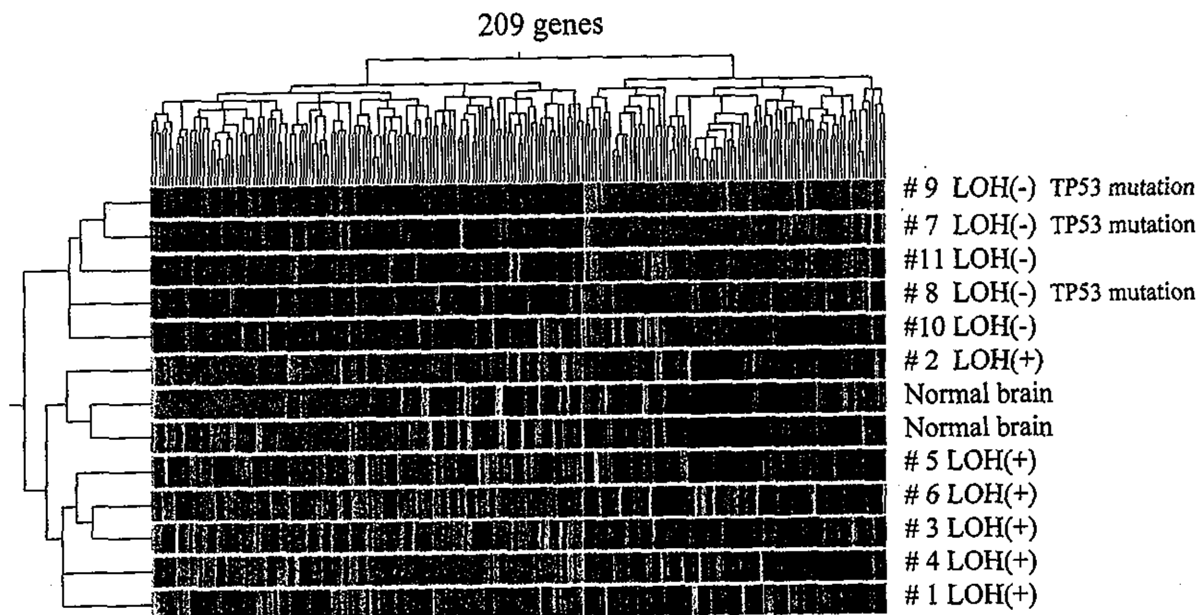

Figure 1 Hierarchical clustering of 11 oligodendroglial tumors and two normal brain samples using the 209 genes selected by *P*-value. Each column represents a gene and each row represents a sample. Red indicates increased expression, and blue indicates decreased gene expression. Expression of each gene is normalized to its median in this figure. The dendrogram indicates the degree of similarity between their expression profiles. Normal brain samples were clustered into the same group with the tumors with 1pLOH. LOH: loss of heterozygosity

Table 2 List of genes showing higher expression in tumors with 1pLOH

				Expression levels			
P-value	*Accession number*	*Gene*	*Symbol*	*1pLOH(+)*	*1pLOH(−)*	*Normal brain*	*Chromosome*
3.02	L12350	Thrombospondin 2	THBS2	167±36	46±9	117±26	6
2.1	AB023202	KIAA0985 protein	KIAA0985	213±77	65±13	519±137	12
2.1	U32439	Regulator of G-protein signaling 7	RGS7	133±32	43±15	320±2	1q
1.86	U79289	Human clone 23695		72±35	22±2	188±11	1q
1.83	S78296	Internexin neuronal intermediate filament protein α	INA	622±182	177±54	825±97	10
1.57	AB020639	Estrogen-related receptor γ	ESRRG	66±21	18±12	68±4	1q
1.51	AB018293	KIAA0750 gene product	KIAA0750	67±59	6±3	630±54	11
1.42	AB029029	*Myelin transcription factor 1-like	MYT1L	125±70	9±8	303±35	2
1.4	U79242	Human clone 23560		97±33	17±13	127±21	11
1.4	L18983	*Protein tyrosine phosphatase, receptor type N	PTPRN	99±81	2±0	628±76	2
1.31	L39833	Potassium voltage-gated channel, shaker-related subfamily	KCNAB1	81±36	23±10	205±29	3
1.25	U01824	Solute carrier family 1, member 2	SLC1A2	52±33	8±7	15±7	11
1.2	M25756	*Secretogranin II (Chromogranin C)	SCG2	269±180	62±35	354±16	2
1.18	U94190	Huntingtin-associated protein interacting protein	HAPIP	42±55	2±0	319±95	3
1.18	U78575	Phosphatidylinositol-4-phosphate 5-kinase, type Iα	PIP5K1A	110±41	34±20	92±6	1q
1.08	AA021140	cDNA clone IMAGE 363856		50±46	3±1	85±15	2
1.08	X96381	Ets variant gene 5	ETV5	280±151	77±58	224±71	3
1.07	AF053136	*Synuclein β	SNCB	145±147	6±5	741±174	5
1.07	D21267	Synaptosomal-associated protein, 25 kD	SNAP25	553±301	169±117	2167±135	20
1.04	K03000	*Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	118±57	16±11	196±86	9
1.02	U52112	*L1 cell adhesion molecule	L1CAM	479±273	56±63	498±58	X
1.02	U71364	Serine proteinase inhibitor, clade B, member 9	SERPINB9	58±31	18±21	59±12	6
1.01	U72936	Alpha thalassemia/mental retardation syndrome X-linked	ATRX	166±82	55±65	131±49	X
1.01	D82343	Olfactomedin 1	OLFM1	762±599	236±56	2109±128	9

P-value: prediction value, which reflects the difference between two groups (the details are described in Materials and methods). Expression level of each gene was demonstrated as a mean value and a s.d. of average differences in each subgroup. Of the 86 genes selected by P-value, 24 genes whose mean average difference had more than threefold difference between the tumors with and without 1pLOH are listed. The genes examined by semi-quantitative RT–PCR are indicated by *

Genes showing lower expression in tumors with 1pLOH and their chromosomal location

Of the 123 genes selected by *P*-value, 61 genes (50%) were mapped to chromosome 1 (58 to 1p, 1 to 1q, and 2 to 1p or 1q) and 12 genes (10%) were mapped to chromosome 19 (11 to 19q, and 1 to 19p), while 50 genes (41%) were mapped to other chromosomes. When we focused on top 30 genes that had an absolute

P-value of more than 1.5, 83% (25 genes) were mapped to chromosome 1 or 19. Relative expressions of the 73 genes, 61 on chromosome 1 and 12 on chromosome 19, in tumors with 1pLOH compared to tumors without 1pLOH were 0.54 ± 0.13 in average. Of the 123 genes, 16 genes whose mean average difference had more than threefold difference between the two groups were listed in Table 3 (whole list of the selected genes would be available on request).

The validation using semi-quantitative RT–PCR

Of the 24 higher expressed and 16 lower expressed genes in 1pLOH tumors, semi-quantitative RT–PCR was performed on nine known genes whose differences were more than fourfold and also more than 40 in mean average difference between the two groups (indicated by * in Tables 2 and 3). The results of RT–PCR corresponded well to the GeneChip data (Figure 2). The additional tumor samples showed similar expression pattern to the same 1p status cases examined by GeneChip, although there were two cases (case 18 and 19) which showed exceptional expression pattern. Those two cases neither had allelic loss on 1p/19q nor had *TP53* mutation. The case 19 was rather similar to the 1pLOH tumors. Case 18 showed lower expression in some of the genes that had higher expression in other tumors without 1pLOH.

Expression of genes on chromosome 1p

The relative expressions of the genes on chromosome 1p ($n = 158$) in tumors with 1pLOH against tumors without 1pLOH were arranged on the chromosome map to see their relationship with chromosomal loci (Figure 3). Genes showing lower expression in tumors with 1pLOH were distributed over the whole chromosome 1p arm. There also were many genes whose expressions were not decreased in 1pLOH tumors, which were also found in various chromosomal loci.

Discussion

Using the oligonucleotide microarray technology, we could identify genes that were differentially expressed between the subgroups of oligodendroglioma by the 1p status. Results of semi-quantitative RT–PCR performed on some of the identified genes were concordant with the chip analysis data, confirming the fidelity of the system in general. Additional oligodendrogliomas studied by RT–PCR showed similar expression pattern to the GeneChip cases according to their 1p status. Of the five tumors without 1pLOH and without *TP53* mutation, however, one additional case was rather similar to the tumors with 1pLOH and another additional case also showed some inconsistency. Such variations of gene expression pattern suggest heterogeneity in tumors without 1pLOH and that more than two subgroups may exist in oligodendroglial tumors, as reported recently (Ino *et al*., 2001). The numbers of cases analysed in our study were still limited, and a larger-scale study would enable detailed classification of oligodendroglial tumors based on gene expression profiles. Nonetheless, our data clearly showed that oligodendrogliomas of different genetic subsets indeed had distinct gene expression pattern, and could identify many differentially expressed genes.

Five of 10 cases without 1pLOH had *TP53* mutation, and the expression patterns of the genes examined by RT–PCR were not significantly different between tumors with and without *TP53* mutation. There was no apparent difference in the expression pattern of the 209 genes among five tumors without

Table 3 List of genes showing lower expression in tumors with 1pLOH

P-value	*Accession number*	*Gene*	*Symbol*	*Expression levels* 1pLOH(+)	1pLOH(−)	Normal brain	*Chromosome*
−1.96	J04177	*Collagen type IX α 1	COL11A1	13±16	230±111	22±7	1p21
−1.94	M97388	Down-regulator of transcription 1	DR1	23±10	82±18	31±5	1p22
−1.87	AB029000	KIAA1077 protein	KIAA1077	2±0	50±39	2±0	8
−1.50	D49493	Growth differentiation factor 10	GDF10	2±0	37±49	2±0	10
−1.49	AB011173	KIAA0601 protein	KIAA0601	47±38	225±79	25±23	1p
−1.48	X74262	*Retinoblastoma-binding protein 4 (RbAp48)	RBBP4	19±17	109±51	49±1	1p
−1.31	AL109671	cDNA clone EUROIMAGE 29222		19±25	103±60	80±6	15q
−1.25	AI806222	Arachidonate 5-lipoxygenase-activating protein	ALOX5AP	7±12	42±34	15±13	13
−1.17	AB028967	*Potassium voltage-gated channel, Shal-related subfamily	KCND2	35±20	165±68	33±9	7q
−1.12	M59830	Heat shock 70 kD protein 1B	HSPA1B	25±22	90±35	119±60	6p
−1.12	AF056490	Phosphodiesterase 8A	PDE8A	103±38	318±133	217±99	15
−1.11	J04111	v-jun avian sarcoma virus 17 oncogene homolog	JUN	62±50	226±116	80±8	1p32-p31
−1.09	S78203	Solute carrier family 15, member 2	SLC15A2	2±0	31±42	2±0	3
−1.08	AF104922	Growth differentiation factor 8	GDF8	6±4	43±37	2±0	2q
−1.06	D11151	Endothelin receptor type A	EDNRA	27±22	94±45	11±2	4
−1.05	U80055	Cystein dioxygenase type I	CDO1	28±24	97±37	2±0	5q

P-value: prediction value, which reflects the difference between two groups (the details are described in Materials and methods). Expression level of each gene was demonstrated as a mean value and a s.d. of average differences in each subgroup. Of the 123 genes selected by P-value, 16 genes whose mean average difference had more than threefold difference between the tumors with and without 1pLOH are listed. The genes examined by semi-quantitative RT–PCR are indicated by *

Figure 2 Results of semi-quantitative RT-PCR. Eleven tumors (#1-11) used in the GeneChip experiments (printed in boldface), nine additional tumors (#12-20), and two normal brain tissues (#21, 22) were analysed. The upper six genes and the middle three genes showed higher and lower expression in 1pLOH tumors in the GeneChip experiment, respectively. GAPDH was used as a control

Figure 3 The relative expressions of the genes on chromosome 1p. Horizontal-axis represents the location of the examined 158 genes demonstrated from telomere (left) to centromere (right). Vertical-axis represents relative expressions with error bar, dividing the mean expressions (average difference) of tumors with 1pLOH by those of tumors without 1pLOH. The value beyond 1.7 is not shown in this figure

1pLOH examined by GeneChip, regardless of the *TP53* mutation status (data not shown). Therefore, the listed genes were most likely extracted by the status of 1pLOH than that of *TP53*.

Of the genes showing higher expression in tumors with 1pLOH (see Table 2), we noticed several genes that were expressed predominantly in normal brain or neural tissue, indicating that those genes are functional in the normal nervous system. Contamination of normal tissue cells was not likely, because allelic losses observed on the microsatellite analysis were almost complete in all cases, indicating that the examined

tissues consisted mostly of tumor cells. For example, *MYT1L* encodes a zinc finger protein which plays a role in the development of neurons in the central nervous system (Kim *et al.*, 1997), and *PTPRN*, which had especially similar expression pattern to *MYT1L* in the RT–PCR analysis, is implicated in neuroendocrine secretory processes. *SNCB* plays a role in neuronal plasticity, *SLC1A2* is a glial high affinity glutamate transporter, and *HAPIP* is also abundantly expressed in the neural tissues. *L1CAM* is an axonal glycoprotein involved in neuronal migration and differentiation (Kenwrick *et al.*, 2000). In combination with the results of clustering, these data may suggest that tumors without 1pLOH are more distant from normal brain, possibly reflecting their differentiation status.

Analysis on the differentially expressed genes provided potentially interesting information on their chromosomal locations. Of the top 123 genes whose expressions were most significantly decreased in tumors with 1pLOH, nearly 60% were located either on chromosome 1 or chromosome 19, with the ratio of expression levels to tumors without 1pLOH around 50%. It was reported that nearly all oligodendrogliomas with 1p and 19q LOH lose the entire arm of 1p and 19q (Bigner *et al.*, 1999; Nigro *et al.*, 2001; Smith *et al.*, 1999), which was also confirmed by our microsatellite analysis on 1p. Therefore, reduced expression of genes in a wide range of 1p is likely to be a consequence of losing one copy of each gene. On the other hand, some genes on chromosome 1p had higher expressions in tumors with 1pLOH, suggesting that the expression regulations of those genes were not simply dependent on the copy number. In a few genes such as *COL11A1* and *RBBP4*, the relative expressions were remarkably low probably because of their overexpression in tumors without 1pLOH, rather than their expression reduction in 1p losing tumors (see Table 3). Despite the rather comprehensive expression analysis, we still could not pinpoint a particular gene that would affect the chemosensitivity of oligodendrogliomas. None of the genes previously suggested to be related with chemosensitivity, such as O^6-methylguanine-DNA methyltransferase (MGMT), multidrug resistance 1 (MDR1), multidrug resistance-associated protein (MRP), glutathione S-transpherase pi, metallothionein and topoisomerase IIα (Nutt *et al.*, 2000; Tanaka *et al.*, 2000), were detected as differentially expressed genes in our study. On the other hand, we showed that significant numbers of genes were differentially expressed between oligodendroglioma subsets, including expression reduction of numerous genes in the chromosome 1p. An interesting question is whether about 50% reduction of such numerous genes in the same chromosomal region could have any biological effect on tumorigenesis or chemosensitivity. Recent studies showed that loss of one copy of a gene and subsequent reduction of its expression level is possibly related to tumorigenesis, a phenomenon called haplo-insufficiency (Fero *et al.*, 1998; Gutmann *et al.*, 1999). Whether similar phenomenon underlies the biological features of oligodendroglioma remains to be investigated.

From a technological point of view, it could be an important observation that the oligonucleotide microarray may be quantitative enough to detect expression reduction caused by the allelic loss in numerous genes. cDNA microarray and serial analysis of gene expression (SAGE) have been tried with some success to detect increase or decrease of expressions of certain genes which were altered by gene amplifications or deletions (Caron *et al.*, 2001; Pollack *et al.*, 1999). Our data indicated that oligonucleotide microarray would be a good system to identify such genes.

In summary, we showed that genetic subsets in oligodendrogliomas by 1p status were reflected in gene expression profile. Some of the interesting genes differentially expressed included genes implicated in the function of nervous tissues, genes on chromosome 1p and 19q. Molecular mechanism of chemosensitivity and chemoresistance may well be represented by those differentially expressed genes, and our data would serve as good baseline data for the future studies to solve that clinically important question.

Materials and methods

Sample preparation

Tumor samples obtained at surgery were snap frozen in liquid nitrogen and stored at −80°C until use. Histological diagnosis was made on formalin-fixed paraffin-embedded tissues processed separately. To minimize the notorious variability of the histological diagnosis in oligodendroglial tumors, the histology slides were reviewed by four independent neuropathologists to make consensus diagnoses following the WHO classification (Kleihues and Cavenee, 2000). Paired blood samples were obtained after written informed consents, and were subjected to DNA extraction for the microsatellite analysis. Of 40 oligodendroglial tumors, six tumors with 1pLOH and five without 1pLOH were selected for expression profiling using GeneChip system (Affymetrix). Total RNA from normal whole brain was purchased from two different providers (Clontech, Palo Alto, CA, USA and Life Technologies, Inc., Rockville, MD, USA), which were used to see the expression profile of the normal neurons and glial cells.

Genetic analysis

LOH assay on chromosomes 1p, 19q and 10q to detect allelic losses were performed using Genetic Analyzer 310 (Applied Biosystems, Foster City, CA, USA) as previously described. The following microsatellite markers located at the commonly deleted in gliomas were used: *D1S244*, *D1S2734*, and *D1S402* for 1p (1p36), *D19S112*, *D19S596*, *D19S412* and *D19S219* for 19q (19q13), *D10S1744*, *D10S1680* and *D10S583* for 10q (10q22-23) (Ueki *et al.*, 2000). For tumors with 1pLOH, four additional 1p markers were further examined to see the range of the deletion: *D1S1166* (1p13), *D1S495* (1p22), *D1S2835* (1p32) and *D1S2657* (1p34). The SSCP assay for exons 5 to 8 of *TP53* was performed using previously published primer pairs (Fults *et al.*, 1992), again using Genetic Analyzer 310. Exons showing migration shift were PCR amplified again and were directly sequenced using BigDye Terminator Kit (Applied Biosystems) following the manufacturer's protocol. Established comparative multiplex

PCR assays were used to detect homozygous deletion of *CDKN2A* (Ueki *et al*., 1996). For RNA extraction, the frozen tumor sample was homogenized in Isogen (Nippon Gene, Osaka, Japan) and total RNA was isolated following manufacturer's instructions.

Gene Chip experiment

Five μg of total RNA from each sample were used to synthesize biotin-labeled cRNA, which was then hybridized to the high-density oligonucleotide array (GeneChip Human U95A array; Affymetrix) following the previously published protocol with minor modifications (Ishii *et al*., 2000). Arrays contain probe sets for approximately 12 626 human genes and ESTs, which were selected from Build 95 of the UniGene Database (derived form GenBank 113, dbEST/10-02-99). After washing, arrays were stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene, OR, USA) and analysed by a Hewlett-Packard Scanner to collect primary data. The GeneChip 3.3 software (Affymetrix) was used to calculate the average difference for each gene probe on the array, which was shown as an intensity value of gene expression defined by Affymetrix using their algorithm. The average difference has been shown to quantitatively reflect the abundance of a particular mRNA molecule in a population (Ishii *et al*., 2000; Lockhart *et al*., 1996). To allow comparison among multiple arrays, the average differences were normalized for each array by assigning the average of overall average difference values to be 100. A value of two was assigned to every average difference below two. Of the total 12 626 probe sets represented on the array, control probes and genes scored as absent (not detected) by the expression algorithm in GeneChip software (Affymetrix) or less than 100 in all 13 samples were excluded from the analysis because of low confidence of scarcely expressed genes, and 5668 probe sets were left.

Selection of differentially expressed genes

For the selection of differentially expressed genes by 1p status, we used prediction value (*P*-value) which reflects the difference between two groups, given by $(\mu 1-\mu 2)/(\sigma 1+\sigma 2)$ when $(\mu 1, \sigma 1)$ and $(\mu 2, \sigma 2)$ denote the means and standard deviations of the log of the expression level of gene for the sample in group 1 and group 2, respectively (Golub *et al*., 1999). Pre-filtering was applied to select probe sets whose maximum and minimum average difference among 11 tumor samples differed by more than 100, and had more than two-fold difference. For the remaining 3875 probe sets, the prediction values were calculated. We also used Mann–Whitney test, which measures whether the distribution of gene expression level between two groups is overlapped.

Clustering analysis

The expression patterns of samples were statistically analysed using GeneSpring 4.0 software (Silicon Genetics, Redwood City, CA, USA). Average differences were converted into logarithm, and hierarchical clustering was carried out using Pearson correlation coefficient of 0.8 (Eisen *et al*., 1998).

Semi-quantitative RT–PCR

Semi-quantitative RT–PCR was performed using 13 samples used for GeneChip analysis and additional nine oligodendroglial tumors. Of the nine additional cases, four cases had combined 1p and 19q LOH, while five cases had neither genetic alteration. Two of the five additional cases without 1pLOH had *TP53* mutation. cDNA was synthesized with oligo-dT primer from 2 μg total RNA, using SuperScript Preamplification System (Life Technologies, Inc.). The concentration of the cDNA was equalized using the *GAPDH* gene expression as a control. PCR was then performed with 2 μl of cDNA for 31–37 cycles, consisted of 30 s of denaturing at 94°C, 30 s of annealing at 63–70°C and 1 min of extension at 72°C. The primer sets used are listed in Table 4. PCR products were separated by electrophoresis on 1.5% agarose gels and were visualized with ethidium bromide staining. Numbers of PCR cycles were optimized to ensure product intensity within the linear phase of amplification. For each primer set, the amplicon was sequenced after subcloned into pGEM-T Easy vector (Promega, Madison, WI, USA) to confirm that the correct target gene was amplified.

Identification of gene location

Chromosomal loci of the genes were identified using the locus information from the web sites of GenBank, UniGene and LocusLink on NCBI, by referring to the corresponding GenBank accession number of each probe set.

Detailed chromosomal locations of 950 genes mapped on 1p were obtained from the web site of Map Viewer (Homo sapiens build 26) on NCBI, in which the gene locations are shown by distances from the telomere of the short arm. These 950 genes were matched to the probe sets on Human U95A array by referring to the LocusLink ID, UniGene ID and GenBank accession number, which identified 502 probe sets represented on U95A array. Genes with expressions (average difference) scored as absent or less than 100 in average of 11 tumors were excluded because of low confidence in evaluating genes with low expression. For the 158 genes remaining, we calculated relative expressions by dividing the mean expressions in tumors with 1pLOH by those in tumors without 1pLOH, which were then arranged on the chromosome map.

Table 4 Primer pairs used in RT–PCR

Gene	*Sense (5′→3′)*	*Antisense (3′→5′)*
MYT1L	AAACAGCGGGCCAGCAACGGTATAG	CAGCAGCAAAAAACAAGAGGCATCC
PTPRN	GTGGAGGATGGTGTCAAGCAGTGTG	GGCTGTCAGGGCAAATTCAAACTGG
SCG2	GTTCTGCCAAGGCTCCCTTATGGTG	GGATTTGCTTGGGGTGGAGGAATG
SNCB	ATGGACGTGTTCATGAAGGGCCTG	GGACAGGGACAGAATTGTGCTGCT
ALDH1A1	CCTCTGACCCCAGGAGTCACTCAA	TTCATGGAAACCGTACTCTCCCAG
L1CAM	TACCACCCGGTCCCCACTTTATTGC	ATGTTGTGTGGTGGGTACCGAAGGC
COL11A1	GCCAAAGGAGAAACCAGGAAGTTGG	CACAAAATGGGTTGGTGGCACCAAG
RBBP4	CCAAACCAAGCCACTCAGTTGATGC	CCTTGTCCTTCTGGATCCACGCTTC
KCND2	TCGAGCCCTGGCTGTGAAAAGAATC	TTATTTGCACAGCCCACCATGGAAC
GAPDH	CATGTGGGCCATGAGGTCCACCAC	AATGCCTCCTGCACCACCAACTGC