発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Narita Y, Shibui S	Strategy of surgery and radiation therapy for brain metastases	Int J Clin Oncol	14	275-280	2009
Shibui S	Treatment of metastatic brain tumors	Int J Clin Oncol	14	273-274	2009
渋井壮一郎	がん薬物療法学, -基礎・臨床研究のアップデート-XI. 各臓器癌に対する薬物療法 脳腫瘍	日本臨牀	67	557-562	2009
渋井壮一郎	脳腫瘍の放射線治療・化学療法	がん看護	12	416-419	2007
渋井壮一郎	脳腫瘍に対する新しい治療	医学のあゆみ	222	953-954	2007
成田善孝, 渋井壮一郎	新しい神経疾患治療薬の動き　グリオーマ	脳神経外科ジャーナル	25	1242-1246	2007
櫻田香, 嘉山孝正	グリオーマ手術時の覚醒下手術	脳 21	12	86－90	2009
Shibahara I, Kumabe T	Imaging of hypoxic lesions in patients with gliomas by using positron emission tomography with 1-(2-[18F] fluoro-1-[hydroxymethyl]ethoxy)methyl-2-nitroimida	J Neurosurg		in press	2009
Sonoda Y, Kumabe T	Analysis of the *IDH1* codon 132 mutations in Japanese glioma patients	Cancer Sci	100	1996-1998	2009
Sonoda Y, Kumabe T	Long-term survivors of glioblastoma: Clinical features and molecular analysis	Acta Neurochir (Wien)	151	1349-1358	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ohta K, Kuratsu J	Decreased expression of germinal center-associated nuclear protein is involved in chromosomal instability in malignant gliomas	Cancer Sci	100	2069-2076	2009
Kudo M, Kuratsu J	Antitumor effect of humanized antiinterleukin - 6 receptor antibody (tocilizumab) on glioma cell proliferation. Laboratory investigation	J Neurosurg	111	219-225	2009
Hide T, Kuratsu J	Sox11 prevents tumorigenesis of glioma-initiating cells by inducing neuronal differentiation	Cancer Res	69	7953-7959	2009
Sawamura Y	Chemotherapy with cisplatin and vincristine for optic pathway/hypothalamic astrocytoma in young children	Jpn J Clin Oncol	39	277-283	2009
Sawamura Y	Role of surgery for optic pathway/hypothalamic astrocytomas in children	Neuro Oncol	10	725-733	2008
Kamada K, Sawamura Y	Expressive and receptive language areas determined by a non-invasive reliable method co-using functional magnetic resonance imaging and magnetoencephalography	Neurosurgery	60	296-306	2007
山崎文之, 杉山一彦	Single voxel proton MR spectroscopy による脳病変の診断	脳神経外科ジャーナル	18	603-608	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yamasaki F, Sugiyama K	Glioblastoma treated with postoperative radio-chemotherapy: Prognostic value of apparent diffusion coefficient at MR imaging	Eur J Radiol	Feb 26. [Epub ahead of print]		2009
Hidaka T, Sugiyama K	The combination of low cytoplasmic and high nuclear expression of p27 predicts a better prognosis in high-grade astrocytoma	Anticancer Research	29	597-603	2009
Aoki T, Takahashi JA	Phase II study of ifosfamide, carboplatin, and etoposide in patients with a first recurrence of glioblastoma multiforme	J Neurosurg	112	50-56	2010
Hirata E, Takahashi JA	Endogenous tenascinc enhances glioblastoma invasion with reactive change of surrounding brain tissue	Cancer Sci	100	1451-1459	2009
Shirahata M, Takahashi JA	Using gene expression profiling to identify a prognostic molecular spectrum in gliomas	Cancer Sci	100	165-172	2009
Suzuki Yu, Tanaka K	Pharmacokinetic investigation of increased efficacy against malignant gliomas of carboplatin combined with Hyperbaric Oxygenation	Neurol Med Chir	49	193-197	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Suzuki Y, Tanaka K	Increased distribution of carboplatin, an anti-cancer agent, to rat brains with the aid of hyperbaric oxygenation	Xenobiotica	38	1471-1475	2008
Yamazaki H, Tanaka K	Increased transendothelial permeability of anti-cancer agent carboplatin with aid of hyperbaric oxygenation	Xenobiotica	38	1298-1304	2008
Kamada K, Todo T	The motor-evoked potential threshold evaluated by tractography and electrical stimulation	J Neurosurg	111	785-795	2009
Ikushima H, Todo T	Autocrine TGF-β signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors	Cell Stem Cell	5	504-514	2009
Todo T	"Armed" oncolytic herpes simplex viruses for brain tumor therapy	Cell Adhesion & Migration	2	208-213	2008
Nagane M	Predominant antitumor effects by fully human anti-TRAIL-receptor2 (DR5) monoclonal antibodies in human glioma cells *in vitro* and *in vivo*	Neuro-Oncology	doi:10.1093/neuonc/nop069		2010

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nagane M	Prolonged and severe thrombocytopenia with pancytopenia induced by radiation-combined temozolomide therapy in a patient with newly-diagnosed glioblastoma-analysis of O^6-methylguanine-DNA methyltransferase status	J Neuro-Oncol	92	227-232	2009
Nagane M	Prognostic significance of O^6-methylguanine-DNA methyltransferase protein expression in patients with recurrent glioblastoma treated with temozolomide	Jpn J Clin Oncol	37	897-906	2007
西川亮	Low-grade glioma の治療における諸問題	脳神経外科ジャーナル	18	418-422	2009
Beppu T	Choice of posterior subtemporal transtentorial approach for tumor resection in deep anteromedical superior cerebellum- Case report	Neurologia Medico Chirugica	49	42-46	2009
Beppu T	Preoperative imaging of superficially located glioma resection using short inversion-time inversion recovery images in high-field magnetic resonance imaging	Clin Neurol Neurosurg	109	327-334	2007
Kashimura H, Beppu T	Three-dimensional anisotropy contrast imaging of pontine gliomas:2 case reports	Surg Neurol	67	156-159	2007

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Muragaki Y	Low-grade glioma on stereotactic biopsy: How often is the diagnosis accurate?	Minim Invasive Neurosurg	51	275-279	2008
Shinohara C, Muragaki Y	Long-term prognostic assessment of 185 newly diagnosed gliomas--gradeIII glioma showed prognosis comparable to that of grade II glioma	Jpn J Clin Oncol	38	730-733	2008
Kato T, Muragaki Y	Metabolic assessment of gliomas using 11C-methionine, [18F] fluorodeoxyglucose, and 11C-choline	Am J Neuroradiol	29	1176-1182	2008
若林俊彦	temozolomide (TMZ) 不応性腫瘍の治療	脳 21	50	50-54	2009
Ohno M, Wakabayashi T	The modulation of microRNAs by type I IFN through the activation of signal transducers and activators of transcription 3 in human glioma	Mol Cancer Res	7	2022-2030	2009
Ito S, Wakabayashi T	Human neural stem cells transduced with IFN-beta and cytosine deaminase genes intensify bystander effect in experimental glioma	Cancer Gene Ther	Nov. 9	[Epub ahead of print]	2009

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Wakabayashi T, Kayama T, Nishikawa R	A multicenter phase I trial of interferon-β and temozolomide combination therapy for high-grade gliomas (INTEGRA study)	Jpn J Clin Oncol	38	715-718	2008
Sanuki-Fujimoto N, Sumi M	Metabolic assessment of gliomas using 11C-methionine, [18F] fluorodeoxyglucose, and 11C-choline positron-emission tomography	Radiotherapy and Oncology	91	433-437	2009
Uno T, Sumi M	Changes in patterns of care forlimited-stage small-cell lung cancer: results of the 99-01 patterns of care study-a nationwide survey in Japan	Int J Radiat Oncol Biol Phys	71	414-419	2008

a median survival of 18.3 months (95% CI = 14.6–22.5 months). When the 60 patients who were 18–70 years old on this trial were compared with the EORTC (RT+TMZ) data, the median survival (20.3 vs. 14.6 months) and percent surviving at 24 months (41.7% vs. 26.5%, $p = 0.02$) appeared superior. Data on MGMT methylation and postprogression treatment with VEGF-targeted therapies for this population will be available for presentation. *Conclusion*: Talampanel was well tolerated and did not appear to increase the known hematologic or nonhematologic toxicities of TMZ. Talampanel can be added to RT+TMZ without significant added toxicity. These encouraging survival results in this study suggest that blocking AMPA receptors may be a useful strategy in glioblastoma.

O66. ONGOING CLINICAL TRIALS AND THE FUTURE DIRECTION OF GLIOMA TREATMENT

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Because of the proposed sensitivity to chemotherapy of oligodendroglial tumors both the RTOG and EORTC had investigated if these tumors benefit from adjuvant PCV chemotherapy. These studies, EORTC study 26951 and RTOG 9402, both showed that the addition of PCV chemotherapy (consisting of procarbazine, CCNU, and vincristine) to 59.4 Gy radiotherapy does increase progression-free survival without improving overall survival in anaplastic oligodendroglioma and anaplastic oligoastrocytoma. A major finding of both studies is the large difference in prognosis of patients with and without combined 1p/19q loss. Based on these differences in survival and the clear different outcome in anaplastic oligodendroglioma with 1p/19q loss, EORTC and the collaborative groups felt that it was no longer rational to treat these patients according to histology without taking the genotype of these tumors into account. For studies in anaplastic gliomas it was therefore proposed to classify into anaplastic glioma without 1p/19q loss and anaplastic oligodendroglial tumors with 1p and 19q codeletion. Another challenge is the definition of a proper end point for these trials. Overall survival seems to be the most relevant outcome parameter, even at progression. The outline and initiatives in grade III gliomas (EORTC 26053/22054, CATNON plus the codeleted trial) are presented. Standard therapy for glioblastoma is surgical resection aimed to be as complete as possible, respecting neurological function followed by chemoirradiation with temozolomide. TMZ given as concomitant and adjuvant therapy to RT has shown to increase progression-free survival (PFS) (rate at 6 months, 53.9% vs. 36.4%) and median survival (14.6 vs. 12.1 months) compared to adjuvant treatment with RT therapy only (EORTC 26981/22981 NCIC CE.3 trial). Still, many patients do not respond to therapy. The resistance of cells against DNA damage caused by nitrosoureas and temozolomide is at least in part mediated by the DNA-repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT). Epigenetic silencing of the MGMT gene by promoter methylation compromises this DNA repair and has been associated with longer survival in (glioblastoma) patients who are treated with alkylating or methylating agents. An analysis of the EORTC 26981/22981 NCIC CE.3 trial showed, that indeed patients with glioblastoma containing a hypermethylated MGMT promoter benefited from TMZ (overall survival [OS] rate at 24 months, 46% vs. 23%), whereas those who did not have a methylated MGMT promoter did have a significantly worse survival rate and less benefit from the addition of temozolomide to RT (OS rate at 24 months, 14% vs.<2%). This raises the question if the small benefit from chemoirradiation observed in this group outweighs the toxicity and costs of the temozolomide treatment, and calls for the development of more effective drug regimens for this specific group of patients. Although there may be small numbers of patients with an unmethylated MGMT promoter that do benefit from combined chemoirradiation, for the entire subgroup of these molecularly defined GBM patients the overall benefit is questionable. Most interestingly, the phase II trial with the integrin inhibitor cilengitide also demonstrated a marked benefit mainly in the patients with glioblastoma containing a methylated MGMT promoter. Consequently, the current Merck/EORTC phase III trial is designated to delineate the role for cilengitide in glioblastoma with methylated MGMT. Even earlier, Eli Lilly took the approach to examine the protein kinase C-β inhibitor, enzastaurin, together with radiotherapy but without TMZ in patients with glioblastoma containing an unmethylated MGMT promoter. This raises the general question whether treatment in glioblastoma trials should not only be stratified according to MGMT but entry into those trials limited by MGMT status. This would call for different approaches of GBM patients, depending on the MGMT promoter gene status. The primary question to address in GBM with unmethylated MGMT promoter gene is the identification of drugs that provide more survival benefit compared to TMZ. The current EORTC trial initiatives are presented.

O67. THE RESULT OF A CLINICAL TRIAL FOR MALIGNANT GLIOMAS BY JCOG BRAIN TUMOR STUDY GROUP (JCOG 0305)

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Purpose: Japan Clinical Oncology Group (JCOG) Brain Tumor Study Group conducted a multiinstitutional randomized controlled trial on malignant gliomas entitled, a randomized controlled phase II study of chemoradiotherapy using ACNU versus procarbazine and ACNU for astrocytoma grade 3 and 4, with the support of the Health and Labour Sciences Research Grants of the Ministry of Health, Labour, and Welfare in order to establish a standard therapy for malignant gliomas in Japan. *Method*: The patients with newly diagnosed supratentorial astrocytoma grade 3 or 4 were enrolled and randomized into two groups. The patients in group A were treated with ACNU (80 mg/m^2 iv) during the postoperative radiotherapy (RT, 60 Gy local), while those in group B received procarbazine (80 mg/m^2 for 10 days per os) preceding administration of ACNU. Each regimen was continued every 8 weeks for 2 years if it was tolerable for the patients and their disease did not progress. The primary end point was the overall survival rate and the secondary end points were the response rate on the MRI and the frequency of the adverse events. Procarbazine is expected to reduce O^6-methylguanine-DNA methyltransferase (MGMT) and enhance the anticancer activity of nitrosoureas. The protocol was activated in April 2004 and 111 patients were registered by the end of August 2006 from 19 collaborating neurosurgical institutes of JCOG-BTSG. *Results*: The overall survival of the patients treated with ACNU+RT was 16.2 months and that of procarbazine+ACNU+RT was 18.7 months, while PFS of both groups were 6 months. CTCAE grade 3/4 was observed in 40–60% of the patients. *Conclusion*: ACNU-based chemoradiotherapy was an effective but toxic treatment.

O68. CURRENT CLINICAL TRIALS OF GLIOMA THERAPY AND SITUATIONS OF NEURO-ONCOLOGY PRACTICE IN KOREA

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There has been no qualified sponsor-investigator clinical trial program, and the standard therapies have been all we could do for the treatment of malignant glioma patients in the Korean Brain Tumor Society. We have just started to join two international clinical trials since 2008. In this article the past and current status of the neuro-oncology field in Korea as well as eastern and northern Asian countries will be introduced, and clinical outcomes of concurrent radiotherapy and temozolomide chemotherapy for 100 patients of four university hospitals of Korea (Advisory Board of S-P Korea) will be presented.

O69. HISTOGRAM ANALYSIS OF PERFUSION MRI DATA FOR THE ASSESSMENT OF TUMOR RESPONSE DURING GLIOMA THERAPY

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Purpose: A recently developed histogram analysis of relative cerebral blood volume (rCBV) from the entire tumor has been reported to offer excellent interobserver agreement for quantitative analysis and demonstrate the heterogeneous morphologic features of glioma vascularity. We aimed to determine whether histogram analysis can be adopted in the assessment of tumor response during glioma therapy. *Methods*: We retrospectively studied 51 dynamic susceptibility contrast 3-T MR imaging data of 29 patients (mean age 50.5 years, range, 18–76) with histologically confirmed gliomas (9 low grade, 20 high grade). rCBV maps were created and normalized to unaffected white matter. Histogram width (HW), peak height position (PHP), and maximum value (MV) of the entire tumor were measured from normalized histogram distribution. *Results*: The values (mean ± SD) of HW, PHP, and MV were 4.64 ± 2.03, 4.58 ± 2.63, and 6.29 ± 2.79 for the preoperative imaging of high-grade gliomas ($n = 8$), and 3.83 ± 1.96, 2.66 ± 1.66, and 4.73 ± 1.96 for the final imaging, which showed definite radiological tumor progression or confirmed tumor recurrence by biopsy ($n = 8$). Thirty-two imaging data obtained during the median imaging follow-up of 3.7 months were divided into two groups (progression vs. stable/radiation necrosis) according to the follow-up result, and three parameters were compared. All three parameters were positively correlated with tumor progression (HW, 3.05 ± 2.18 vs. 1.02 ± 0.50; PHP, 2.39 ± 1.71 vs. 0.94 ± 0.28; MV, 4.13 ± 2.83 vs. 1.56 ± 0.52) and MV was the most predictive with multivariate analysis. *Conclusion*: Our results suggest that histogram analysis of rCBV can be a more objective and useful diagnostic

脳 腫 瘍

Chemotherapy for malignant brain tumors

渋井壮一郎

Key words：glioblastoma, astrocytoma, oligodendroglioma, ACNU, temozolomide

はじめに

従来，悪性神経膠腫(グリオーマ)に代表される悪性脳腫瘍は化学療法の効果が期待できない疾患とされてきた．1970年代から行われている欧米での臨床試験では，BCNU，CCNUなどのnitrosourea系抗癌剤の有用性が指摘され，それ以来20年以上にわたって，これらの薬剤を中心に治療が行われてきた．2005年にEuropean Organisation for Research and Treatment of Cancer(EORTC)から発表されたtemozolomideによる臨床試験結果は，その点，画期的なものであり，初めて薬剤の有効性が統計的有意差をもって証明されたもので，これにより悪性グリオーマ治療の新しいスタンダードが作られたといっても過言ではない．当面，この薬剤を中心に悪性グリオーマの治療開発が行われていくものと考えられる．

1. 悪性星細胞腫

a. 退形成性星細胞腫および膠芽腫の治療の歴史

悪性星細胞腫は，グリオーマの半数以上を占め，通常，WHO分類の退形成性星細胞腫(anaplastic astrocytoma)および膠芽腫(glioblastoma)のことを示す．いずれも浸潤性発育をするため，MRIにより造影病変として描出される部分より数cm先まで腫瘍細胞があるとされている(図1)．そのため，手術による治癒切除(全摘)は不可能であり，後治療が必須であるが，放射線治療のみでは効果が不十分であり，化学療法剤を併用することで，その効果を高める工夫がされてきた．米国を中心に1970年代後半より，幾つかの大規模臨床試験が行われ，第III相試験の結果が報告されている[1-4]．それらの報告によれば，照射量については，線量を上げることで生存期間の延長を得られたが，過度の照射は放射線壊死の発生を高め，必ずしも生存期間を延長させないため，60 Gyというのが，ほぼ一致した見解になっている．多分割照射や定位放射線治療の併用で腫瘍線量を上げる試みもなされているが，現在のところ腫瘍本体に2 cm程度のマージンをつけた領域を計画標的体積(planning target volume)として60 Gyの照射計画を立てる施設が多い．更に60 Gyの照射にBCNUを併用することで，放射線単独に比べ，若干の生存期間の延長をもたらし，それが標準治療として扱われてきた．一方，国内においては，欧米のBCNUの有効性を参考にしてACNUを併用した化学放射線治療が広く行われてきたが，ACNUを併用した群と併用しない群との間で行われた第III相試験では，症例数の蓄積が不十分だったことも相俟って，奏効率で差は出たものの，生存率では有意差が出るに至らなか

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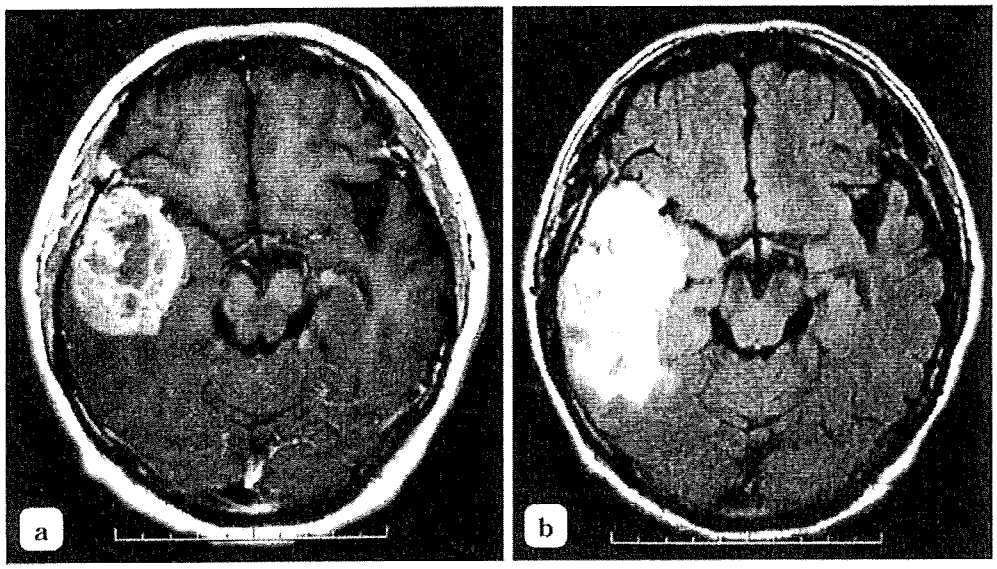

図1 膠芽腫(glioblastoma)のMRI画像
a: gadolinium-DTPA造影T1強調画像. b: FLAIR法. FLAIR法にて高輝度領域には腫瘍の浸潤があるとされている.

った[5]. しかしながら, 欧米でのBCNUによる治療成績および国内でのACNUを用いた臨床試験結果から, 手術による可及的摘出後, nitrosourea系抗癌剤を併用した放射線治療が悪性グリオーマに対する標準治療とされてきた. これを裏づける形で, high grade glioma(退形成性星細胞腫および膠芽腫)に対し, nitrosourea系抗癌剤を併用した放射線治療を行った群と併用しなかった群との12のランダム化臨床試験結果のメタアナリシスでは, 1年生存率でわずか6%ではあるが, 有意に併用群の治療成績が優っていたという結果であった[6]. ただし, 薬剤の使用量, 使用期間などについては, 一定のものがなく, 維持療法の効果についてもその効果は証明されていない.

b. Temozolomide併用放射線治療

temozolomide(TMZ)はStuppら[7]によるEORTC臨床試験結果の発表以来, 悪性グリオーマに対する治療薬として, 最も注目されている薬剤の一つである. 経口的に服用するアルキル化剤で, 従来のnitrosourea系抗癌剤と同様あるいはそれ以上の効果をもちながら, 悪心・嘔吐や骨髄抑制などの副作用が軽微で, 外来での治療が可能なことから, 利便性という点でも優れている. Stuppらの臨床試験は, 膠芽腫を対象とした第III相試験であり, 組織診断確定後6週間以内に, 無作為に放射線単独治療(1回2Gyで週5日, 総線量60Gyの分割照射)または放射線+TMZ併用療法(1日75mg/m^{2}を放射線治療期間中最長49日服用し, その後更に6コースの化学療法(28日ごとに150-200mg/m^{2}を5日間))に割り付け, 1週以内に治療を開始するというものである(**図2**). 15カ国85施設から573例(放射線単独群286例, 放射線+TMZ併用群287例)が登録され, その結果, 28カ月の平均観察期間で, 生存期間中央値は併用群14.6カ月(95%信頼区間13.2-16.8), 放射線単独群12.1カ月(95%信頼区間11.2-13.0), 2年生存率は併用群26.5%(95%信頼区間21.2-31.7%), 単独群10.4%(95%信頼区間6.8-14.1%)であり, 無増悪生存期間中央値は併用群6.9カ月, 放射線単独群5.0カ月であった. 併用群において死亡についてのハザード比0.63(95%信頼区間0.52-0.75, log-rank test $p<0.001$)であった(**図3**). 国内でも退形成性星細胞腫再発例に対し, 28日ごとに5日間のTMZ治療の第II相試験[8]が行われ, 2006年9月より使用可能となったことから広く用いられるようになり, 世界標準としての地位を築いたといって過言ではない.

TMZはかなり有望な薬剤ではあるが, まだ

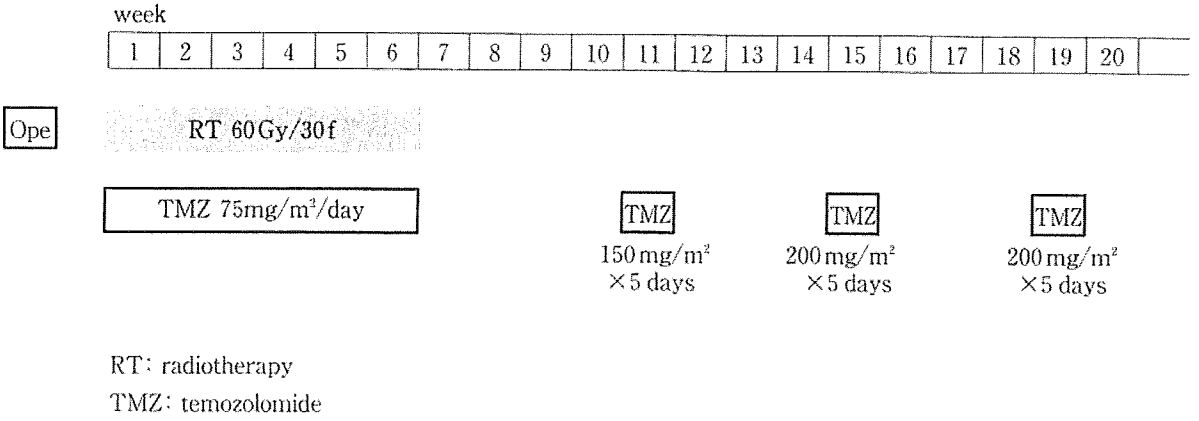

図2　悪性神経膠腫に対するtemozolomide併用放射線治療スケジュール(Stupp ら[7]より引用)

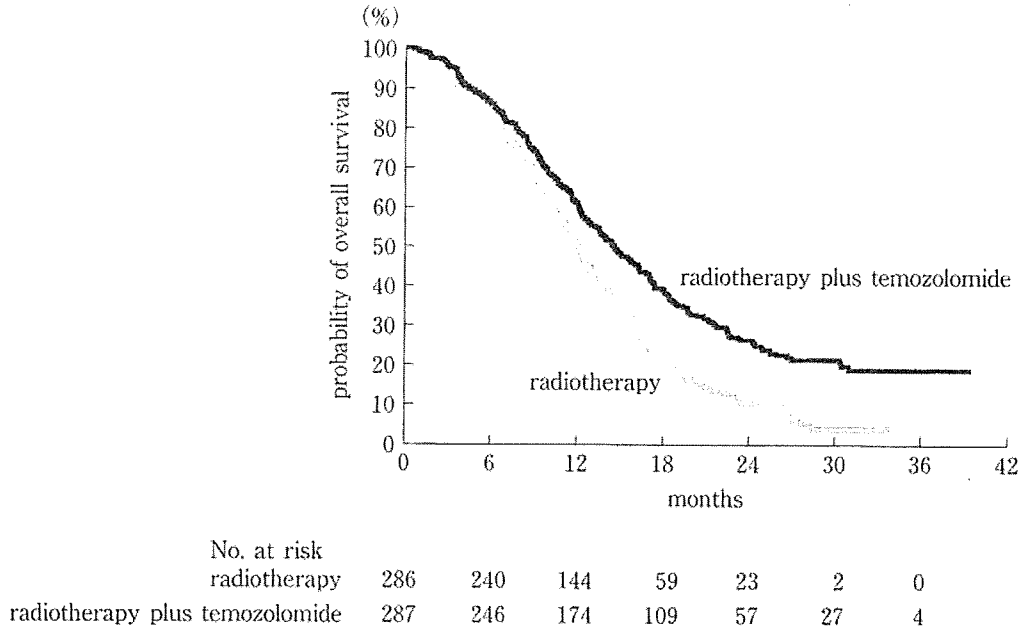

図3　膠芽腫に対する放射線単独治療とtemozolomide併用放射線治療との比較試験結果(Stupp ら[7]より引用)

まだ治療効果としては不十分である．特にアルキル化剤に対する薬剤耐性機構と考えられているO^6-methylguanine-DNA methyltransferase (MGMT)の影響を受けることが知られている．Hegi ら[9]によれば，TMZ併用の放射線治療を行った膠芽腫患者で，MGMT promoterがメチル化されていない(MGMTが発現している)症例の生存期間中央値は12.7カ月であったのに対し，メチル化されている(MGMTが発現していない)症例は21.7カ月と有意に延長しており，無増悪生存期間もそれぞれ5.3カ月，10.3カ月であり，MGMTがTMZ治療に及ぼす影響を指摘している(**図4**)．MGMTによる耐性を克服する意味でも，現在，多くの化学療法剤との組み合わせによる臨床試験が実施されているが，TMZを7日間投与/7日休薬を繰り返す方法や21日間投与/7日間休薬を繰り返す方法など，TMZの投与量を増加する治療も試みられており，今後しばらくは，TMZを主体とする治療法の開発が続けられるものと考えられる．

一方，TMZは血液毒性を含む有害事象が少ないことも特徴の一つであるが，ニューモシスチス肺炎の合併が国内でも数件報告されている．特に，TMZに特有な有害事象の一つであるリ

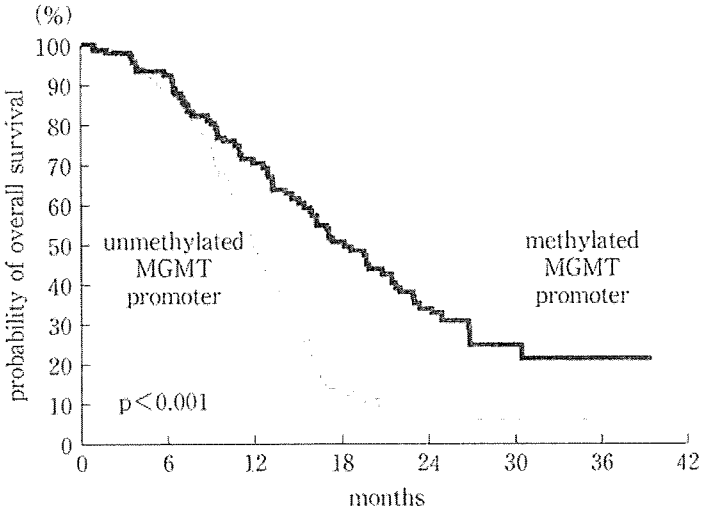

図4 MGMT promoterのメチル化の有無による膠芽腫生存率
(Hegiら[9]より引用)

ンパ球減少時，ステロイド併用時，放射線治療期間中などに発生する可能性があるため，血液状態に十分注意を払いながらST(sulfamethoxazole/trimethoprim)合剤(バクタ®，バクトラミン®1日1錠)の併用が推奨されている．

c. JCOG脳腫瘍グループによる臨床試験

国内においても質の高い臨床試験を実施するためには，信頼できるデータ管理機構が必要であるが，脳腫瘍の分野においてはそのような組織が構築されてこなかった．2002年に厚生労働科学研究費'効果的医療技術の確立推進臨床事業(現がん臨床研究事業)'の一つとして，'悪性脳腫瘍の標準的治療法の確立に関する研究(主任研究者渋井壮一郎)'班が組織され，日本臨床腫瘍研究グループ(Japan Clinical Oncology Group: JCOG)の13番目の臓器グループとして認可された．2003年には同研究事業'転移性脳腫瘍の標準的治療法の確立に関する研究(主任研究者嘉山孝正)'班も加わり，国立がんセンター中央病院に事務局を置き，全国の大学病院脳神経外科を中心とした31施設によってJCOG脳腫瘍グループを構築している．

グループとして最初の臨床試験は，2004年に開始した'星細胞腫grade 3・4に対する放射線化学治療としてのACNU単独療法とprocarbazine+ACNU併用療法とのランダム化第II/III相試験(JCOG 0305)である[10]'．脳腫瘍全国統計[11]によれば，膠芽腫および退形成性星細胞腫の5年生存率は，それぞれ7%，23%にすぎず，その原因の一つが有効な化学療法が存在しないことにあると考えられている．従来，nitrosourea系抗癌剤が悪性神経膠腫に対する標準治療薬として用いられてきたが，これらの薬剤に対する耐性機構も前述のMGMTである．一方，procarbazineは，腫瘍内のMGMTを消費し低下させる作用をもっていることが知られており，これをnitrosourea系抗癌剤に先行して投与することにより，MGMTによる耐性を克服し，抗腫瘍効果を高める可能性がある．臨床的には，Brandesら[12]がBCNUに先行してprocarbazineを投与する方法での治療を行っており，58例の再発膠芽腫においてcomplete response 10%，partial response 19%，奏効率29%という優れた成績を発表している．

JCOG 0305臨床試験の対象は，20-69歳までの初発星細胞腫grade 3(退形成性星細胞腫)およびgrade 4(膠芽腫)で，手術による組織診断後2週間以内にランダム化し，3週以内に治療を開始する．A群としては，放射線治療第1日目および第36日目にACNU 80 mg/m²を静脈内投与し，60 Gyの局所照射を行う．それ以降は56日ごとに同様にACNUの静脈内投与を12コース行う(**図5**)．B群では放射線治療第1日目より10日間procarbazine 80 mg/m²を経口投

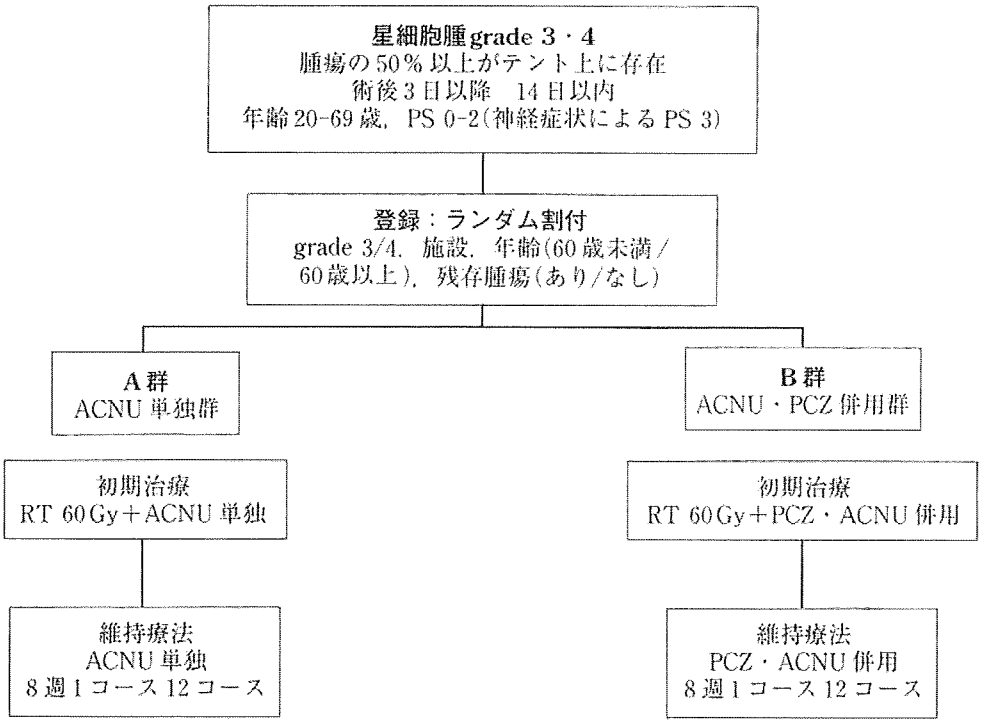

図5　JCOG 0305星細胞腫grade 3および4に対するランダム化比較試験シェーマ

与し，第8日目にACNU 80 mg/m^{2}を静脈内投与する．第36日目から再びprocarbazineを10日間経口投与し，第42日目ACNUを静脈内投与する．この治療も56日ごとに12コース繰り返す．本臨床試験は，B群の安全性有効性が確立していないため，第II/III相試験として計画され，登録を開始したが，2005年にStuppらによりEORTCのTMZを用いた臨床試験結果の発表があり，2006年9月には，国内でのTMZ使用も認可されたため，第II相終了条件であるB群56例が登録された2006年9月に登録を中止し，現在，経過観察を行っている．

2. 退形成性乏突起膠腫

退形成性乏突起膠腫(anaplastic oligodendroglioma)は，星細胞腫に比べ化学療法に反応する脳腫瘍とされていたが，分子生物学的知見と治療効果の関連性を示した最初の悪性神経膠腫ということで注目されている．特にprocarbazine，CCNU，vincristineによる化学療法(PCV療法)が有効とされ，次のような投与方法が用いられている．day 1にCCNU 110 mg/m^{2}経口投与，day 8–21にprocarbazine 60 mg/m^2/day 14日間経口投与，day 8および28にvincristine 1.4 mg/m^{2}静注が欧米での治療法であるが，国内では，CCNUが使用できないため，ACNU静注をこれに代用する治療法(PAV療法)が行われている．その後，退形成性乏突起膠腫の中でも染色体1番の短腕(1p)および19番長腕(19q)の欠失を認める症例において，この治療法が有効であることが指摘され，手術によって採取された腫瘍での1p，19qの欠失を調べ，それが認められる症例においては積極的にPCV療法を行っていくテーラーメイド治療ともいえる治療が可能となった[13]．しかしながら，2006年にEORTC[14]およびRTOG[15]の2つのグループから，PCV療法は無増悪生存期間を延長するものの，全生存期間を改善しなかったという趣旨の発表があり，染色体1p，19q欠失を示す腫瘍はPCV療法が有効であるだけなく，放射線治療にも同様に反応するいわゆる予後良好群の形質であることが示された．PCV療法に特異的に反応を示す形質ではないにせよ，分子生物学的知見が治療との関連性を示すという意味では，TMZ治療におけるMGMT promoterのメチル化同様に極めて有用な情報といえる．

おわりに

TMZの出現により，30年来変わらなかった悪性グリオーマの治療が一歩前進したといえる．5年生存率が10％以下というあらゆる臓器の悪性腫瘍の中でも最も予後の悪い膠芽腫の一部に，長期間再発のみられない症例も時に経験されるようになったのは事実である．しかしながら，TMZについてはまだまだ使用経験も浅く，長期使用時の血液毒性，ニューモシスチス肺炎の合併，二次がん発生の危険性，更には高額な医療費など，今後解決すべき問題も少なくない．これらを一つずつ解決するとともに，国内においてもエビデンスレベルの高い臨床試験を実施し，更に有効な治療法が開発されていくことが望まれる．

文献

1) Walker MD, et al: Randomized comparisons of radiotherapy and nitrosoureas for the treatment of malignant glioma after surgery. N Engl J Med 303: 1323-1329, 1980.
2) Chang CH, et al: Comparison of postoperative radiotherapy and combined postoperative radiotherapy and chemotherapy in the multidisciplinary management of malignant gliomas. Cancer 52: 997-1007, 1983.
3) Green SB, et al: Comparisons of carmustine, procarbazine, and high-dose methylprednisolone as additions to surgery and radiotherapy for the treatment of malignant glioma. Cancer Treat Rep 67: 121-132, 1983.
4) Walker MD, et al: An analysis of dose-effect relationship in the radiotherapy of malignant gliomas. Int J Radiat Oncol Biol Phys 5: 1725-1731, 1979.
5) Takakura K, et al: Effects of ACNU and radiotherapy on malignant glioma. J Neurosurg 64: 53-57, 1986.
6) Stewart LA, Meta-analysis Group: Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet 359: 1011-1018, 2002.
7) Stupp R, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
8) 西川 亮ほか：初回再発の退形成性星細胞腫患者に対するtemozolomide単剤投与の有効性および安全性の検討 多施設共同第II相試験．癌と化学療法 33: 1279-1285, 2006.
9) Hegi M, et al: MGMT gene silencing and benefit from temozolomide in glioblastoma. N Engl J Med 352: 997-1003, 2005.
10) Shibui S: Randomized controlled trial on malignant brain tumors—activities of the Japan Clinical Oncology Group (JCOG)-Brain Tumor Study Group (BTSG). Neurol Med Chir (Tokyo) 44: 220-221, 2004.
11) The Committee of Brain Tumor Registry of Japan: Report of brain tumor registry of Japan (1969-1996). Neurol Med Chir (Tokyo) 43 (Suppl): 1-111, 2003.
12) Brandes AA, et al: A multidrug combination designed for reversing resistance to BCNU in glioblastoma multiforme. Neurology 58: 1759-1764, 2002.
13) Ino Y, et al: Molecular subtypes of anaplastic oligodendroglioma: Implications for patient managament at diagnosis. Clin Cancer Res 7: 839-845, 2001.
14) van den Bent MJ, et al: Adjuvant procarbazine, lomustine, and vincristine improves progression-free survival but not overall survival in newly diagnosed anaplastic oligodendrogliomas and oligoastrocytomas: a randomized European Organisation for Research and Treatment of Cancer phase III trial. J Clin Oncol 24: 2715-2722, 2006.
15) Cairncross G, et al: Phase III trial of chemotherapy plus radiotherapy compared with radiotherapy alone for pure and mixed anaplastic oligodendroglioma: Intergroup Radiation Therapy Oncology Group Trial 9402. J Clin Oncol 24: 2707-2714, 2006.

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The Modulation of MicroRNAs by Type I IFN through the Activation of Signal Transducers and Activators of Transcription 3 in Human Glioma

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Abstract

Type I IFNs are involved in double-stranded RNA responses. Here, we investigated the possibility that IFN-β may induce or downregulate cellular microRNAs (miRNA) in human neoplasms and thereby use the RNA interference system to show antitumor effects. Because of its known connection to glioma biology, we focused on miR-21 among seven miRNAs influenced by IFN-β. We analyzed the effect of IFN-β treatment on miR-21 expression in glioma cells and intracranial glioma xenografts. IFN-β treatment reduced miR-21 expression in glioma cells markedly, and IFN-β administration suppressed the growth of glioma-initiating cell–derived intracranial tumors. The levels of primary *miR-21* gene transcripts, precursor miR-21, and mature miR-21 decreased 6 hours after the addition of IFN-β, indicating that the reduction in miR-21 levels was due to transcriptional suppression. We did reporter assays to elucidate the IFN-β–mediated suppression of miR-21; the addition of signal transducers and activators of transcription 3 (STAT3)–expressing vectors induced the IFN-β–mediated suppression of miR-21, whereas STAT3-inhibiting agents inhibited the miR-21 suppression. Thus, the results of our study show that the downregulation of miR-21 contributes to the antitumor effects of IFN-β and that miR-21 expression is negatively regulated by STAT3 activation. These results highlight the importance of understanding the transcriptional regulation of the miRNAs involved in oncogenesis. (Mol Cancer Res 2009;7(12):OF1–9)

Introduction

MicroRNAs (miRNA) are small noncoding RNAs consisting of 20 to 22 nucleotides that participate in the posttranslational regulation of gene expression by means of RNA interference (RNAi). The miRNA genes are transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. These pri-miRNA transcripts are processed by Drosha to release the pre-miRNA precursor product, which is less than 70 nucleotides in length. After the pre-miRNA is transported into the cytoplasm, Dicer processes the intermediate to generate a mature 22-nucleotide miRNA. This mature miRNA is integrated into the RNA-induced silencing complex (1) and forms double-stranded RNA with complementary mRNAs (mRNA). Depending on the degree of homology between the miRNA and the mRNA, the RNA-induced silencing complex could inhibit mRNA function by either promoting its cleavage or by inhibiting its translation (2, 3). Emerging evidence suggests that miRNAs are involved in crucial biological processes, including the development, differentiation, apoptosis, and proliferation of mammalian cells (4). In humans, miRNAs have been proposed to contribute to oncogenesis because they possess multifaceted functions either as tumor suppressors or as oncogenes (5).

RNA interference induces a multitude of responses in addition to the knockdown of a gene. This is best understood in the context of an antiviral immune response. In particular, double-stranded RNA, a nucleic acid associated with viral replication, is involved in numerous interactions contributing to the induction, activation, and regulation of antiviral mechanisms. It is especially responsible for stimulating important protective responses such as the activation of dicer-related antiviral pathways, induction of type I IFN (IFN-α/β), and stimulation of double-stranded RNA–activated protein kinase and oligoadenylate synthase (6). IFN-α/β regulates the levels of crucial mediators of the antiviral response, such as protein kinase R, the 2′-5′ oligoadenylate synthase/RNase L system, the adenosine deaminase ADR1, or the Mx GTPase (7, 8). Thus, RNA interference might be involved in the IFN-mediated antiviral response. It was recently reported that the levels of liver-specific miRNA, i.e., miR-122, and several other miRNAs are regulated by IFN-β in human hepatoma cells, and that IFN-β rapidly modulates the expression of miRNAs, which target the hepatitis C virus genomic RNA, and thus, inhibits viral replication (9, 10).

In addition to its ability to interfere with viral replication, IFN-β is also known for its antiproliferative effects in a variety of neoplasms such as hepatocellular carcinoma, chronic myeloblastic leukemia, melanoma, renal cancer, and glioma (11, 12). However, the possibility that IFN-β might induce or downregulate cellular miRNAs in human neoplasms and thereby use the RNA interference system in its action against tumor progression has been left unexplored.

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Malignant glioma represents ~20% of all intracranial tumors. Despite the advances in radiation therapy and chemotherapy administered after the surgical resection of the tumor, the prognosis of malignant glioma remains poor, with a median survival time of <10 months (13). In the treatment of malignant gliomas, IFN-β exhibits pleiotropic biological effects such as antiproliferation, immunomodulation, induction of differentiation from glioma-initiating cells (GIC), and drug sensitization by classically activating Janus kinase/signal transducers and activators of transcription (STAT) pathways (14, 15). However, there is no report on IFN-mediated modulation of cellular miRNAs as an antitumor mechanism.

In the present study, we test whether IFN-β can alter the expression of cellular miRNAs in human glioma cells by using the data obtained from genomewide microarray technology. On the basis of the initial screening efforts identifying several increased or attenuated miRNAs, we show that in cultured glioma cells and orthotopic glioma xenograft, IFN-β treatment leads to STAT3-mediated reduction in the expression of miR-21, an antiapoptotic miRNA that has been shown to be overexpressed in gliomas (16).

Results

Differential miRNA Expression in Human Glioma Cells Treated with IFN-β

To investigate which miRNAs are induced or downregulated by IFN-β, we used a microarray containing 662 mammalian miRNAs. We identified a total of two overexpressing and five underexpressing human miRNAs in the IFN-β–treated glioma cells. The expression of miRNAs, including miR-187 and miR-194, was increased >2-fold, whereas that of miR-100, let-7a, let-7b, let-7c, and miR-21 was decreased <0.5-fold in the T98 cell line treated with IFN-β as compared with the expression levels in cells without any treatment (Fig. 1A; Supplementary Fig. S1 and Table S1). To confirm the accuracy of microarray data, we examined the changes in the expression of these miRNAs following IFN-β treatment by performing quantitative reverse transcription-PCR (qRT-PCR). The findings were similar to the pattern of expression observed in the miRNA microarray analysis (Table 1). Among these miRNAs influenced by IFN-β treatment, miR-21 was pursued because of its known connection to glioma biology. Indeed, IFN-β treatment of T98 glioma cells recovered the expression of programmed cell death 4 (PDCD4), a well-known target of miR-21 (refs. 17-19; Fig. 1B).

miR-21 Overexpression in Glioma Cells, Particularly in the GICs

Previously, miR-21 was suggested to be aberrantly expressed and to be one of the major antiapoptotic factors in malignant gliomas (16, 18, 20-22). To our knowledge, we have shown, for the first time, the overexpression of miR-21 in a surgical specimen of glioblastoma by performing *in situ* hybridization (Fig. 2A-F). The *in situ* hybridization was optimized to distinguish between the areas of high (*blue*) and low expression of miR-21. The locked nucleic acid–enhanced miR-21–specific probe clearly stained the glioblastoma tissue but did not stain the normal cortex tissue. Tumor cells expressed significant amounts of miR-21, as seen at high magnification, whereas nontumoral tissue showed no expression of miR-21 (Fig. 2C and E). In contrast, neither tumor nor nontumoral tissues in the section adjacent to that hybridized with the miR-21 probe showed positive staining with the scramble probe (Fig. 2D and F). Next, we compared the miR-21 expression levels in glioma cell lines, GICs, and the normal brain tissue. The miR-21 was overexpressed in glioma cells compared with the normal brain. Notably, the amount of miR-21 was greater in GICs than in the glioma cell lines (Fig. 2G). This finding may indicate that miR-21 plays a crucial role in the initiation and progression of glioma.

IFN-β Downregulates miR-21 Transcription

Previous evidence reported by our group and others has shown that IFN-β induced growth inhibition or apoptosis in a variety of neoplasms (12, 23-25). Recently, we reported that IFN-β induced the differentiation of GICs to cells with an oligodendrocyte-like phenotype (26). In this study, we investigated the possibility that IFN-β might downregulate oncomir miR-21 in human gliomas. We compared the expression of miR-21 in IFN-β–treated and untreated glioma cells by using qRT-PCR. IFN-β treatment reduced the expression of miR-21 by ~40% to 60% in all the glioma cells tested, including the SKMG1, T98, U87, U251 glioma lines, and 0222-GIC (Fig. 3A). On the other hand, miR-21 was not affected by temozolomide, an alkylating agent commonly used in the treatment of glioma (Supplementary Fig. S2). At 4 weeks after the intracerebral inoculation of GICs, the mice received either PBS or IFN-β i.p. We previously showed that IFN-β suppressed the growth of GIC-derived intracranial tumor (26). In this study, the systemic delivery of IFN-β reduced the level of miR-21 in the tumor (Fig. 3B). The regulation of miRNA expression has been documented at the transcriptional level and RNase-mediated posttranscriptional processing (19). Therefore, we next analyzed the time course of reduction of primary *miR-21* gene transcripts (pri-miR-21), precursor miR-21 (pre-miR-21), and mature miR-21 in response to IFN-β treatment (Fig. 3C). The pri-miR-21 transcript levels decreased 6 hours after the addition of IFN-β, and began to recover at ~48 hours. Similar to these findings, the reduction of pre-miR-21 and mature miR-21 occurred as early as 6 hours. However, the recovery of pre-miR-21 began later than that of pri-miR-21. These results indicate that the decrease in the levels of miR-21 is the result of transcriptional suppression.

STAT3 Negatively Regulates miR-21 Transcription

To examine the molecular mechanisms involved in miR-21 expression, we analyzed the structure of the *miR-21* gene by studying its promoter and primary transcripts. As previously reported (27), several conserved enhancer elements were found in the consensus sequence upstream of the transcription start site of the *pri-miR-21* on the basis of TRANSFAC matrices, including Ets/PU.1, activator protein-1, serum response factor, CAAT/enhancer-binding protein-α, p53, and STAT3.[4] This suggests that highly conserved transcriptional regulatory mechanisms may operate on the *pri-miR-21* promoter (Fig. 4A). Of all these transcription factors, we focused on STAT3 in this study because IFN-β phosphorylates the tyrosine and, in part,

[4] http://www.gene-regulation.com/pub/databases.html

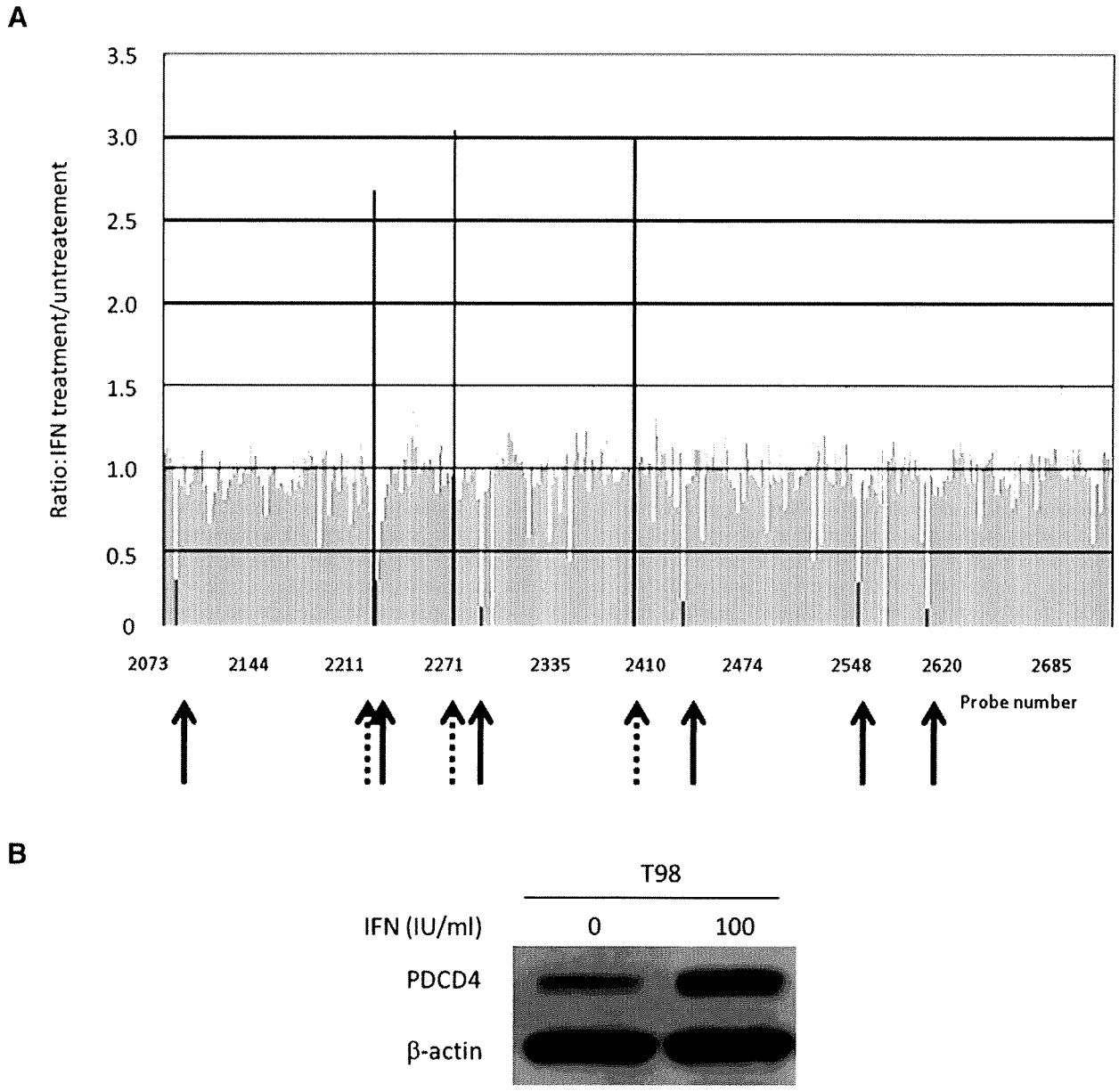

FIGURE 1. Differential miRNA expression in human glioma cells treated with IFN-β. **A.** Of 662 mammalian miRNAs spotted onto the microarray, a total of 3 overexpressing (*broken arrows*) and 6 underexpressing miRNAs (*solid arrows*) was identified in the IFN-β–treated glioma cells (see also Table 1; Supplementary Fig. S1 and Table S1). **B.** IFN-β treatment of T98 glioma cells recovered the expression of PDCD4, a well-known target of miR-21.

the serine of STAT3 in both T98 and SKMG1 glioma cells (Fig. 4B). Furthermore, a STAT3-specific inhibitory peptide increased the level of miR-21 expression and inhibited IFN-β–mediated suppression of miR-21 (Fig. 4C).

We constructed a reporter plasmid in which the full-length *pri-miR-21* promoter was fused to the 5′-end of the luciferase gene. The plasmid was transfected into T98 and SKMG1 cells. The reporter gene system displayed high basal activity in untreated cells and clearly reduced activity in response to IFN-β treatment. In contrast, the addition of the STAT3 inhibitor prior to IFN-β treatment returned the activity of the promoter to the basal level (Fig. 5A). To further determine if STAT3 is responsible for the reduction of the promoter activity, the reporter construct harboring the *pri-miR-21* promoter was cotransfected into SKMG1 cells with a STAT3-expressing plasmid, which was found to increase the total amount as well as the phosphorylation of STAT3 (Fig. 5B). As shown in Fig. 5C, the STAT3-expressing vector significantly reduced the promoter activity, and the addition of IFN-β further suppressed it.

Discussion

In this study, we hypothesized that type I IFN might regulate the expression of specific miRNAs in gliomas and that these

Table 1. Differentially Expressed miRNAs in T98 Glioma Cells Treated with IFN-β

Probe no.	MiRNA	Fold change (microarray)	Mean fold change (q-PCR*)	Chromosome location	No. of putative targets†	Connection to cancer in previous reports
Upregulated						
2269	*hsa_miR_187*	3.05	1.37 ± 0.13	18q12.2	0	↑ Thyroid tumor
2395	*mmu_miR_187*	3.00	NE	18A2	NA	NA
2218	*hsa_miR_194*	2.68	1.74 ± 0.34	1q41, 11q13.1	35	↓ Oral cancer, ↑ prostate cancer
Downregulated						
2603	*hsa_miR_100*	0.16	0.7 ± 0.15	11q24.1	6	↓ Oral cancer, ↓ ovarian cancer, ↑ hepatocallular
2285	*hsa_let_7b*	0.17	0.57 ± 0.08	22q13.31	12	↓ Lung cancer, ↓ colon cancer, ↓ breast cancer
2431	*hsa_let_7c*	0.20	0.6 ± 0.02	21q21.1	6	
2556	*hsa_let_7a*	0.32	0.66 ± 0.07	9q22.32, 22q13.31	164	
2220	*mmu_let_7a*	0.32	NE	13A5, 9A5.1	NA	NA
2083	*hsa_miR_21*	0.33	0.44 ± 0.14	17q23.1	46	↑ Glioblastoma, ↑ breast cancer

*The exprssion changes in mature miR-21, miR-187, miR-194, miR-100, and let-7a,b,c were validated in triplicate by using the LightCycler TaqMan Master and TaqMan MicroRNA assays. Values expressed as mean ± SD.
†Putative target genes were investigated by the prediction software programs, Targetscan (http://genes.mit.edu/tscan/targetscan2003.html; ref. 41), Miranda (http://www.microrna.org/microrna/getMirnaForm.do; ref. 42) and PicTar (http://www.pictar.org/) software (43). The name of common genes is listed in Supplementary Table S1.

modulations lead to antiproliferative effects. By performing initial screening by the microarray method, we observed an increase in the miR-187 and miR-194 levels and a decrease in the levels of miR-100, miR-21, and let-7 family miRNAs in response to IFN-β treatment. The biological functions and putative targets of each miRNA, except the let-7 family miRNAs and miR-21, in cancer remain unclear (Table 1). miR-187 was reported to be overexpressed in thyroid tumors (28). The level of miR-194 was decreased in colon cancer and oral cancer, but increased in prostate cancer. Therefore, it is unclear whether miR-194 acts as an oncomir or a tumor suppressor. Similarly, miR-100 was reported to be highly expressed in ovarian carcinoma and hepatocellular carcinoma, but its expression was lower in oral cancer. Let-7 is one of the first identified miRNAs. The biological functions of let-7 in animals include the regulation of stem cell differentiation, organ development, and cell proliferation and differentiation. Moreover, many let-7 family members function as tumor suppressors in a variety of cancers (29). However, there is no report suggesting that let-7 functions as a tumor suppressor in gliomas. Of the miRNAs regulated by IFN-β, we focused on miR-21 because it is one of the most well known miRNAs associated with tumorigenesis and progression in gliomas. miR-21 also modulates tumorigenesis through the regulation of genes, such as *bcl-2, PTEN, tropomyosin-1*, and *PDCD4* (17, 30-32). Indeed, IFN-β treatment of T98 glioma cells recovered the expression of PDCD4 (Fig. 1B). These results suggest that miR-21 is one of the major antiapoptotic factors. Our results showed miR-21 overexpression in a glioblastoma surgical specimen by performing *in situ* hybridization with the miR-21–specific probe, and in glioma cell lines and GICs by performing qRT-PCR. To our knowledge, this is the first report to show the expression of miR-21 *in situ* in a human glioma surgical specimen.

We found that IFN-β downregulated miR-21 in cultured glioma cells and intracranial glioma xenograft in mice. In the time course experiments, miR-21 expression was reduced as early as 6 hours after IFN-β treatment. The IFN-β treatment showed a relatively fast response in reducing miR-21 levels, suggesting that the negative regulation of miR-21 might be mediated directly by IFN-β, for example, through phosphorylation of Janus kinase/STAT. Our finding that IFN-β also suppressed the expression of pri-miR-21 and pre-miR-21 suggests that it regulates miR-21 transcription. The putative regulatory region of the *miR-21* gene is located within an intron of the overlapping transmembrane protein 49 (*TMEM49*) gene, and contains two consensus STAT3-binding sites at ~800 bp upstream from the transcription start site (33). The results of a recent study (26), similar to our findings, showed that IFN-β induces the phosphorylation of STAT3 in glioma cells and thereby activates STAT3-mediated miR-21 transcription in a luciferase reporter gene system. Our findings support the hypothesis that STAT3 activation exerts a cytostatic or antiproliferative effect in some types of cells (34-37); however, the role of STAT3 activation is debatable because its overactivation has been reported to be oncogenic in some cell lines (38, 39). Loffler et al. showed that IL-6–dependent STAT3 activates the transcription of miR-21 in multiple myeloma cells. Whereas IL-6 induces proliferation of myeloma cells, IFN-β reduces the growth of glioma cells or induces apoptosis in these cells (33). The possible explanation of this seemingly paradoxical role of STAT3 activation is that the STAT pathway is context-dependent and that various intracellular and/or environmental cues play a pivotal role in determining the outcome of pathway activation. This discrepancy may arise from the difference in cytokine stimulus and cell type (33).

An unresolved question that needs to be addressed is why the recovery of mature miR-21 occurred earlier than that of pri-miR-21 and pre-miR-21, as shown in Fig. 3C. miR-21 may form a family and possess isoforms similar to those of let-7 family miRNAs, in which the mature let-7a sequence is produced by three separate precursors (pre-let-7a-1, pre-let-7a-2, and pre-let-7a-3). Similarly, mature miR-21 could be produced by precursor(s) as well as the known pre-miR-21. One other possibility is that the maturation of miR-21 may be involved in the recovery of mature miR-21 after IFN-β treatment.

In conclusion, the downregulation of miR-21 in response to IFN-β treatment contributes to the antitumor effects of this cytokine. This is the first report demonstrating that an oncomir miR-21 is downregulated in cancer by endogenous stimulation

with a cytokine or a growth factor. This finding adds a new dimension to the anticancer mechanism of IFNs. In addition, although there is little evidence supporting a direct or immediate transcriptional regulation of miRNAs by IFNs, this study shows for the first time that miR-21 expression is negatively regulated by STAT3 activation. Our results highlight the importance of understanding the transcriptional regulation of miRNAs, which would be involved in oncogenesis.

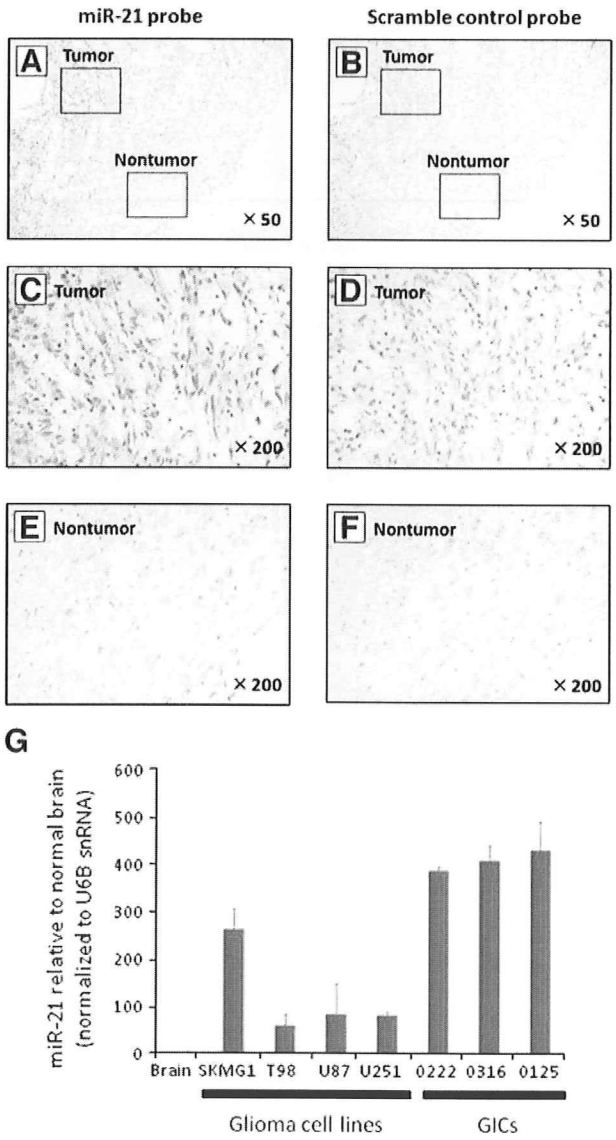

FIGURE 2. miR-21 overexpression in glioma. **A** to **F.** miR-21–specific probe and scramble control probe were hybridized *in situ* with glioblastoma tissue obtained from a surgical specimen. The miR-21–specific probe clearly stained the glioblastoma tissue but did not stain the normal cortex tissue (**A**). Tumor cells expressed significant amounts of miR-21, as observed under high magnification (**C**), whereas nontumorous tissue did not express miR-21 (**E**). In contrast, in the section adjacent to that hybridized with the miR-21 probe, neither tumor nor nontumorous tissues showed positive staining with the scramble probe (**B, D**, and **F**). **G.** qRT-PCR showed that miR-21 was overexpressed to a great extent in glioma cells than in normal brain cells. Notably, the amount of miR-21 was greater in GICs than in the glioma cell lines. Columns, mean; bars, SD (normal brain expressed as 1).

Materials and Methods

Glioma Cell Lines and Primary Tumor Sphere Cultures

We used human glioma cell lines (T98, SKMG1, U251MG, and U87MG) in this study. The source of the cell lines and the culture conditions have been reported previously (23). Glioma tissue samples were obtained from patients (nos. 0222, 0316, and 0125) undergoing surgical treatment at the Nagoya University Hospital, Nagoya, Japan, after they provided written informed consent. The procedures for the derivation of GICs were described in our recent article (26). Briefly, dissociated tumor cells were cultured in NBE media comprising neurobasal medium, with N2 and B27 supplements (Invitrogen), and human recombinant basic fibroblast growth factor and epidermal growth factor (20 ng/mL each; R&D Systems). We have previously shown that as few as 10^3 GICs could form a tumor in the brain of nonobese diabetic/severe combined immunodeficient mice (26).

Treatment

Human IFN-β (kindly provided by Toray, Kamakura, Japan) was added to the culture medium at 12 h after 2×10^5 cells were placed in a 25 cm^2 cell culture flask (BD Falcon). To examine the effect of STAT3 inhibition on miR-21 expression, we treated the cells with 50 μmol/L of the STAT3 inhibitor (PpYLKTK-mts; Calbiochem) 30 min before the addition of IFN-β. We confirmed that the STAT3 inhibitor specifically inhibited the IFN-β–mediated phosphorylation at tyrosine-705 of STAT3 (data not shown).

miRNA Microarray Analysis

We used mirVana miRNA Bioarray V2 (Ambion) containing 662 miRNAs in four copies. We treated T98 cells with IFN-β at a concentration of 100 IU/mL or with PBS for 12 h. To isolate miRNA fractions from total RNA, we fractionated and cleaned up 30 μg of total RNA by using the flashPAGE fractionator system and reagents (Ambion). Chemically synthesized oligoribonucleotides (Ambion) or purified miRNAs were labeled by using the mirVana miRNA Labeling Kit (Ambion) and amine-reactive dyes as recommended by the manufacturer. Poly(A) polymerase and a mixture of unmodified and amine-modified nucleotides were first used to append a poly-nucleotide tail. The amine-modified miRNAs were then cleaned up and coupled to *N*-hydroxysuccinimide ester–modified Cy3 or Cy5 dye (GE Healthcare). We removed the unincorporated dyes by using a second glass fiber filter–based cleaning procedure. A 3× miRNA hybridization buffer (Ambion) was added to the fluorescently labeled miRNAs, and the solution was heated at 95°C for 3 min. Slides were hybridized from 12 to 16 h at 42°C in sealed cassettes by using a water bath. Following hybridization, the slides were washed and dried before performing a high-resolution scan on a GenePix 4000B (Axon Instruments). Raw data were normalized and analyzed using Array-Pro Analyzer Version 4.5 (Media Cybernetics) to determine the signal intensity of each spot and its local background on the microarrays. We calculated the net intensity by subtracting the mean intensity of all pixels within the local background area from the mean intensity of all pixels within the spot areas. We normalized differences in net intensity between the two fluorescent dye channels in a microarray by using the local regression (loess) normalization. We selected

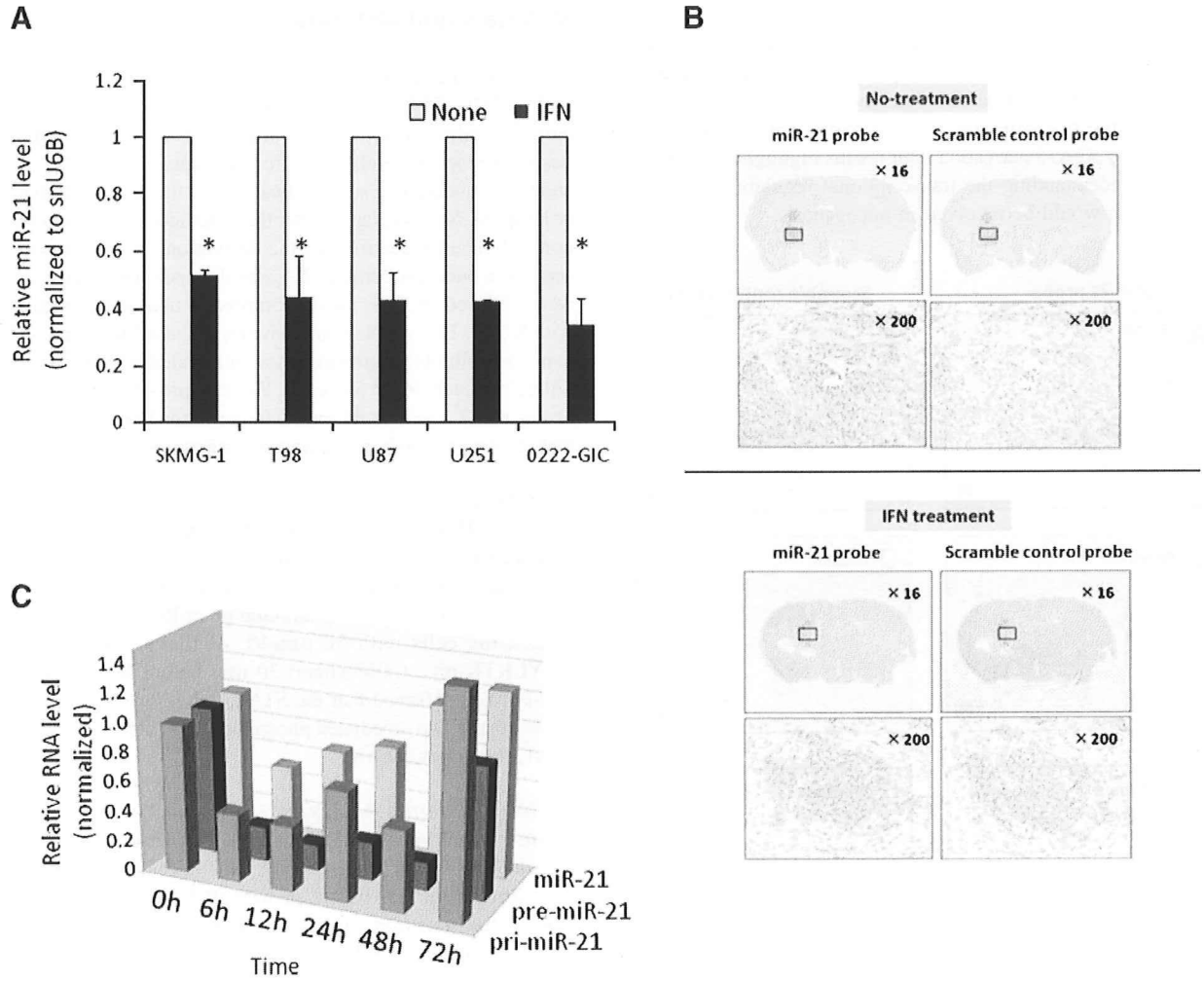

FIGURE 3. IFN-β downregulates miR-21 transcription. **A.** The expression of miR-21 in IFN-β–treated and untreated glioma cells was compared by using qRT-PCR. In all the glioma cells analyzed, IFN-β treatment reduced the expression of miR-21 by ~40% to 60%, including the cells of the SKMG1, T98, U87, and U251 glioma lines, and 0222-GIC cells. **B.** At 4 wk after intracerebral inoculation of GICs, the mice received i.p. injection of either PBS or IFN-β. IFN-β suppressed the growth of the GIC-derived intracranial tumor. In particular, the systemic delivery of IFN-β reduced the level of miR-21 in the tumor. **C.** We analyzed the time course of reduction of the levels of *pri-miR-21* gene, pre-miR-21, and mature miR-21 transcripts in response to IFN-β treatment. The levels of pri-miR-21 transcript were 0.46 ± 0.02, 0.44 ± 0.05, 0.74 ± 0.33, 0.55 ± 0.17, and 1.37 ± 0.82; pre-miR-21 levels were 0.23 ± 0.03, 0.17 ± 0.01, 0.26 ± 0.04, 0.19 ± 0.02, and 0.89 ± 0.32; and miR-21 levels were 0.53 ± 0.04, 0.69 ± 0.03, 0.78 ± 0.05, 1.11 ± 0.09, and 1.26 ± 0.16 at 6, 12, 24, 48, and 72 h, respectively. Columns, mean; bars, SD (*, $P < 0.05$).

the analyzed data by using the MicroArray Data Analysis Tool (Filgen).

qRT-PCR

Total RNA, including miRNA, was isolated by using the mirVana RNA Isolation Kit (Ambion). The analyses of mature miR-21, miR-187, miR-194, miR-100, and let-7a, let-7b, and let-7c were carried out in triplicate by using the LightCycler TaqMan Master (Roche) and TaqMan MicroRNA assays (Applied Biosystems) on a LightCycler ST300 (Roche). PCR conditions were as follows: 95°C for 10 min and 45 cycles of 95°C for 10 s, 60°C for 30 s, and 40°C for 30 s. The relative level of miRNA expression was calculated by the 2-ΔΔCt method. The data were normalized to the expression of U6B small nuclear RNA. In some assays, samples from the normal brain tissue were used as a calibrator, whereas in others, untreated samples were used.

To quantify the pri-miR-21 and pre-miR-21 expression, we treated the isolated RNA with DNase and reverse-transcribed it using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The LightCycler-FastStart DNA Master SYBR Green I kit (Roche) was used for real-time PCR applications. The primer sets for pri-miR-21 and pre-miR-21 qRT-PCR were 5′-TTTTGTTTTGCTTGGGAGGA-3′ and 5′-AGCAGACAGTCAGGCAGGAT-3′, and 5′-TGTCGGGTAGCTTATCAGAC-3′ and 5′-TGTCAGACAGCCCATCGACT-3′, respectively. The PCR conditions were as follows: 95°C for 15 min, and 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s.

Western Blotting

Cell lysis and immunoblotting were done as described previously (23). Antibodies against the following proteins were used: PDCD4 (ab51495; Abcam), phosphorylated STAT3 (p-Ser727