that wild-type *p53* and *p16* function as tumor suppressors preventing tumor angiogenesis. Moreover, the combined transfer of *p53* and *p16* has led to efficient induction of apoptosis, which could not be achieved by *p53* on its own in Rb-positive tumor cells [27]. In this respect, in malignant gliomas which have highly inactivated *p53* and *p16*, the wild-type *p53* and *p16* may also appear to be appropriate target genes for a gene therapy. Therefore, our investigation of the biological effects of restoring wild-type *p53* and *p16* on angiogenesis in gliomas seems to provide a basis for future clinical trials.

Acknowledgements

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Midkine promoter-based conditionally replicative adenovirus for malignant glioma therapy

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Abstract. Little is known concerning promoters or gene therapy specific for malignant glioma. To explore the potential use of *midkine* promoter in gene therapy for malignant glioma, we constructed a *midkine* promoter-based conditionally replicating adenovirus (Ad-*MK*). *Midkine* was overexpressed in malignant glioma tissues but *cyclooxygenase-2* was not. The *midkine* promoter activity of the 600-bp fragment was 2 orders of magnitude higher in midkine-positive glioma cells than in midkine-negative primary normal brain cells. Ad-*MK* showed strong oncolytic effects in midkine-positive glioma cells but did not exhibit cytotoxicity in midkine-negative primary normal brain cells. The cell-killing effect was evident in E3-intact Ad-*MK* more than in E3-deleted Ad-*MK*. In an animal experiment, Ad-*MK* completely eradicated midkine-positive glioma xenografts. These findings indicate that *midkine* promoter-based conditionally replicative adenovirus might be a promising new modality of gene therapy for malignant glioma.

Introduction

Malignant glioma is characterized by rapidly growing and aggressively invasive neoplasm. Due to the lack of an effective treatment, the median survival associated with this diagnosis continues to be around 1 year (1), suggesting a pressing need for novel therapeutic strategy. Gene therapy has shown promise as a new approach for this malignancy, but the results of clinical trials with replication-deficient viral vectors and suicide gene therapy have been unsatisfactory with regard to therapeutic outcome (2). The main reason for these disappointing results is related to the limited spread of the vectors in the tumor mass. The new therapeutic modality provided by oncolytic virotherapy with conditionally replicative adenovirus (CRAd) is expected to advance the treatment of malignant glioma. The strategy is replacement of adenovirus promoters with tumor-specific promoters to control the expression of viral genes essential for replication. The tumor-specific promoters allow the expression of viral genes preferentially in cancer cells, so that the virus only replicates in and kills those cells. Tumor-specific promoters such as *α-fetoprotein*, *prostate-specific antigen*, *MUC1* and *cyclooxygenase-2* (*cox-2*) have been widely used experimentally to drive viral genes expression (3). However, an appropriate promoter for malignant glioma has not yet been identified. We are interested in the gene encoding midkine (MK), because it has been reported that MK expression closely correlates to carcinogenesis (4). MK is a heparin-binding growth factor identified as a product of a retinoic acid-responsive gene, and promotes growth, survival, migration and other activities of various cells. Overexpression of MK has been observed in several types of malignant neoplasms such as gastrointestinal cancer, ovarian cancer and breast cancer (5,6). It has also been reported that MK expression is elevated in malignant glioma, but not in normal brain tissue (7). These findings indicate that MK promoter might be a potential candidate for oncolytic gene therapy of malignant glioma.

In the present study, we examined the potency of *MK* promoter in a tumor-specific promoter-based replicative adenovirus constructed for oncolytic gene therapy of malignant glioma. First, we determined the MK expression levels in human glioma and normal brain tissues. Subsequently, we evaluated the *in vitro* and *in vivo* antitumor effect of CRAd in which expression of the *E1A* gene is driven by the *MK* promoter.

Materials and methods

Surgical specimens, cell lines and cell culture. Human specimens were surgically obtained from 12 glioblastomas (WHO grade IV), 4 anaplastic astrocytomas (WHO grade III), 3 diffuse astrocytomas (WHO grade II) and 5 normal brain tissues. Malignant glioma was defined as including glioblastoma and anaplastic astrocytoma. Primary normal brain cells were established in our laboratory. The human malignant

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glioma cell line U87MG was obtained from the American Type Culture Collection (Manassas, VA); U251MG and U373MG were generously provided by Dr N. Arita (Hyogo College of Medicine, Hyogo, Japan); and LN319 was generously provided by Dr M. Tada (Hokkaido University School of Medicine, Sapporo, Japan). Cells were maintained in DMEM with 10% heat-inactivated fetal bovine serum. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Real-time quantitative RT-PCR. RNA samples (100 ng) were used in reverse transcription and real-time PCR for RNA expression studies. The reaction was carried out with the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA) in a total volume of 50 µl that contained TaqMan one-step RT-PCR master-mix (Applied Biosystems), 0.3 µM of each forward and reverse primer, and 0.21 µM of TaqMan probe. The forward and reverse primer and TaqMan probe were, respectively, 5'-GCGCGCTACAA TGCTCAGT-3', 5'-TGGCTTTGGCCTTTGCTTT-3' and 5'-CAGGAGACCATCCGCGTCACCAA-3' for *MK*, and 5'-GGTTGCTGGTGGTAGGAATGTT-3', 5'-CATAAAGCGT TTGCGGTACTCA-3' and 5'-CCGCAGTACAGAAAGTAT CACAGGCTTC CA-3' for *cox-2*. The reaction was performed with the following thermal cycling method: 30 min at 48°C for reverse transcription, 5 min at 95°C for AmpliTaq Gold activation, 15 sec at 95°C and 60 sec at 60°C for 40 cycles. *GAPDH* was chosen as a housekeeping gene to be tested as an endogenous control.

Western blot analysis. Cell lysate protein (10 µg) from each sample was subjected to 13.5% SDS-Tris glycerine gel electrophoresis and was then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were incubated with primary antibodies, rabbit anti-human MK monoclonal antibody generously provided by Dr H. Shimada (Chiba University Graduation School of Medicine, Chiba, Japan), rabbit anti-adenovirus-2 E1A polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and human anti-ß-actin monoclonal antibody (Sigma-Aldrich Fine Chemical, St. Louis, MO). Membranes were also incubated with secondary antibodies, horseradish peroxidase-conjugated sheep anti-rabbit IgG (Dako, Glostrup, Denmark) and horseradish peroxidase-conjugated sheep antimouse IgG (Amersham, Piscataway, NJ).

Assay for MK promoter activity. The *MK* promoter of 600 bp (-562/33) or 2300 bp (-2285/33) in length, was cloned into the pGL3-basic vector (Promega, Madison, WI), which contained the firefly luciferase gene. Both DNA fragments were transfected into target cells with DOTAP Liposomal Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany). Dual luciferase assays were performed according to the manufacturer's protocol (Promega). Luciferase activity in each plasmid was evaluated as the ratio to activity in the control plasmid (pGL3-SV40) driven by the *SV40* enhancer/promoter.

Construction of the adenovirus vector. The replication-competent Ad-*MK* including the adenoviral *E1A* region under the control of human *MK* promoter containing 600 bp or 2300 bp of the 5'-flanking region of the human *MK* gene was constructed. The pXC1 plasmid has adenovirus 5 sequences from 22 to 5790 bp containing the *E1* gene (Microbix Biosystems Inc., Toronto, Ontario, Canada). The 492-552 bp region of pXC1 was deleted to obtain pXC1-491. The *MK* promoter was ligated to pXC1-491 plasmid to obtain pXC1-MK. To construct the Ad-*MK*, homologous recombination was performed between pXC1-MK plasmid and the right hand side of pBHGE3 adenovirus DNA with the *E3* region and pBHG10 adenovirus DNA without the *E3* region in 293 cells by standard techniques (8). Wild-type adenovirus (Ad-*Wild*) and the *E1*-deleted AdCMV-*LacZ* virus were constructed as previously described (9). All of the viruses were purified with double cesium chloride gradients using standard method. Serial dilutions of viruses were plated on 293 cells for plaque assay, and the titer was expressed as plaque forming unit (pfu)/ml (multiplicity of infection, m.o.i.).

Cell count assay. A total of 3000 cells were plated in 12-well plates, and after 24 h, were infected with Ad-*MK600*, Ad-*MK2300*, Ad-*Wild* or AdCMV-*LacZ* viral control. Culture medium alone was used as a mock-infection control. Ten days after infection, IC_{50} was determined by counting the number of viable cells after staining with Trypan-blue.

Animal experiment. Nude mice 5-6 weeks of age were purchased from Charles River Laboratories (Tsukuba, Japan). Ten million each of U87MG and U373MG cells were inoculated s.c. into the flanks of mice in 200 µl of DMEM. The tumor growth was assessed by measuring bidimensional diameters every 3 days with calipers. The tumor volume was determined by using the simplified formula of rotational ellipse (1 x width^3 x 0.5). When the tumor reached a volume of approximately 100 mm^3, animals were randomly assigned to treatment groups. Animals were treated with a single intratumoral injection of adenovirus at $1x10^9$ pfu suspended in 100 µl of PBS. Each treatment group consisted of 5 animals.

Results

MK and Cox-2 gene expression in glioma tissues and cell lines. To measure the mRNA levels of the *MK* and *cox-2* gene, we performed real-time quantitative RT-PCR. We demonstrated the mRNA expression level of the *MK* and *cox-2* gene of each sample relative to that of *GAPDH* (Fig. 1). As shown in Table I, *MK* mRNA expression of malignant glioma tissues (glioblastoma and anaplastic astrocytoma) was 12 times that of diffuse astrocytoma ($P<0.05$, unpaired t-test) and 40 times that of normal brain tissues ($P<0.01$, unpaired t-test). However, *cox-2* gene expression did not significantly differ among the glioma samples and normal brain tissues. In diffuse astrocytomas and normal brain tissues, there was not significant difference between *MK* and *cox-2* mRNA expression. However, in malignant glioma tissues, mRNA expression of *MK* was 4 times that of *cox-2* ($P<0.01$, paired t-test).

To determine MK gene and protein expression in human glioma cell lines and primary normal brain cells, we performed real-time quantitative RT-PCR and Western blot analysis. The microscopic observation revealed that primary normal brain cells mainly consisted of glial cells. We determined the

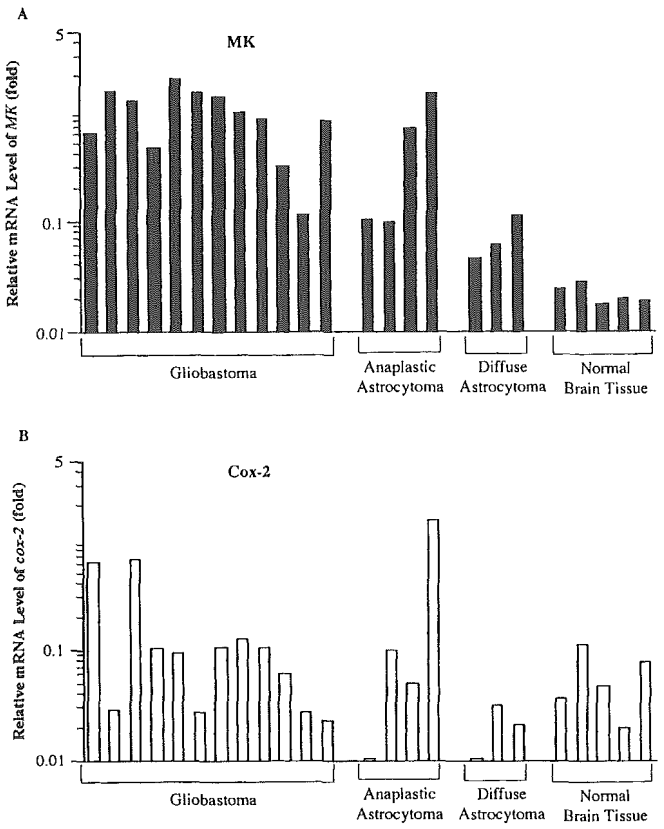

Figure 1. Expression of human *MK* and *cox-2* mRNA in glioma and normal brain tissues. Real-time quantitative RT-PCR was carried out to detect (A) *MK* or (B) *cox-2* mRNA expression in 12 glioblastomas, 4 anaplastic astrocytomas, 3 diffuse astrocytomas and 5 normal brain tissues.

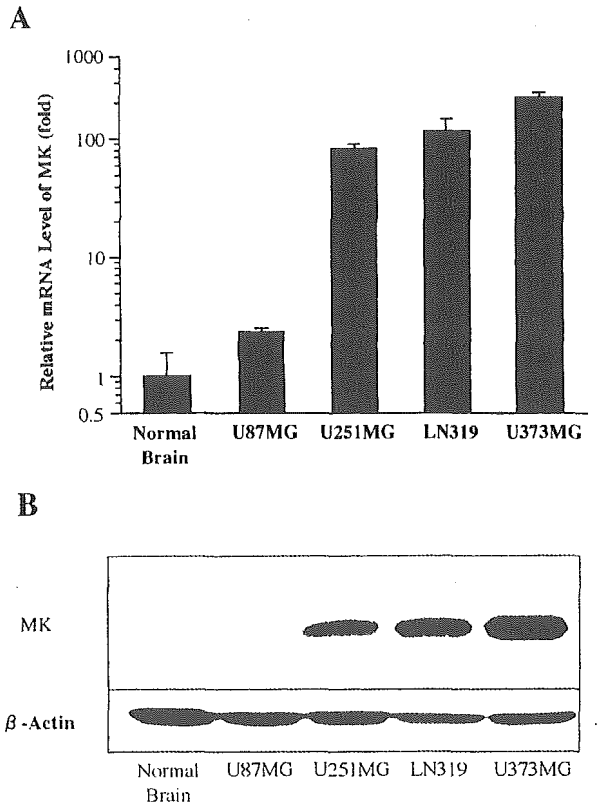

Figure 2. Expression of human MK. (A), *MK* mRNA expression of the glioma cell lines (U87MG, U251MG, LN319 and U373MG) and primary normal brain cells. The expression levels were determined by real-time quantitative RT-PCR. Bars, means ± SD; (B), Western blot analysis used to assay MK protein expression in the glioma and primary normal brain cells. Top panel shows the MK protein (16 kDa), and the ß-actin control is shown in the bottom panel.

Table I. Midkine and cox-2 expression in human glioma.

Tissue specimens	(n)	Midkine mRNA[a]	Cox-2 mRNA[a]
Malignant glioma (Glioblastoma + Anaplastic astrocytoma)	16	0.8585±0.544	0.2258±0.378
Diffuse astrocytoma	3	0.0722±0.028	0.0210±0.008
Normal brain tissue	5	0.0214±0.004	0.0578±0.032

[a]All values are expressed as the mean ± SD

mRNA expression level of the *MK* gene of each sample relative to that of primary normal brain cells after adjusting all samples for *GAPDH* gene expression. The mean value of *MK* mRNA of U251MG, LN319 and U373MG was 60 times that of U87MG (P<0.001, unpaired t-test) and 142 times that of primary normal brain cells (P<0.001, unpaired t-test; Fig. 2A). U251MG, LN319 and U373MG cells strongly expressed MK protein, whereas primary normal brain cells and U87MG did not (Fig. 2B). Therefore, we proceeded with subsequent experiments using U251MG, LN319 and U373MG as MK-positive cells, and U87MG and primary normal brain cells as MK-negative cells.

Transcriptional activity of MK promoter in glioma cell lines and primary normal brain cells. To compare the transcriptional activity of human *MK* promoter between MK-positive and negative cells, *MK* promoter activity was estimated with *SV40* promoter activity designated as 1 (Fig. 3). The promoter activity of the 600-bp fragment of the *MK* gene positively correlated with expression levels of *MK* mRNA (r=0.9; P<0.005, Pearson's correlation coefficient) and MK protein (r=0.9; P<0.005, Pearson's correlation coefficient). The transcriptional activity of the 600-bp fragment of the *MK* promoter in MK-positive glioma cells was 105 times that in primary normal brain cells (P<0.05, unpaired t-test) and 26 times that in U87MG (P<0.05, unpaired t-test). Moreover, transcriptional activity of pGL3-MK600 was 1.5 times that of pGL3-MK2300 in MK-positive cells (P<0.05, paired t-test), suggesting the presence of a negative regulatory element in the region from -2285 to -562 bp.

Selectivity of Ad-MK to adenovirus E1A expression in glioma cells. To confirm the activity and selectivity of Ad-*MK* on E1A

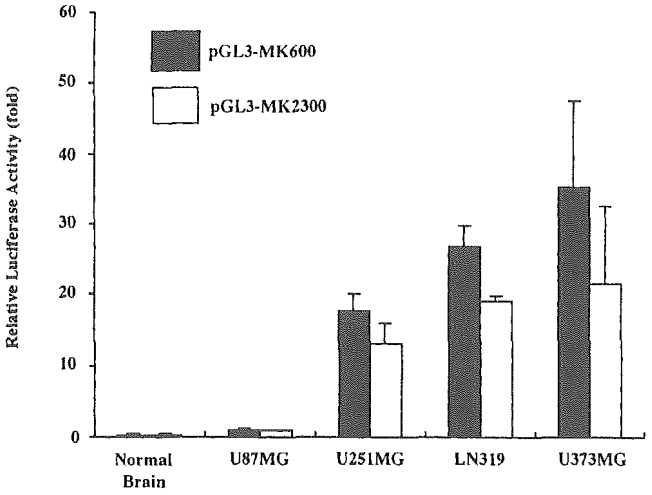

Figure 3. Transcriptional activity of the *MK* promoter. Luciferase activity of reporter plasmids with the *MK* promoter was assayed in MK-positive glioma cells (U251MG, LN319 and U373MG), and MK-negative cells (primary normal brain cells and U87MG). The 600-bp and 2300-bp fragments of the *MK* promoter were inserted into luciferase reporter plasmid (pGL3-MK600, pGL3-MK2300). Bars, means ± SD.

expression in human glioma cell lines and primary normal brain cells, cells were infected with Ad-*MK600* at 10 m.o.i. for 36 h. Expression of E1A protein was assessed by Western blot analysis as shown in Fig. 4. Ad-*MK600* produced obvious expression of E1A in the MK-positive cells, but not in the MK-negative cells. Although there was no detectable E1A expression in cells infected with AdCMV-*LacZ*, E1A was clearly expressed in all cells infected with Ad-*Wild*.

Selective cytotoxicity of Ad-MK on glioma cells in vitro. To evaluate the cytotoxicity of the *MK* promoter-dependent adenovirus *in vitro*, we evaluated IC_{50} (m.o.i.) of Ad-*MK600* and Ad-*MK2300* on glioma cell lines and primary normal brain cells. As shown in Fig. 5A, the IC_{50} of Ad-*MK600* was 0.037, 0.0035 and 0.0014 m.o.i. for U251MG, LN319 and

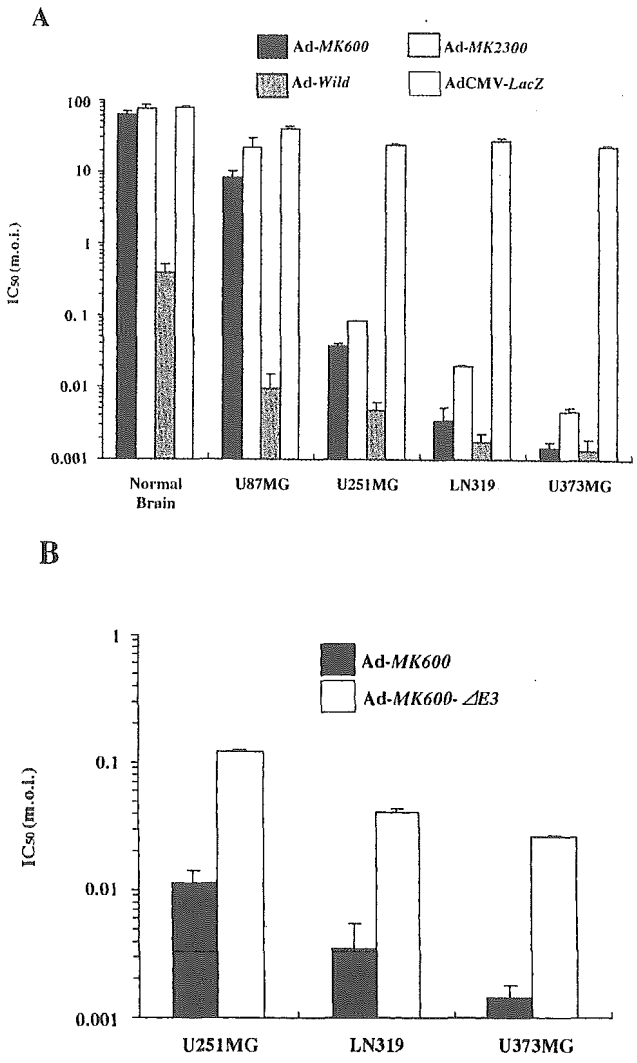

Figure 5. Cytotoxic effect induced by the infection with Ad-*MK* in glioma cells. (A), Cells were infected with AdCMV-*LacZ*, Ad-*MK600*, Ad-*MK2300* or Ad-*Wild* for ten days. The IC_{50} (m.o.i.) was calculated. Bars, means ± SD. (B), Cytotoxic effect of adenoviral *E3* region on Ad-*MK600*. Three MK-positive cell lines were infected with Ad-*MK600* or Ad-*MK600-ΔE3*. The IC_{50} of each adenovirus was determined. Bars, means ± SD.

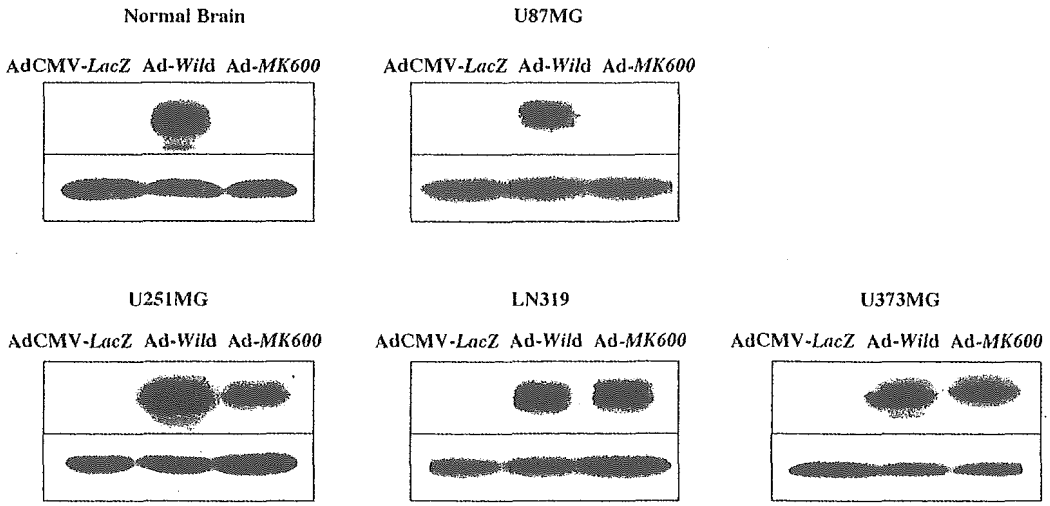

Figure 4. Expression of E1A in glioma cells. Cells were infected with AdCMV-*LacZ*, Ad-*Wild*, or Ad-*MK600*. Cell lysates from infected cells were subjected to Western blot analysis with anti-E1A antibodies. Top panel shows the band position of E1A protein and the ß-actin control is shown in bottom panel.

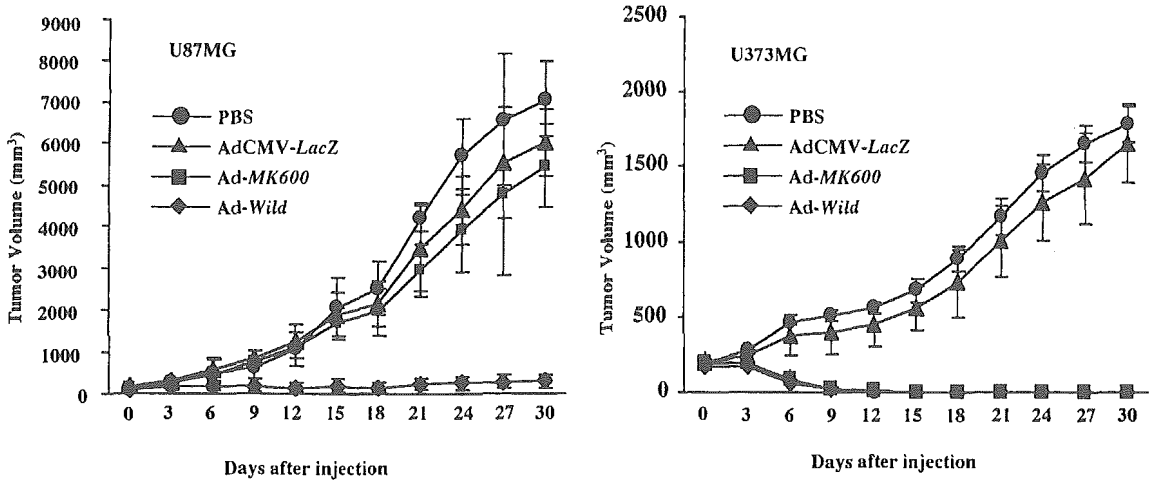

Figure 6. Effect on tumor growth from treatment with no virus (PBS), AdCMV-*LacZ*, Ad-*MK600* or Ad-*Wild*. U87MG or U373MG glioma cells ($1x10^7$) were injected s.c. into flanks of nude mice, followed by the intra-tumoral injection of adenoviruses. MK-positive U373MG tumor xenografts were effectively treated by a single dose of Ad-*MK600* (right), whereas MK-negative U87MG tumor xenografts were unaffected (left). Bars, means ± SD.

U373MG cells, respectively, with a mean value of 0.014 m.o.i., which was respectively 4500 times and 600 times lower than those for primary normal brain cells ($P<0.01$, unpaired t-test) and U87MG ($P<0.01$, unpaired t-test). In MK-positive cells, the IC_{50} of Ad-*MK600* was 4 times lower than that of Ad-*MK2300* ($P<0.01$, paired t-test). In contrast, the IC_{50} of AdCMV-*LacZ* did not differ significantly between cell lines. In MK-positive cells, the IC_{50} of Ad-*MK600* was 2100 times lower than that of AdCMV-*LacZ* ($P<0.01$, paired t-test). In MK-negative primary normal brain cells, the IC_{50} of both Ad-*MK* viruses were not significantly different from those of AdCMV-*LacZ*.

To explore the oncolytic role of the *E3* region in the context of Ad-*MK600*, we analyzed the difference of cytotoxic capacity between *E3*-intact Ad-*MK600* and *E3*-deleted Ad-*MK600-ΔE3* in MK-positive cells. Cytotoxic potency of Ad-*MK600* was 14 times that of Ad-*MK600-ΔE3* ($P<0.05$, unpaired t-test; Fig. 5B).

Treatment of glioma xenografts with Ad-MK600. To evaluate the therapeutic efficacy of Ad-*MK600 in vivo*, no vector (PBS), AdCMV-*LacZ*, Ad-*MK600* or Ad-*Wild* was injected into U87MG and U373MG glioma xenografts on day 0. As shown in Fig. 6, Ad-*MK600* and Ad-*Wild* completely eradicated the U373MG tumors ($P<0.001$, χ^2 test). In contrast, Ad-*MK600* did not significantly reduce the sizes of U87MG tumors compared with AdCMV-*LacZ*. However, Ad-*Wild* significantly reduced the size of U87MG tumors by 98% compared with AdCMV-*LacZ* ($P<0.001$, unpaired t-test).

Discussion

Tumor-selective oncolysis is key to the use of replicating viruses as cancer therapeutics. An appropriate promoter for malignant glioma has not yet been identified. Only nestin promoter and myelin basic protein promoter have reportedly been used to construct viral vectors for treatment of malignant glioma, but these vectors lack adequate tumor specificity and therapeutic effect for malignant glioma (2). Recently, the regulatory sequences of the human *MK* and *cox-2* genes have been used for transcriptional targeting of gene expression in the context of gene therapy, because overexpression of these genes has reportedly been observed in various types of malignant tumors, such as gastrointestinal, ovarian and breast cancer (6,10). In malignant glioma, it has been reported that MK and cox-2 expression correlate with malignancy in immunohistochemical, Western blot and Northern analysis (7,10). These reports involved qualitative analysis; there have been no reported quantitative analyses of expression of MK and cox-2 in malignant glioma. We report here, for the first time, the quantitative analysis of *MK* and *cox-2* expression in malignant glioma. In the present study, *MK* was overexpressed in malignant glioma tissues but *cox-2* was not. These findings indicate that transcriptional upregulation of the *MK* gene might be highly specific for malignant gliomas, compared with the *cox-2* gene. Furthermore, it has been reported that MK has good potential as a tumor marker in esophageal cancer, gastrointestinal cancer, pancreatic cancer and lung cancer (6). Although serum and cerebral spinal fluid levels of MK have not yet been identified in the patient with malignant glioma, our results indicate that MK might be feasible for a marker of malignant transformation in human glioma. Further analysis is required to determine the reliability, sensitivity and precision of MK as a marker of malignant transformation.

It has been reported that Ad-*MK* without the *E3* region in which E1A expression is driven by 2300 bp of the 5'-flanking region of the human *MK* gene, has been used for the treatment of neuroblastoma (11). However, the present results indicated that a 600-bp fragment of the *MK* promoter region activates transcription of the luciferase gene to a greater degree than a 2300-bp fragment. Furthermore, the oncolytic effect of Ad-*MK600* was 4 times that of Ad-*MK2300*. Yoshida *et al* (12) have reported that a 559-bp fragment of the *MK* gene activates transcription of the reporter gene more efficiently than a 2300-bp fragment in pancreatic cancer. These findings regarding *MK* promoter activity are consistent with the present results. In the present study, Ad-*MK600* had a

significant oncolytic effect that was 14 times that of Ad-*MK600-ΔE3*, indicating that *E3* is responsible for augmentation of the antitumor potency of CRAd.

The present is the first study of CRAd in which the cell-type specificity of the *MK* promoter was used to target malignant glioma. We found that Ad-*MK* selectively killed MK-positive glioma cells, but did not kill MK-negative primary normal brain cells. Additionally, in animal models, Ad-*MK600* completely eradicated MK positive-glioma xenografts. AdCMV-*LacZ* had minimum effect suggesting that the therapeutic effect of Ad-*MK600* was associated with viral replication, cell lysis and viral spread. In previous studies, replication-selective adenovirus without the *E1B 55-kDa* sequence (ONYX-015) (13) and replicative adenoviral vector expressing herpes simplex virus-thymidine kinase (14), which were used for treatment of malignant glioma, did not completely eradicate tumors, although they inhibited tumor growth. Since the *E1B 55-kDa* sequence is thought to play a crucial role in adenovirus replication, the oncolytic effect of the adenovirus Ad-*MK600* might be related to overexpression of E1B 55-kDa protein. The present findings suggest that Ad-*MK600* has a significantly enhanced therapeutic window for malignant glioma. It has been reported that there is synergy between CRAd (Ad5-Delta24RGD, ONYX-015) and radiotherapy in glioma xenograft models (15,16). Consequently, adjuvant conventional therapy in combination with Ad-*MK* might enhance the oncolytic effect of Ad-*MK* for the treatment of malignant glioma.

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頭蓋内胚細胞性腫瘍おける増殖因子受容体 c-Met 発現の検討

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はじめに

頭蓋内 Germ cell tumors (GCTs) は稀な腫瘍であり、様々な組織形態を示す腫瘍群の総称で、手術および放射線・化学療法による治療成績の違いにより、予後良行群の germinoma, teratoma、中間群の immature teratoma, germinoma with syncytiotrophoblastic giant cells (STGC)、予後不良群の embryonal carcinoma, yolk sac tumor, choriocarcinoma, 及びこれらの混合腫瘍、の3群に大別される[1]。Germinoma は放射線、化学用法に対する感受性が極めて高く、5年生存率は95%に達するが、予後不良群に属する GCTs では手術、放射線、化学療法による治療にも拘わらず、5年生存率は20%以下である[1]。従って、GCTs の腫瘍組織型を分類することは治療法の選択を決定するうえで重要であり、組織の鑑別に有用な腫瘍マーカーの存在は不可欠であると考えられる。C-kit はレセプター型チロシンキナーゼであり、germ cell の生存や分化に重要な役割を果たしていることがこれまでの研究により示されている[2]。また、精巣 GCTs での発現が検討され、seminoma では高い発現を認め、腫瘍形成に関与していることが示唆されている[3]。さらに頭蓋内 germinoma での発現も、精巣腫瘍同様に認められ、その分泌型である s-kit は腫瘍マーカーとして診断に役立つことが期待されている[4]。一方、中間群、予後不良群に属する組織型では c-kit の発現は認められず[3),4]、c-kit system 以外のレセプター型チロシンキナーゼが腫瘍化に関与している可能性があると考えられる。Hepatocyte growth factor (HGF) のレセプターである c-Met は、これまで種々の癌腫で発現が亢進し、腫瘍の増大、浸潤、転移などを促進すること、また予後と相関していることが報告されている[5]。さらに c-Met は、胚細胞の分化や、胎盤の形成に重要な役割を果たしていることが示されていることより[6),7]、我々は頭蓋内 GCTs の手術摘出標本を用い、c-Met の発現を検討した。

1．対象と方法

免疫組織化学染色

当院で手術された GCTs 26例（germinoma 12例、mixed germ cell tumor で主に teratoma のもの2例、germinoma with STGC 2例、immature teratoma 3例、mixed GCTs の構成組織が主に embryonal carcinoma のもの3例、主に yolk sac tumor のもの2例、choriocarcinoma 2例）を用いた。Informed consent を得た上で、一部の症例では腫瘍摘出後、ただちに凍結した腫瘍をたんぱく抽出に使用した。腫瘍組織はホルマリン固定後パラフィン包埋切片を作成した。0.01M クエン酸（pH 6.0）によるマイクロウエーブ処理後、1次抗体として、抗ヒト c-kit 抗体（Dako)、抗ヒト c-Met 抗体（C-28, Santa Cruz)、Phospho-Met (Tyr1349, Tyr1234/1235) 抗体（Cell Signaling Technology)を使用し、ABC法で免疫組織化学染色を行った。

免疫沈降・ウェスタンブロット法

腫瘍組織よりたんぱくを抽出し定量後、抗ヒト c-Met 抗体と protein A-Sepharose beads を加え免疫複合体を形成させ、wash buffer で洗浄後、7.5% SDS-ポリアクリルアミドゲルに電気泳動、ニトロセルロース膜に転写し、1次抗体、2次抗体と反応、洗浄後 ECL キット (Amersham) で検出した。

2．結　果

1）GCTs における c-Met 発現の免疫組織化学的検討 (Table 1, Figure 1)

Table 1 に腫瘍組織型と c-Met の染色結果を示す。また、Fig. 1 には、各組織型における c-Met, c-kit の代表的な免疫組織染色例を提示する。C-Met は germinoma では12例中9例で陰性で、残る3例ではごく一部の腫瘍細胞の細胞質が非常に淡く陽性を示すのみであった（Fig. 1A)。一方、c-kit 抗体では germinoma 全例で腫瘍細胞膜が強く染色された (B)。Germinoma with STGC では2例中2例で STGC が c-Met 強陽性を示すほか、一部の腫瘍細胞も陽性を示した（C）が、C-kit は germinoma 細胞成分のみ陽性であった（D)。Immature teratoma では3例いずれも、未熟な腺上皮細胞膜に c-Met の発現が強く認められたが（E)、C-kit は陰性であった（F)。Embryonal carcinoma では3例すべてで、多層性管状に増殖した上皮様腫瘍細胞膜に c-Met の強い陽性所見が認められ、間質細胞は陰性であった（G)。Yolk sac tumor では2例中2例で、網目状に配列する腫瘍細胞が陽性であり（I)、choriocarcinoma では2例いずれも、syncytiotrophoblastic cell, cytotrophoblas-

Table 1. Expression of c-Met and c-kit in intracranial germ cell tumors

Prognosis	Histology	C-Met +	C-Kit +
Good	Germinoma	±3/12 (−9/12)	12/12
	Teratoma	1/2 (±1/2)	1/2 (−1/2)
Intermediate	Germinoma with STGC	2/2	2/2
	Immature teratoma	3/3	0/3
Poor	Embryonal carcinoma	3/3	0/3
	Yolk sac tumor	2/2	0/2
	Choriocarcinoma	2/2	0/2

tic cell 共に主に細胞膜を中心に c-Met 陽性を示した(K)。C-kit はこれらの予後不良群 GCTs でいずれにおいても陰性であった (H, J, L)。

2）C-Met リン酸化状態の検討

Phospho-Met 抗体を用いた免疫染色法で腫瘍組織中の c-Met の活性化を検討した。C-Met の発現があった embryonal carcinoma では一部の上皮様腫瘍細胞の細胞膜に phospho-Met の陽性所見を認めた(Figure 1-M)。

3）C-Met 発現レベルの検討 (Figure 2)

GCTs 腫瘍組織よりタンパクを抽出し、c-Met の発現を免疫沈降・ウェスタンブロット法にて確認した。Germinoma では発現がみられず、embryonal carcinoma, yolk sac tumor では c-Met が高発現していることが確認された。

3．考　察

増殖因子／レセプターの質的、量的な発現異常は、その下流のシグナル伝達系の活性化をもたらし、腫瘍細胞の増殖、分化、細胞死の抑制、血管新生などを介し腫瘍形成、進展、浸潤、転移などに深く関わると考えられている。GCTs における増殖因子／レセプターの発現を検討した研究は少なく、特に頭蓋内 GCTs においてはほとんど研究されていないのが現状である。精巣 GCTs においてこれまで報告されているものとして、fibroblast growth factor (FGF) のメンバーである HST-1 や FGF4, 8、FGF receptor 1 が主に non-seminomatous GCTs に発現していること[8]、c-kit が seminoma に発現し[9]、c-kit の変異活性型が約 25%で発現していること[10]、血管新生に関わる VEGF の発現が GCTs で高いこと、EGFR が choriocarcinoma の syncytiotrophoblastic cell で発現し、TGFαとオートクラインループを形成し、腫瘍細胞の増殖に関わる可能性があること[11]、動物モデルでは GDNF のトランスジェニックマウスで seminoma が発生することが示されている[12]。しかし、これまでに GCTs 組織において c-Met の発現及び機能を調べた報告はない。C-Met／HGF は種々の癌で、発現亢進がみられ、オートクラインあるいはパラクラインループを形成し、腫瘍細胞の増殖、遊走促進による浸潤、転移、血管新生などに関与し、悪性度や予後とも相関することが知られている[5]。また、c-Met を高発現させると HGF の関与なくとも腫瘍細胞運動能が亢進する[13]。C-Met は胎盤では trophoblasts に発現しており[7]、*in vitro* で cytotrophoblast の増殖や浸潤に関与していることが示されている[14]。また、最近、choriocarcinoma 細胞株において c-Met は恒常的に活性化されていることが示された[15]。今回の我々の検討でも、choriocarcinoma の腫瘍細胞で c-Met の強い発現がみられたことから、c-Met は腫瘍細胞の増殖や浸潤に関係している可能性がある。EGFR からのシグナル伝達系が活性化されると c-Met の発現、活性化がおこることが甲状腺癌細胞などで示されており[16]、choriocarcinoma では TGFα／EGFR システムが活性化している[11]ことから、EGFR からのシグナルが c-Met の発現を亢進させている可能性がある。

C-Met は癌化だけでなく、胎児発生期の組織分化や形態形成にも関わっている[17]。P19 マウス embryonal carcinoma 細胞株は、retinoic acid 添加で neuroectodermal に、DMSO 添加で mesodermal 細胞に分化するため、*in vitro* のモデルとして頻用される。この系では、c-Met は分化とともに発現とそのリン酸化の増強が起る[18]。また、c-Met は胎児形成発達の際、管腔構造誘導にも関わっており、我々の免疫組織染色の検討で、embryonal carcinoma や、immature teratoma では、分化傾向を示す腺管を形成した上皮細胞に発現が高く見られたことは、これらの腫瘍細胞での分化や腺管形成に

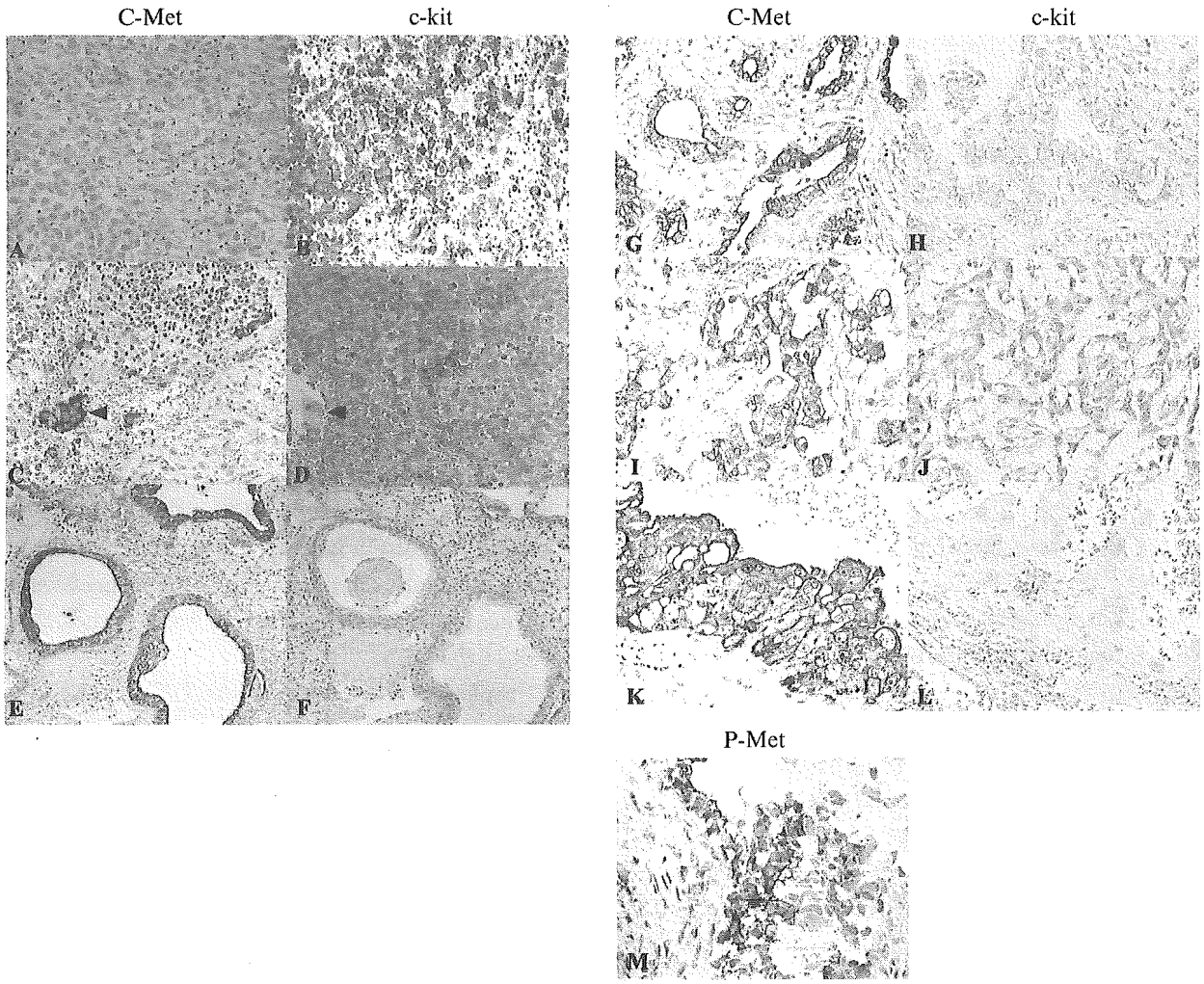

Figure 1. Immunohistochemical findings in different intracranial germ cell tumors
A. B. Germinoma. Tumor cells were negative for c-Met (A) and positive for c-kit (B). X200
C. D. Germinoma with STGC. Syncytiotrophoblastic giant cells (arrow heads) and surrounding tumor cells were positive for c-Met (C). Syncytiotrophoblastic giant cells were negative for c-kit (D). X200
E. F. Immature teratoma. Immunoreactivity for c-Met showed positive in tumor cells displaying epitherial differentiation. C-Met was negative in the mesenchymal component (E). Immunoreactivity for c-kit was not detected in any components (F). X100
G. H. Embryonal carcinoma. C-Met was strongly expressed on the cell surface of the epithelial tumor cells (G). Immunoreactivity for c-Kit was not detected in any components (H). X200
I. J. Yolk sac tumor. C-met was strongly expressed on the tumor cell surface membrane (I). Tumor cells displayed negative reaction for c-kit (J). X200
K. L. Choriocarcinoma. The membrane and cytoplasm of both syncytiotrophoblastic giant cells and cytotrophoblasts were strongly positive for c-Met (K). Tumor cells displayed negative reaction for c-kit (L). X200
M. Phospho-c-Met expression. The membrane expression of the activated form of c-Met (phosphorylated c-Met) was detected in c-Met-positive embryonal carcinoma. X200

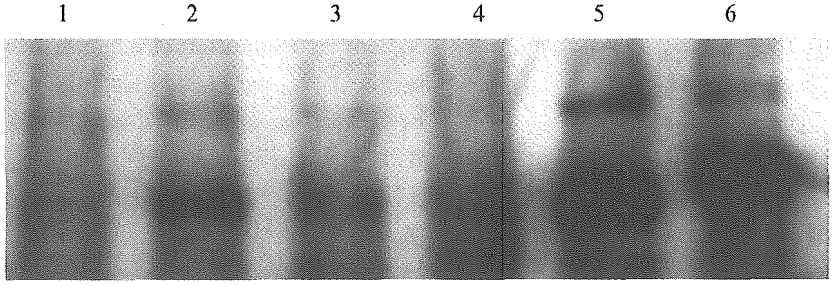

Figure 2. Western blot analysis of c-Met in GCTs
The Met protein was highly expressed in the yolk sac tumors (lanes 2-4) and embryonal carcinomas (lanes 5, 6), but not expressed in germinoma (lane 1).

関与している可能性があると考えられる。今回のリン酸化c-Metに対する抗体を用いた検討で、c-Metの発現が高いembryonal carcinomaではc-Metが活性化されている可能性が示唆されたが、c-MetのGCTsにおける機能をさらに解析するためには、頭蓋内GCTsから腫瘍細胞株を樹立し、*in vitro*での解析をすすめる必要があろう。

前述のように、C-kit/SCFはprimordial germ cellの遊走や生存に関係しており、c-kitの発現亢進が精巣seminomaで認められている[3]。今回の我々の検討でもc-kitは頭蓋内germinomaに発現が確認された。リンパ球や組織球が主体をなす肉芽腫様変化の強いgerminomaでは診断に苦慮することがあり、腫瘍細胞を検出し、確定診断をくだすのにc-kitは有用であると考えられる。一方、臨床的には、GCTsの腫瘍組織内にnon-germinomaの成分を見つけだすことは、治療法の選択、予後を知る上で重要である。C-Metは、我々の検討では予後不良群、中間群に属する腫瘍組織に高い発現が認められ、これらの組織型の検出のためのマーカーの1つとなる可能性がある。

近年、チロシンキナーゼ阻害剤を用いた分子標的治療法が開発され、c-kitを標的としたSTI571の臨床試験が悪性gastrointestinal stromal tumorや化学療法抵抗性の転移性seminomaで行われている[10]。C-Metのチロシンキナーゼ阻害薬であるK252aやSU11271を用いた治療法の開発も進められており[5),19)]、今後頭蓋内germinomaに対するc-kit、non-germinomatous GCTsに対するc-Metを標的とした治療応用の可能性があると考えられる。

結　語

C-Metはc-kitと異なり、予後の不良なGCTsにおいて高発現しており、GCTsの悪性化や腫瘍組織の形態形成に関与している可能性が示唆された。c-Metはnon-germinomatous GCTsの新たな腫瘍マーカーとなる可能性があると考えられた。

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SPECIAL ARTICLE

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Immunohistochemical analysis of the mutant epidermal growth factor, ΔEGFR, in glioblastoma

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Abstract The naturally occurring mutated form of the epidermal growth factor receptor, ΔEGFR (also named EGFRvIII and de2-7EGFR), greatly enhances glioblastoma (GBM) cell growth in vivo through several activities, such as down-regulating p27 and up-regulating BclX(L) while increasing signaling through the RAS-MAPK and PI3-K cascades. More than half of GBMs, especially of the de novo type, overexpress EGFR, and 50%–70% of these express ΔEGFR. However, little is known about the distribution of ΔEGFR-expressing tumor cells within surgical specimens. In order to address this clinically important issue, we performed immunohistochemical analyses of 53 GBMs obtained during surgery using the anti- ΔEGFR monoclonal antibody, DH8.3. We also simultaneously analyzed wild-type EGFR expression in these tissues using the anti-EGFR monoclonal antibody, EGFR.113. ΔEGFR and wild-type EGFR expression were observed in 20/53 (38%) and 29/53 (55%), respectively. Nineteen (95%) of the ΔEGFR-positive tumors also expressed wild-type EGFR; one case was ΔEGFR-positive but wild-type EGFR-negative. In 13/20 (65%) of the ΔEGFR-positive tumors, tumor cells were scattered diffusely within the tumors, 6/20 showed geographical distribution of ΔEGFR-positive tumor cells, and one case showed homogeneous staining. In the wild-type EGFR-positive cases, almost all tumor cells expressed EGFR. The differential distribution of cells expressing the two receptors observed here may suggest either that ΔEGFR arises at a low frequency from wild-type EGFR-expressing cells, perhaps during the process of gene amplification, or that there is a paracrine-type of interaction between them.

Key words Epidermal growth factor receptor · Glioblastoma · Immunohistochemistry

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Indroduction

The epidermal growth factor receptor (EGFR) gene is amplified at the DNA level and overexpressed at the level of mRNA or protein expression in tumor tissues of about 40%–50% of human glioblastoma (GBM) cases.[1–3] This EGFR gene amplification is often followed or accompanied by further gene rearrangement. About two-thirds of such rearrangements result in a particular mutant form called-ΔEGFR, de2-7EGFR, or EGFRvIII, an in-frame deletion of exons 2–7 resulting in a deletion of 267 amino acids.[4–6] The resulting mutant protein is ligand independent, constitutively phosphorylated, and localized primarily to the cell surface.[7–9] ΔEGFR promotes the tumorigenesis of GBM cells in vivo by increasing cellular proliferation,[10] decreasing cellular apoptosis,[11] and promoting tumor cell invasion.[12] However, these conclusions have derived from the analysis of the behavior of xenografts that arose after inoculation of cells expressing relatively homogeneous levels of ΔEGFR, and little is known about the distribution of ΔEGFR-expressing tumor cells in surgical specimens. Since this limits understanding of the clinical significance of ΔEGFR, we performed immunohistochemical analysis of ΔEGFR and wild-type EGFR expression in primary GBM tissues. Our results revealed differential distribution of tumor cells expressing these receptors, suggesting potential interactions between them.

Materials and methods

Brain tumor samples

Fifty-three GBM specimens that were surgically resected between 1989 and 2002 at Saitama Medical School were subjected to investigation. All tumors were diagnosed according to the World Health Organization (WHO) classification of brain tumors.[13] Tissue samples were fixed in 10% formalin and embedded in paraffin for histological as well as immunohistochemical examination. For Western blotting, samples were snap-frozen in liquid nitrogen in the operating rooms and stored at −80°C until use. Twenty samples were available for this series of studies.

Immunohistochemistry

The sections were deparaffinized, rehydrated, and incubated in hydrogen peroxide to block endogenous peroxidase activity, followed by antigen retrieval in a steam cooker and preincubation in normal goat serum. An anti-ΔEGFR monoclonal antibody, DH8.3, raised against a synthetic peptide spanning the unique junctional sequence of the deleted part of ΔEGFR,[14] was diluted 1:50 and applied to the samples for 1 h at room temperature. DH8.3 does not react with wild-type EGFR.[14] A standard ABC method was performed according to the manufacturer's recommendations (Vectastain, Vector Laboratories, Burlingame, CA, USA), and diaminobenzidine tetrahydrochloride was used to visualize the immunoreactivities. The slides were lightly counterstained with hematoxylin. Anti-EGFR monoclonal antibody, EGFR.113, raised against the extracellular domain of the wild-type EGFR was purchased from Novocastra (Newcastle, UK) and used for immunohistochemistry according to the manufacturer's recommendations.

Western blotting

The tissue specimens were lysed in extraction buffer (50 mM Tris-HCl [pH 7.6], 50 mM NaCl, 2% NP-40, 0.5% deoxycholic acid, 0.2% sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5 mM Na_3VO_4) and sonicated. The lysates were centrifuged at 8000*g* for 5 min, and the supernatants were collected. Each protein sample (20 μg) was separated with 7.5% polyacrylamide/SDS gels and electroblotted onto nitrocellulose membranes (ECL membrane, Amersham Pharmacia Biotech, Piscataway, NJ, USA). After blocking with 5% skim milk in Tris-buffered saline with 0.05% Tween 20, the membranes were incubated with an anti-EGFR monoclonal antibody, C13, which reacts with both wild-type EGFR and ΔEGFR (a kind gift from Dr. Gordon Gill, University of California at San Diego), and then incubated with a horseradish peroxidase-conjugated antimouse secondary antibody (Vector Laboratories), and subjected to chemiluminescence detection (ECL, Amersham Pharmacia Biotech).

Image intensification

The images from immunohistochemistry and Western blotting were captured with a digital camera and processed with Adobe Photoshop 7.0 on an Apple Macintosh computer.

Results

Immunohistochemistry

Positive immunoreactivities for DH 8.3 and EGFR.113 were observed in 20/53 (38%) and 29/53 (55%), respectively. Of those 20 cases positive for DH8.3, 19 were also positive and 1 was negative for EGFR.113. Typical examples of DH8.3-immunoreactivity are shown in Fig. 1A–E. Immunoreactivities for DH8.3 were observed on the cell membrane or in the cytoplasm of tumor cells that were similar to those for EGFR.113 (Fig. 1A and F). Immunoreactive cells were scattered diffusely in the 13 of 20 (65%) specimens positive for DH8.3 (Fig. 1A and B). In six specimens (30%), cells immunoreactive for DH8.3 were geographically distributed (Fig. 1C). The remaining specimen showed a homogeneous distribution of immunoreactive cells. In one specimen, DH8.3-immunoreactive cells were observed adhering to tumor vessels (Fig. 1D and E). On the other hand, in the tumors immunoreactive for EGFR.113, almost all tumor cells had homogeneous immunoreactivities, as shown in Fig. 1F, except for one case that showed geographical distribution of EGFR-expressing cells. Figure 1F is from the same area of the same specimen shown in Fig. 1B.

Western blotting

Significant amounts of ΔEGFR and full-length EGFR were detected in 9 of 20 (45%; lanes 5, 6, 8, 9, 10, 11, 13, 17, and 20 in Fig. 2) and 12 of 20 (60%, lanes 2, 5, 6, 7, 8, 9, 10, 11, 13, 15, 17, and 20 in Fig. 2) samples by Western blotting, respectively. Three samples (lanes 2, 11, and 15 in Fig. 2) were negative for EGFR.113 by immunohistochemistry but positive for full-length EGFR by Western blotting. None of the samples negative for full-length EGFR by Western blotting was positive for EGFR.113 by immunohistochemistry, and both results were concordant in 17/20 (85%) of the samples. Four samples (lanes 5, 9, 11, and 17 in Fig. 2) were negative for DH8.3 by immunohistochemistry and positive for ΔEGFR by Western blotting, and both results were concordant in 16/20 (80%).

Discussion

Expression of ΔEGFR was observed in 20/53 (38%) of GBM samples by immunohistochemistry and 9/20 (45%) by Western blotting. Those frequencies were similar to those

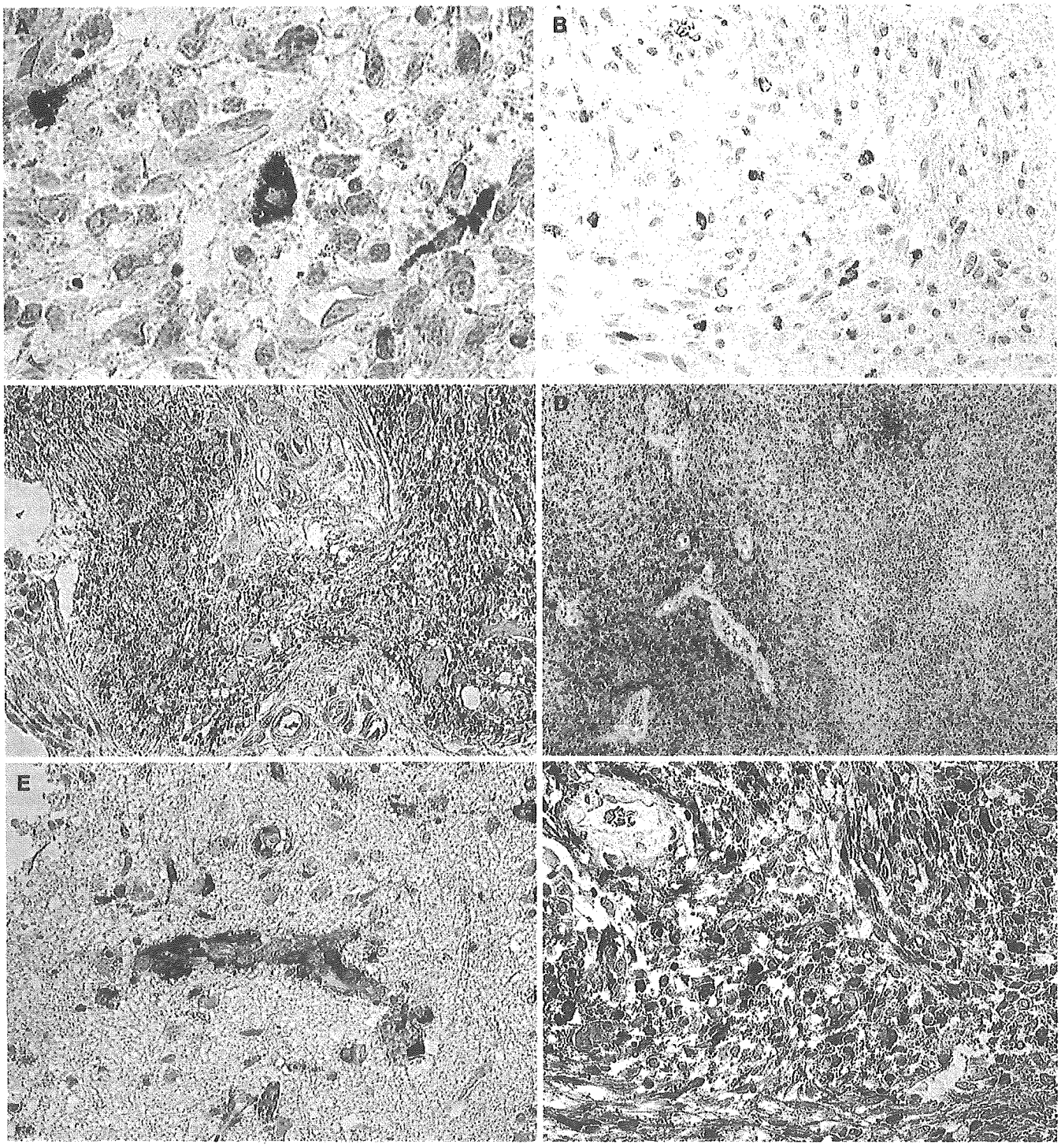

Fig. 1. Immunohistochemical detection of mutated epidermal growth factor receptor (ΔEGFR) (**A–E**) and wild-type EGFR (**F**) expression in glioblastoma specimens. Original magnifications are (**A**) ×1000, (**B**, **C**, **E**, **F**) ×400, and (**D**) ×100

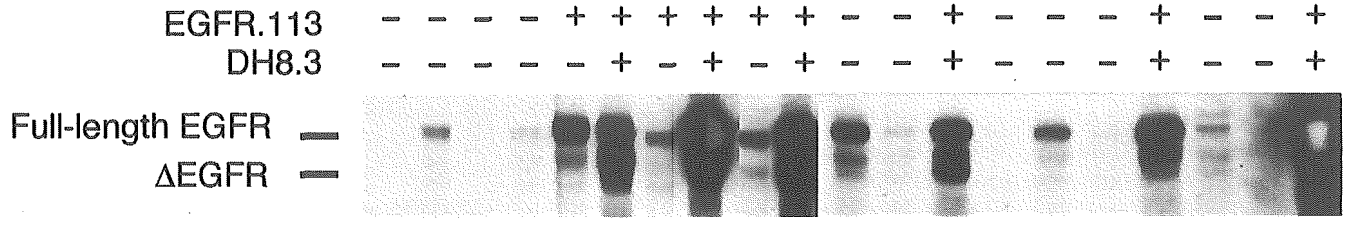

Fig. 2. Western blotting analysis of *ΔEGFR* and wild-type *EGFR* expression in glioblastoma specimens. + or − indicates immunoreactivities for *DH8.3* and *EGFR.113* by immunohistochemistry

in reported series: 7/12 (58%) by Western blotting,[5] and 8/12 (67%)[5] or 12/21 (62%)[4] by immunohistochemistry. Among the 20 cases in which frozen samples were available for Western blotting, the results of Western blotting and immunohistochemistry were concordant in 16 cases (80%). Expression of ΔEGFR in the remaining four cases was positive by Western blotting but negative by immunohistochemistry, which would indicate either that Western blotting was more sensitive for the detection of low levels of ΔEGFR expression, or that there were sampling errors due to the heterogeneous location of positive cells.

Each of the 20 cases that were immunoreactive for DH8.3 by immunohistochemistry was also immunoreactive for EGFR.113, with one exception. Among the 29 cases positive for EGFR.113, 19 (66%) were positive for DH8.3. It has been reported that GBMs with ΔEGFR mutation always harbor increased *EGFR* gene dosage, and frequency of ΔEGFR found in GBMs with *EGFR* amplification was 67%.[6] These data suggest that amplification of the *EGFR* gene precedes *EGFR* mutation.

GBM cells expressing ΔEGFR have a remarkable in vivo growth advantage.[7] One consequence of this could be a clonal expansion of the cell population. Consonant with this notion are reported experiments showing that injection of a mixture of U87MG.ΔEGFR (a GBM cell line expressing ΔEGFR) and parental U87MG at a 1:50000 ratio into nude mouse brains showed an outgrowth of U87MG.ΔEGFR cells, with the proportion reaching 83% in the tumors that developed.[11] The geographical distribution of ΔEGFR-expressing tumor cells observed in surgical specimens has been believed to be the result of an analogous clonal expansion. However, our results here show that GBM tumor cells expressing ΔEGFR prefer to distribute diffusely in a scattering pattern in the majority of cases. In contrast, tumor cells expressing wild-type EGFR appeared to distribute in a more homogeneous fashion (Fig. 1F). The differential distribution of ΔEGFR- and wild-type EGFR-expressing tumor cells, together with the fact that almost all tumors expressing ΔEGFR expressed wild-type EGFR, appears to support the hypothesis that ΔEGFR arises at a low frequency from wild-type EGFR-expressing cells. The significantly enhanced biological aggressiveness of the tumor cells expressing ΔEGFR, together with their often diffuse occurrence among other tumor cells, also raises the possibility that they might provide a positive field effect on surrounding tumor cells that overexpress wild-type EGFR. The nature of such interactions requires further study.

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Case Report

Stereotactic radiosurgery for nodular dissemination of anaplastic ependymoma

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Summary

Dissemination of primary intracranial ependymoma occurs in 10% of all cases and is difficult to treat, so this may be one of the major reasons for the poor prognosis. Two patients with nodular dissemination of anaplastic ependymoma were treated with repeated stereotactic radiosurgery using the gamma knife (GK), resulting in tumour control over 21 months. GK radiosurgery is a safe and effective treatment option for providing good local control in patients with nodular dissemination of ependymoma.

Keywords: Anaplastic ependymoma; dissemination; gamma knife; stereotactic radiosurgery.

Introduction

Intracranial ependymomas are rare neuro-ectodermal tumours arising from the ependymal cells of the ventricular system, and predominantly occur in children and young adults. Intracranial ependymomas constitute approximately 3% of all intracranial neoplasms and about 10% of all childhood brain tumours [6, 11]. Aggressive multimodality management including surgery, radiation therapy, booster irradiation and chemotherapy have extended the survival time, but the overall survival in most series still does not exceed 60% at 5 years [3, 13, 15, 18]. The pattern of recurrence may be local and/or dissemination to remote sites, and the prognosis after recurrence is quite poor. Dissemination occurs in about 10% of cases of primary intracranial ependymoma [2, 16]. The correlation between prognosis and histological grade of the tumour remains controversial [3, 4, 6, 15, 16, 22], but leptomeningeal dissemination occurs more frequently with high-grade ependymoma than with low-grade ependymoma [16]. The mean survival time after dissemination is 6 months [2].

We treated two patients with anaplastic ependymoma, who manifested multiple nodular dissemination in the course of their disease, by stereotactic radiosurgery (SRS) using the gamma knife (GK). The tumours were controlled for 21 months.

Case reports

Case 1

A 14-year-old girl first presented with headache, nausea and vomiting. She was admitted for treatment of a right lateral ventricular tumour in October 1992. Magnetic resonance (MR) imaging revealed a cystic mass with ring enhancement in the right lateral ventricle, involving the cingulate gyrus (Fig. 1). She underwent subtotal removal of the tumour. Histological examination revealed anaplastic ependymoma. She received 60 Gy of local irradiation and chemotherapy using nimustine hydrochloride (ACNU). She made a complete recovery and radiological study showed no residual tumour. ACNU maintenance therapy was given on an outpatient basis for 1.5 years. She led a normal school life after discharge from our hospital. However, MR imaging performed 4 years and 5 months after the initial treatment revealed a nodular enhanced mass at the cingulate gyrus.

She was treated with GK radiosurgery on four occasions, surgery on three occasions and chemotherapy using cisplatin and etoposide for local recurrences (Table 1). During these treatments, multiple intracranial nodular disseminations occurred. She underwent GK radiosurgery on six occasions for these disseminations in various locations (Table 1, Fig. 2). Fourth ventricular dissemination which first occurred 7 years and 10 months after the initial treatment was treated twice by GK radiosurgery. Although the tumour was controlled for 21 months, MR imaging performed 9 years and 7 months after the initial treatment revealed progression of the tumour. Subtotal removal of the fourth ventricular mass was performed, followed by whole brain irradiation (30 Gy). Follow-up MR imaging performed 9 years and 10 months after

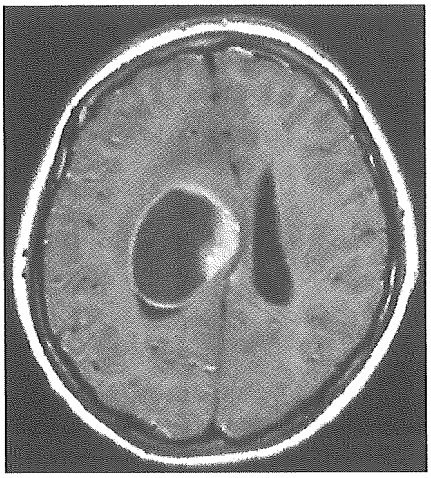

Fig. 1. Case 1: Axial T1-weighted MR image with gadolinium-diethylenetriaminepenta-acetic acid on admission demonstrating a cystic mass with ring enhancement in the right lateral ventricle

the initial treatment showed new dissemination in the anterior horn of the right lateral ventricle, but no enhanced lesion was observed at the primary site and the other nodular disseminations treated by GK radiosurgery were controlled (Fig. 3).

Although the patient developed transient left hemiparesis after the second operation, hemiparesis gradually improved and she began to walk with assistance of mechanical aids. She developed no additional neurological deficits after the third and fourth operations and repeated GK radiosurgery. During these treatments, the patient led a normal school life, entered a nursing school, and passed the national board examination. Her mental status and consciousness was normal, and Karnofsky scale was 70% at the last follow up.

Case 2

A 14-year-old boy presented with headache and nausea persisting for a month. He was admitted for treatment of a fourth ventricular tumour in January 1998. MR imaging revealed a heterogeneous enhanced mass in the fourth ventricle and hydrocephalus (Fig. 4). He underwent subtotal removal of the tumour. Histological examination revealed anaplastic ependymoma. Chemotherapy using cisplatin and etoposide, and local irradiation (33 Gy) by the hyper-fractionated method and craniospinal irradiation (30 Gy) by even-fractionated method were given as adjuvant therapy. Although he suffered from transient lower cranial nerve paresis after the surgery, his neurological condition gradually improved. His first relapse occurred 1 year and 7 months after the initial treatment. MR imaging revealed a small enhanced mass in the fourth ventricle.

GK radiosurgery was performed on three occasions for local recurrence in the fourth ventricle and lower vermis and on three occasions for intracranial nodular dissemination (Table 2, Fig. 5). Multiple nodular disseminations first occurred at 2 years and 4 months after the initial treatment. Nodular dissemination at the thoracic spine occurred 3 years and 10 months after the initial treatment and caused paralysis of the right lower limb and gait disturbance. Surgical removal of the spinal tumour, chemotherapy using ifosfamide, cisplatin and etoposide, and local spinal irradiation (36 Gy) were performed. MR imaging performed 5 months after the surgery showed no residual spinal tumour. MR imaging performed 4 years and 3 months after the initial treatment revealed that the intracranial disseminations were well controlled (Fig. 6). However, follow-up MR imaging performed 4 years and 5 months after the initial treatment revealed diffuse craniospinal dissemination.

During his treatment, the patient led a normal school life and was employed in the computer company after finishing high school. Paraparesis occurred after surgery for the spinal dissemination. His mental status and consciousness was normal, and Karnofsky scale was 60% at the last follow up.

Discussion

The extent of surgical resection is the most consistent factor affecting outcome in cases of intracranial ependymoma [3, 6, 13, 15, 17, 18, 22]. Complete resection can lower the risk of recurrence. The dismal prognosis after recurrence emphasises the importance of the extent of resection at the initial operation. However, complete removal is often not possible and the recurrence rate remains high. The predominant site of relapse is local

Table 1. *Recurrence pattern and treatment for case 1*

Date	Intervals after the initial treatment	Location	Recurrence pattern	Treatment
1992.10.	primary	rt. lateral ventricle of body cingulate gyrus	primary	subtotal removal, LB (60 Gy), ACNU
1997.8.	4 y 5 mo	cingulate gyrus	local 1	GK (25 Gy)
1998.10.	5 y 5 mo	cingulate gyrus	local 2	total removal, CDDP + VP-16
1999.2.	5 y 11 mo	cingulate gyrus	local 3	GK (16 Gy)
1999.10.	6 y 7 mo	cingulate gyrus	local 4	total removal
2000.3.	7 y	cingulate gyrus	local 5	GK (18 Gy)
2001.1.	7 y 10 mo	cingulate gyrus	local 6	GK (22 Gy)
		fourth ventricle	dissemination 1	GK (22 Gy)
2001.2.	7 y 11 mo	cingulate gyrus	local 7	total removal
2001.12.	8 y 9 mo	rt. trigone	dissemination 2	GK (22 Gy)
2002.1.	8 y 10 mo	fourth ventricle	dissemination 3	GK (23 Gy)
2002.5.	9 y 2 mo	septum pellucidum	dissemination 4	GK (23 Gy)
2002.8.	9 y 5 mo	lt. lateral ventricle of body	dissemination 5	GK (23 Gy)
		rt. trigone	dissemination 6	GK (22 Gy)
2002.12.	9 y 9 mo	fourth ventricle	dissemination 7	subtotal removal, WB (30 Gy)

GK Gamma knife radiosurgery; *LB* local brain irradiation; *CDDP* cisplatin; *VP-16* etoposide; *WB* whole brain irradiation.

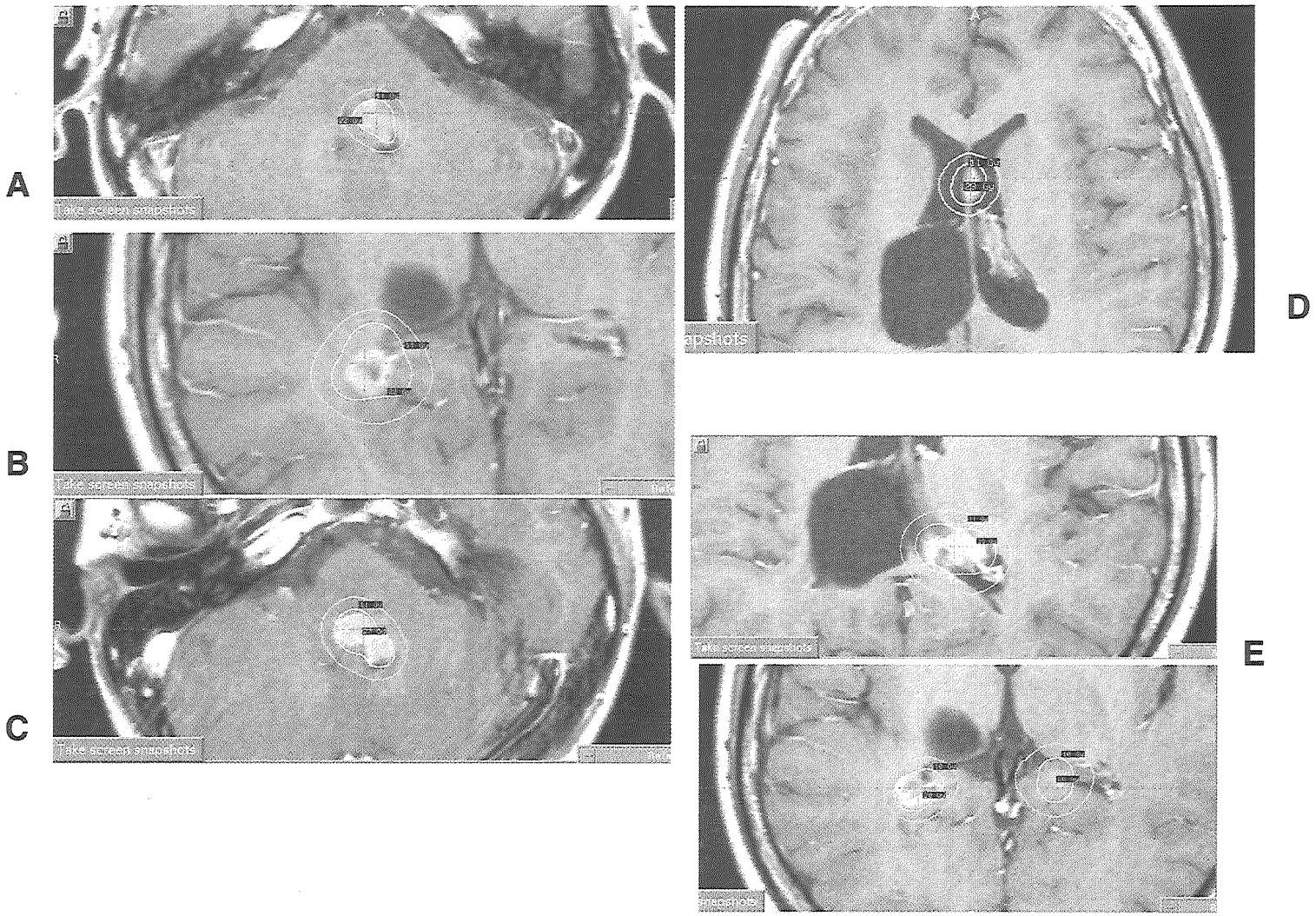

Fig. 2. Case 1: Axial T1-weighted MR images with gadolinium-diethylenetriaminepenta-acetic acid displayed on workstations running the Leksell GammaPlan program. (A) First Gamma knife (*GK*) radiosurgery for fourth ventricular dissemination. (B) First GK radiosurgery for dissemination in the right trigone. (C) Second GK radiosurgery for fourth ventricular dissemination. (D) GK radiosurgery for dissemination in the septum pellucidum. (E) Second GK radiosurgery for dissemination in the right trigone and GK radiosurgery for left lateral ventricular dissemination