Vaccination with a tumor-specific antigen peptide is one of the most relevant strategies for tumor immunotherapy, and it is essential to identify the tumor antigen peptides that will elicit maximal immunological reactions in an MHC Class I/peptide complex–restricted manner.[2] The *HLA-A24* allele is highly expressed in Asians; in particular, 60% of the Japanese population has this allele.[3] A significant number of people of other ethnicities also express this antigen. Therefore, the identification of an HLA-A24–restricted CTL epitope would contribute to the development of immunotherapies for use in patients worldwide. In the present study, we investigated the suitability of IL-13Rα2 as a target antigen for malignant gliomas by examining its expression in normal tissues and gliomas. We then identified a potential HLA-A24–restricted peptide derived from IL-13Rα2 that can effectively induce HLA-A24–restricted and IL-13Rα2–specific CTLs, and demonstrated that the CTLs induced by this novel antigenic peptide can kill freshly isolated HLA-A24$^+$ IL-13Rα2$^+$ human-derived glioma cells.

Methods

Cells and Cultures

The glioma cell lines SKMG-1, KNS42, T98, U87MG, U251MG, and AO2 were grown in Eagle minimal essential medium (Nissui) containing 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The HLAs in these cell lines were genetically typed by SRL, Inc.

The peptide transporter-negative BxT hybrid cell line 174×CEM (referred to as T2), was transfected with a plasmid encoding HLA-A2402. The transfected cell line was cloned by limiting dilution and designated T2-A24.[13] This cell line was grown in RPMI1640 supplemented with 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.8 mg/ml G418 (GIBCO).

Tumor tissues obtained in 3 patients who had undergone resection for high-grade gliomas at Nagoya University Hospital, Nagoya, Japan were placed in primary culture. Immediately after the extraction, the tissues were homogenized and digested with 1% DNase and 0.1% trypsin for 30 minutes at 37°C, and then centrifuged at 800 rpm for 5 minutes. The cells were seeded at a density of 2×10^6 cells per 100-mm dish and maintained in Dulbecco modified Eagle's medium (GIBCO) supplemented with 10% FBS, 5-mM L-glutamine, 2-mM nonessential amino acids, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were then incubated in a standard tissue culture incubator (at 100% relative humidity and 5% CO_2 in air). After achieving 80–90% confluence, the cells were subcultured onto a new 100-mm plate at a density of 2×10^6. The established cell lines were designated as NNS03, NNS04, and NNS08, and their HLAs were typed as HLA-A24/A33, A2, and A2/A26, respectively. All cells were confirmed to be glioma cells based on their expression of glial fibrilliary acidic protein.

Ribonucleic Acid Isolation and RT-PCR Analysis of IL-13Rα2 Expression

Reverse transcription was performed using total cellular RNA extracted by the guanidinium thiocyanate/cesium chloride method in the presence of random hexamers and SuperScript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. The primers used for the amplification of human IL-13Rα2 were 5′-TGGTCAGAAGTGTGCCTGTC-3′ (sense) and 5′-TCTGCCCAGGAACTTTAAC-3′ (antisense). In the Takara Thermal Cycler, 25 amplification cycles, comprising denaturation for 30 seconds at 94°C, annealing for 30 seconds at 57°C, and extension for 1 minute at 72°C were conducted using AmpliTaq-gold DNA polymerase (Applied Biosystems). The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, and stained with ethidium bromide. Band intensities were quantified by densitometric scanning using the National Institutes of Health IMAGE software. The results were evaluated semiquantitatively by comparison with the relative amounts of β-actin PCR products, and classified into groups of high (++: IL-13Rα2/β-actin > 0.5), moderate (+: < 0.5), or no expression (−: 0).

Peptide Synthesis

We used the binding-prediction software program BIMAS (http://bimas.dcrt.nih.gov/molbio/hla_bind/) to identify potential HLA-A24–binding peptides within IL-13Rα2. All peptides were synthesized by Thermo Electron GmbH and purchased from Greiner Bio-One Japan. The purity of the peptides was shown to be $> 90\%$.

Major Histocompatibility Complex Stabilization Assay

The 5 synthesized peptides were used in an MHC stabilization assay by means of T2-A24 cells as described elsewhere.[13] Briefly, T2-A24 cells (3×10^5) were incubated with 200 μl of RPMI1640 containing 0.1% FBS and 10-μM peptides at 26°C for 16 hours, followed by incubation at 37°C for 3 hours. Surface HLA-A24 molecules were then indirectly stained with the anti–A24 monoclonal antibody (A11.1; One Lambda, Inc.) and fluorescein isothiocyanate-labeled anti–mouse immunoglobulin G. Expression was measured in a FACS Calibur (Becton Dickinson), and the mean fluorescence intensity was recorded.

Generation of DCs

All DCs derived from patients were found to be HLA-A24$^+$ cells on whole HLA typing conducted by SRL, Inc. The DCs were generated and matured as reported in other studies with minor modifications.[18] Briefly, peripheral blood mononuclear cells were isolated using Ficoll-Paque (Amersham Biosciences), and the monocyte fraction was enriched by plastic adherence. After a 2-hour incubation period at 37°C, the nonadherent cells were removed, and the adherent cells were cultured with 500 U/ml of rhGM-CSF and 500 U/ml of rhIL-4 (Strathmann Biotech AG) in AIM-V medium (GIBCO). On Day 6, 500 U/ml of rhGM-CSF, 500 U/ml of rhIL-4, 1000 U/ml of rhIL-6, 10 U/ml of human recombinant tumor necrosis factor–α, and 10 U/ml of rhIL-1β (Strathmann Biotech AG) were added to the wells. On Day 8, the matured DCs were harvested from the wells by vigorous washing.

Peptide Pulsing of DCs and Induction of IL-13Rα2–Specific CTL Lines

The mature DCs generated were resuspended in AIM-V at a density of 10^6 cells/ml and pulsed with 10 μg/ml of peptides for 4 hours at 37°C. The peptide-pulsed DCs were then treated with mitomycin C for 45 minutes, washed, and resuspended in AIM-V medium containing 10% human AB serum. The autologous $CD8^+$ T-cell population was magnetically isolated from peripheral blood mononuclear cells by using CD8 MicroBeads (Miltenyi Biotech). Next, 1 million $CD8^{++}$ T cells were cocultured in each well of a 24-well plate with 10^5 peptide-pulsed DCs in 2 ml of AIM-V medium supplemented with 10% human AB serum, 1000 U/ml rhIL-6, and 10 ng/ml rhIL-12 (Strathmann Biotech AG). On Day 7, the lymphocytes were restimulated with autologous peptide-pulsed DCs treated with mitomycin C in AIM-V medium supplemented with 10% human AB serum, 10 U/ml rhIL-2 (PeproTech EC), and 10 U/ml rhIL-7 (Strathmann Biotech AG). Restimulation of the lymphocytes by peptide-pulsed DCs was repeated once a week to establish CTL lines.

Cytotoxic T-Lymphocyte Assay

Target cells were labeled with $Na_2{}^{51}CrO_4$ (Perkin-Elmer), and the labeled cells (100 μl) were incubated with effector cells (100 μl) in a U-bottomed 96-well microtiter plate. After incubation for 4 hours at 37°C, the supernatants were collected and their radioactivity was measured with a gamma counter. The percentage specific lysis was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). Monoclonal antibodies against HLA-ABC (W6/32) and HLA-DR (HDR-1) were kindly provided by Dr. K. Itoh, Kurume University, and were used for antibody-blocking tests.

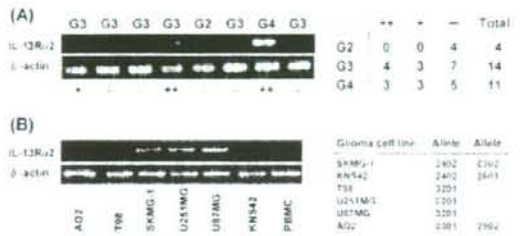
Statistical Analysis

The statistical significance of the difference between groups was determined by analysis of variance, using the Bonferroni correction for the multiple post-hoc analyses performed. The statistical analysis was conducted using commercially available software (Systat 9).

Results

Expression of IL-13Rα2 in Glioma Tissue Samples and Cell Lines

To assess the frequency of IL-13Rα2 expression in glioma cells, we performed RT-PCR on histopathologically confirmed glioma samples obtained in 29 patients. The gliomas examined in this analysis included 25 high-grade and 4 low-grade gliomas. A sample of nonglioma tissue was also examined for comparison. Interleukin-13Rα2 expression was observed in 14 (56%) of the high-grade gliomas (World Health Organization Grades 3 and 4), and high- and moderate-level expression was observed in 7 cases each. One low-grade glioma (Grade 2) and the nonglioma tissue sample did not express IL-13Rα2 (Fig. 1A, data not shown). Interleukin-13Rα2 was highly expressed in 3 of 6 glioma cell lines (Fig. 1B). We also examined the



	++	+	−	Total
G2	0	0	4	4
G3	4	3	7	14
G4	3	3	5	11

Glioma cell line	Allele	Allele
SKMG-1	2402	2902
KNS42	2402	2601
T98	3201	
U251MG	3201	
U87MG	3201	
A07	3301	7902

FIG. 1. Detection of IL-13Rα2 in glioma cells by RT-PCR on agarose gel electrophoresis. A: Representative results in a surgical specimen. The *table* represents the summarized data showing that IL-13Rα2 is expressed only in high-grade glioma cells. The expression intensity was semiquantitatively determined by comparison with the relative amounts of β-actin PCR products that were used as an internal control, and classified into groups of high (++), moderate (+), and no expression (−), based on the band intensities quantified by densitometric scanning using the National Institutes of Health IMAGE program. B: Results in human glioma cell lines. High expression of IL-13Rα2 was observed in 3 of 6 glioma cell lines. The *table* shows the results of the HLA typing of all glioma cell lines and shows that SKMG-1 and KNS42 have the A2402 allele (*underlined*).

expression of IL-13Rα2 in other cancer cell lines such as medulloblastoma, colon cancer, melanoma, and pancreatic cancer, and found that only 1 of 2 medulloblastoma types expressed IL-13Rα2.

Selection of Potential HLA-A24–Binding Peptides Within the IL-13Rα2 Protein

Using computer-based algorithms, the following 5 peptide sequences with a high predictive score on BIMAS were selected for synthesis: WYEGLDHAL, LYLQWQPPL, VYYNWQYLL, TYPKMIPEF, and EYELKYRNI (Table 1). These sequences were designated P174, P49, P146, P368, and P68, respectively. Human leukocyte antigen stabilization assays were performed to test their affinity to the HLA-A2402 molecule by using T2-A24 cells. As shown in Fig. 2, P146 bound most strongly to the HLA-A24 molecule. Despite having lower affinities than P146, the other 4 peptides also showed positive values. Therefore, we used all the 5 peptides for the subsequent experiments.

Induction of HLA-A24–Restricted and IL-13Rα2–Specific CTL Lines

To determine which peptide could best induce HLA-A24–restricted and IL-13Rα2–specific CTLs, we examined the cytotoxic activities of CTL lines induced by repetitive stimulation with autologous DCs pulsed with the peptides listed in Table 1. After we performed the CTL assays using at least 3 separately obtained CTL lines for each peptide, we confirmed that 3 peptides (P174, P49, and P146) could induce CTL lines cytotoxic to the HLA-$A24^+$ IL-$13R\alpha2^+$ glioma cell line SKMG-1 (Fig. 3). Of these, the P174-induced CTL lines consistently showed the strongest cytotoxicity in several independent experiments in glioma tissues obtained in 2 patients.

To confirm whether the cytotoxicity of the CTL lines induced by IL-13Rα2–derived peptides was truly restrict-

TABLE 1

Characterization of IL-13Rα2–derived peptides used for CTL induction

Peptide Designation	Position*	Amino Acid Sequence	Predictive Score†
P174	174–182	WYEGLDHAL	360.000
P49	49–57	LYLQWQPPL	300.000
P146	146–154	VYYNWQYLL	200.000
P368	368–376	TYPKMIPEF	165.000
P68	68–76	EYELKYRNI	75.000

* Numbers indicate the position of the peptide in the amino acid sequence of IL-13Rα2.

† Estimated half-time of dissociation from HLA-A24 molecules (in minutes).

ed to both HLA-A24 and IL-13Rα2, we assessed their lytic activity against 6 glioma cell lines that differentially express IL-13Rα2 and HLA-A24. Representative results with a CTL line induced by the most immunogenic peptide (P174) are shown in Fig. 4A. Only the SKMG-1 cell line showed remarkable lysis by the P174-induced CTL line. The specificity of the lytic activity of this CTL line against the SKMG-1 cell line was statistically significant compared with its activity against all other 5 glioma cell lines. Furthermore, an antibody-blocking assay revealed that anti–HLA-Class I but not Class II antibodies could reduce the lytic activity of P174-induced CTLs in a dose-dependent manner. Taken together, these results clearly demonstrate that CTL with a desirable specificity, such as being HLA-A24–restricted and IL-13Rα2–specific, can be effectively induced by P174-pulsed DCs.

Cytotoxicity of P174-Induced CTLs Against Surgically Removed Glioma Cells

To assess the possibility of P174 use in patients with gliomas, we tested the cytotoxicity of P174-induced CTLs against glioma cells in primary cultures that had been freshly isolated from 3 patients. As shown in Fig. 5, only NNS03 cells expressing both HLA-A24 and IL-13Rα2 were significantly lysed by the P174-induced CTLs, suggesting that P174 can be applied in patients with glioma cells that express both HLA-A24 and IL-13Rα2.

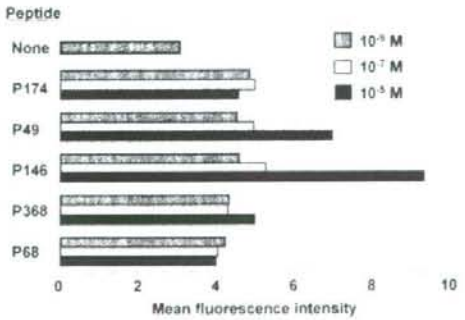


FIG. 2. Bar graph of MHC stabilization assay. The T2-A24 cells were incubated with the indicated peptides and HLA-A24 expression was analyzed by flow cytometry. The results are presented as the mean fluorescence intensity.

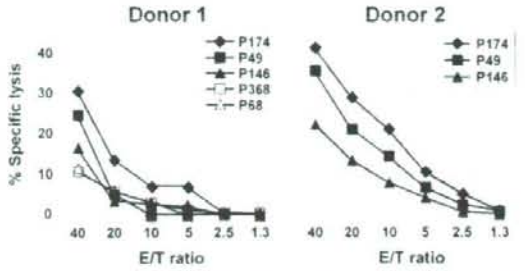


FIG. 3. Graphs comparing the cytotoxic activity of CTL lines induced by DCs pulsed with IL-13Rα2–derived peptides. The cytotoxic activity of the CTL lines was tested using the standard 4-hour ^{51}Cr release assay by using SKMG-1 cells (HLA-A24$^+$ and IL-13Rα2$^+$) as a target. E/T ratio = effector to target ratio.

Discussion

Debinski et al.[5–7] have reported that almost all human glioblastoma multiforme cells express IL-13R. They further demonstrated that IL-13Rα2, which binds IL-13 in an IL-4–independent manner, is the restricted binding site for IL-13 on malignant gliomas.[16] Another study by Joshi et al.[10] also has demonstrated high specificity of IL-13Rα2 for glioblastoma multiforme. In the present study, we first investigated the IL-13Rα2 expression in a series of resected glioma tissues and demonstrated that more than half of the high-grade gliomas expressed IL-13Rα2. This high expression in glioma cells was also confirmed on immunostaining with anti–IL-13Rα2 antibody. In contrast, none of the samples from Grade 2 gliomas expressed the antigen. These results suggest that IL-13Rα2 expression is restricted to malignant lesions and might be a prognostic marker in low-grade gliomas, as suggested previously.[7] Our data could render this protein a very promising target of glioma-specific immunotherapy. However, the frequency of IL-13Rα2 expression observed in the present study is not as high as that reported in previous studies. The difference might be related to the methods for examination or the backgrounds of the patients included in the studies. The availability of IL-13Rα2 as a target antigen should be confirmed by further investigations in a larger population. For reference, we conducted RT-PCR analysis of the expression of IL-13Rα2 in other cancer cell lines including colon cancers, melanomas, pancreatic cancers, and medulloblastomas, and found that only the medulloblastoma cell line expressed IL-13Rα2. However, ovarian or renal cancers have also been reported to express IL-13Rα2,[1,12] which may imply that IL-13Rα2 is associated with malignancy.

As a mainstay of this study, we investigated peptides derived from IL-13Rα2 that have the highest potential to elicit cellular immune responses against glioma cells expressing IL-13Rα2 and HLA-A24. First, 5 candidate peptides from the IL-13Rα2 protein were predicted using a bioinformatics approach. Second, we pursued the most promising peptide that can induce CTLs specific to IL-13Rα2 together with HLA-A24, and found P174 to be the most immunogenic. Finally, we tested the specificity of the P174-induced CTLs to IL-13Rα2 and HLA-A24, and their cytotoxicity against HLA-A24$^+$ IL-13Rα2$^+$ primary glioma cells as well as an established glioma cell line.

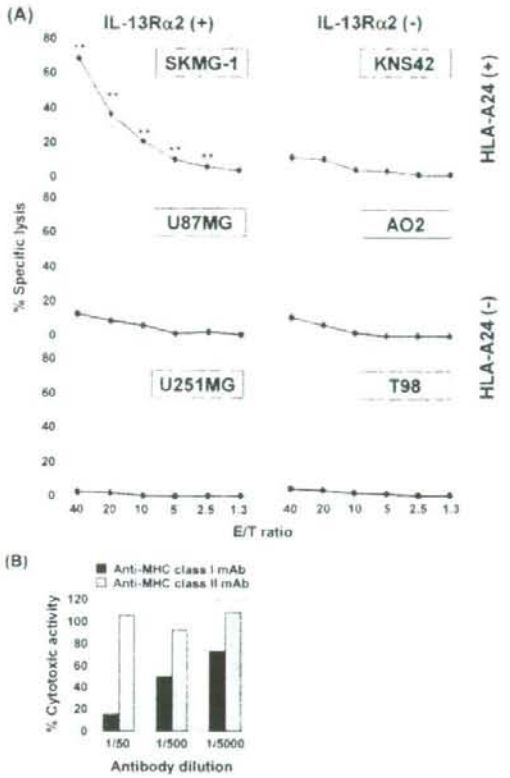


FIG. 4. Graphs demonstrating the specificity of P174-induced CTL line. A: Cytotoxic activity of P174-induced CTLs against various glioma cell lines. The cytotoxic activity of P174-induced CTLs was tested using the standard 4-hour ^{51}Cr release assay to confirm their HLA restriction and antigen specificity. Results are shown as the means ± standard deviations. **The lytic activity of P174-induced CTLs against the SKMG-1 cell line at the E/T ratio is significantly different than those against the other glioma cell lines ($p < 0.05$). B: Bar graph demonstrating the antibody blocking assay. The cytotoxic activity of P174-induced CTLs was tested using the standard 4-hour ^{51}Cr release assay in the presence or absence of anti–MHC Class I or Class II monoclonal antibody (mAb); SKMG-1 cells were the target. The results are expressed in terms of percent specific activity, and cytotoxicity without monoclonal antibodies is defined as 100%.

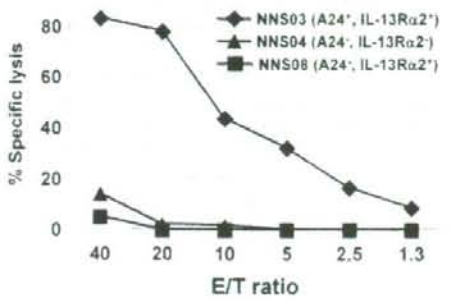


FIG. 5. Graph demonstrating cytotoxicity of P174-induced CTLs against primary-cultured glioma cells from surgically removed tissues. The cytotoxic activity of P174-induced CTLs was tested using the standard 4-hour ^{51}Cr release assay.

Substantial time and effort in the laboratory is generally required to identify new CTL epitopes. To improve the identification process, online bioinformatics algorithms are commonly used to predict which epitope will bind to a particular HLA. There are some algorithms available on the internet that use a scoring system to predict the binding affinity of a given peptide,[19] and we selected 5 peptides for this study using the BIMAS algorithm. We also performed an MHC stabilization assay to evaluate each peptide's affinity for the HLA-A24 molecule.[13] The results of our MHC stabilizing assay showed that P146, which had the third highest score on BIMAS analysis, has the greatest affinity for the HLA-A24 molecule. In generating CTLs from DCs pulsed with the candidate peptides, we found that the P174 peptide was the most potent epitope and induced CTLs with a fine-tuned specificity to IL-13Rα2 and HLA-A24. However, this result appears to be inconsistent with those of the MHC stabilization assay because the P146-derived CTLs were less efficient in causing lysis in the SKMG-1 cells. Proteasomal cleavage of the mother protein is an important process for antigen presentation by MHC Class I molecules, and we speculate that the sequences of the predicted antigenic peptides are not always in accordance with the predicted proteasome cleavage sites.[22] Based on a proteosomal cleavage site prediction system, we also confirmed that among the 5 candidates, only the ends of the P174 peptide are the cleavage sites.[17] In addition, it has been reported that peptide-binding assays might not be sufficiently sensitive to detect the binding of all natural ligands.[8] To confirm that cytotoxicity of the P174-derived CTLs is truly restricted to IL-13Rα2 and HLA-A24, we conducted CTL assays in 6 glioma cell lines that have different levels of IL-13Rα2 and HLA-A24 expression. The results showed the remarkable cell lytic activity of the P174-derived CTLs against SKMG-1 cells. The cytotoxicity observed with cells that had undergone DC stimulation 4 times was much higher (~ 70%) than that in the cells that had undergone DC stimulation 3 times (~ 35%). This indicates that repeated DC pulsation leads to an increase in the specific cytotoxicity of CTLs. This finding has also been described in a previous study,[11] and it could be speculated that the repetition of DC pulsation contributes to higher purification of the CTLs specific to the peptide. The restriction of the CTLs to both IL-13Rα2 and HLA-A24 was verified by the minimal cytotoxicity to the other 5 kinds of glioma cells, and the effective inhibition of tumor cell lysis in the presence of anti–MHC Class I antibody. In addition, it is noteworthy that the lytic activity of P174-derived CTLs against the primary glioma cells obtained from the surgical specimens was also restricted to both HLA-A24 and IL-13Rα2; this raises the possibility future application in patients.

To our knowledge, this is the first study in which a novel HLA-A24–restricted CTL epitope peptide derived from the IL-13Rα2 protein has been identified. The P174 peptide was confirmed to be the most effective in inducing CD8$^+$ CTLs to destroy glioma cells expressing both HLA-A24 and IL-13Rα2. In addition to its high prevalence in Asians,

HLA-A24 is also expressed in 17% of Caucasians, 27% of Hispanics, and 9% of African-Americans.[3] Another HLA-A allele, HLA-A2, is the most common HLA-A allele in the world, occurring in 40% of Japanese, 60% of Caucasians and Chinese, 69% of Hispanics, and 31% of African-Americans.[9] Therefore, large number of patients with gliomas all over the world would be expected to show either HLA-A24 or HLA-2 expression. Indeed in the 14 patients with gliomas who we examined for HLA typing, 7 carried the *HLA-A24* allele and 6 the *HLA-A2* allele. Coupled with an HLA-A0201–restricted peptide associated with IL-13Rα2 that has been identified,[18] the majority of gliomas could potentially be treated with immunotherapy targeting IL-13Rα2 in a peptide or DC-based vaccination.[23,24] Although our study is in its first phase and further studies are needed, our data could contribute to the development of the immunotherapy for malignant gliomas.

Conclusions

Our results demonstrate that IL-13Rα2 could be a very promising antigen for the treatment of high-grade gliomas. We first identified a novel HLA-A24–restricted CTL epitope peptide derived from the IL-13Rα2 protein using glioma cell lines and primary glioma cells. The P174 peptide could eventually provide a peptide-based immunotherapy against malignant gliomas in patients with HLA-A24 expression.

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References

1. Bernard J, Treton D, Vermot Desroches C, Boden C, Horellou P, Angevin E, et al: Expression of interleukin 13 receptor in glioma and renal cell carcinoma: IL13Ralpha2 as a decoy receptor for IL13. **Lab Invest 81:**1223–1231, 2001
2. Celis E, Sette A, Grey HM: Epitope selection and development of peptide based vaccines to treat cancer. **Semin Cancer Biol 6:** 329–336, 1995
3. Date Y, Kimura A, Kato H, Sasazuki T: DNA typing of the HLA-A gene: population study and identification of four new alleles in Japanese. **Tissue Antigens 47:**93–101, 1996
4. Debinski W, Gibo DM: Molecular expression analysis of restrictive receptor for interleukin 13, a brain tumor-associated cancer/testis antigen. **Mol Med 6:**440–449, 2000
5. Debinski W, Gibo DM, Hulet SW, Connor JR, Gillespie GY: Receptor for interleukin 13 is a marker and therapeutic target for human high-grade gliomas. **Clin Cancer Res 5:**985–990, 1999
6. Debinski W, Gibo DM, Slagle B, Powers SK, Gillespie GY: Receptor for interleukin 13 is abundantly and specifically over-expressed in patients with glioblastoma multiforme. **Int J Oncol 15:** 481–486, 1999
7. Debinski W, Slagle B, Gibo DM, Powers SK, Gillespie GY: Expression of a restrictive receptor for interleukin 13 is associated with glial transformation. **J Neurooncol 48:**103–111, 2000
8. Hebart H, Daginik S, Stevanovic S, Grigoleit U, Dobler A, Baur M, et al: Sensitive detection of human cytomegalovirus peptide-specific cytotoxic T-lymphocyte responses by interferon-gamma-enzyme-linked immunospot assay and flow cytometry in healthy individuals and in patients after allogeneic stem cell transplantation. **Blood 99:**3830–3837, 2002
9. Imanishi T, Akazawa T, Kimura A, Tokunaga K, Gojobori T: Allele and haplotype frequencies for HLA and complement loci in various ethnic groups, in Tsuji K, Aizawa M, Sasazuki T (eds): **HLA 1991.** New York: Oxford University Press, 1992, Vol 1, pp 1065–1220
10. Joshi BH, Plautz GE, Puri RK: Interleukin-13 receptor alpha chain: a novel tumor-associated transmembrane protein in primary explants of human malignant gliomas. **Cancer Res 60:**1168–1172, 2000
11. Kiessling A, Fussel S, Schmitz M, Stevanovic S, Meye A, Weigle B, et al: Identification of an HLA-A*0201-restricted T-cell epitope derived from the prostate cancer-associated protein trp-p8. **Prostate 56:**270–279, 2003
12. Kioi M, Kawakami M, Shimamura T, Husain SR, Puri RK: Interleukin-13 receptor alpha2 chain: a potential biomarker and molecular target for ovarian cancer therapy. **Cancer 107:**1407–1418, 2006
13. Kuzushima K, Hayashi N, Kimura H, Tsurumi T: Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. **Blood 98:**1872–1881, 2001
14. Miloux B, Laurent P, Bonnin O, Lupker J, Caput D, Vita N, et al: Cloning of the human IL-13R alpha1 chain and reconstitution with the IL4R alpha of a functional IL-4/IL-13 receptor complex. **FEBS Lett 401:**163–166, 1997
15. Mintz A, Gibo DM, Madhankumar AB, Debinski W: Molecular targeting with recombinant cytotoxins of interleukin-13 receptor alpha2-expressing glioma. **J Neurooncol 64:**117–123, 2003
16. Mintz A, Gibo DM, Slagle-Webb B, Christensen ND, Debinski W: IL-13Ralpha2 is a glioma-restricted receptor for interleukin-13. **Neoplasia 4:**388–399, 2002
17. Nussbaum AK, Kuttler C, Hadeler KP, Rammensee HG, Schild H: PAProC: a prediction algorithm for proteasomal cleavages available on the WWW. **Immunogenetics 53:**87–94, 2001
18. Okano F, Storkus WJ, Chambers WH, Pollack IF, Okada H: Identification of a novel HLA-A*0201-restricted, cytotoxic T lymphocyte epitope in a human glioma-associated antigen, interleukin 13 receptor alpha2 chain. **Clin Cancer Res 8:**2851–2855, 2002
19. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: SYFPEITHI: database for MHC ligands and peptide motifs. **Immunogenetics 50:**213–219, 1999
20. Rosenberg SA: Cancer vaccines based on the identification of genes encoding cancer regression antigens. **Immunol Today 18:** 175–182, 1997
21. Sikorski CW, Lesniak MS: Immunotherapy for malignant glioma: current approaches and future directions. **Neurol Res 27:**703–716, 2005
22. Tanaka K, Tanahashi N, Tsurumi C, Yokota KY, Shimbara N: Proteasomes and antigen processing. **Adv Immunol 64:**1–38, 1997
23. Yajima N, Yamanaka R, Mine T, Tsuchiya N, Homma J, Sano M, et al: Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant glioma. **Clin Cancer Res 11:**5900–5911, 2005
24. Yamanaka R, Yajima N, Abe T, Tsuchiya N, Homma J, Narita M, et al: Dendritic cell-based glioma immunotherapy. **Int J Oncol 23:**5–15, 2003
25. Zurawski G, de Vries JE: Interleukin 13 elicits a subset of the activities of its close relative interleukin 4. **Stem Cells 12:**169–174, 1994
26. Zurawski SM, Vega F Jr, Huyghe B, Zurawski G: Receptors for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. **EMBO J 12:** 2663–2670, 1993

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CLINICAL STUDY - PATIENT STUDY

Immunohistochemical profiles of brain metastases from breast cancer

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Abstract The aim of present study is to explore the immunohistochemical profiles of brain metastases from breast cancer. We retrospectively performed immunohistochemical staining for estrogen receptor (ER), progesterone receptor (PgR), human epidermal growth factor receptor type 2 (HER2/neu), and cytokeratin (CK) 5/6 in 29 patients with resected tumor specimens of brain metastases. Immunohistochemical staining for ER, PgR and HER2/neu was performed in 24 patients with primary tumors. The positive frequency of immunohistochemical profiles of ER, PgR, HER2/neu, and CK5/6, in the brain metastases were 13.8%, 6.9%, 37.9%, and 24.1%, respectively. The immunohistochemical profiles including ER, PgR, and HER2/neu of the primary tumor and the brain metastasis differed in seven patients (29.2%, N = 7/24). Interestingly, the biological characteristics of brain metastasis sometimes changed which were represented by immunohistochemical staining. Therefore, the changes in the biological features of breast cancer should be taken into account when developing treatment strategies, including new molecular-targeted drugs, for brain metastases.

Keywords Immunohistochemical staining · Discordance · Hormone receptor · HER2/neu · Breast cancer · Brain metastasis

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Introduction

Brain metastasis is the most common type of malignancy found in the brain and is responsible for a substantial fraction of the total morbidity and mortality of metastatic breast cancer patients. Brain metastasis is generally a late feature in the history of metastatic breast cancer. The incidence of symptomatic brain metastases in metastatic breast cancer ranges from 10% to 16% and has been reported to be even higher in human epidermal growth factor receptor type 2 (HER2/neu)-positive tumors [1]. Current systemic therapy, including hormone therapy, chemotherapy and molecular-targeted drug therapy, is not effective for the treatment of brain metastasis, and the development of treatment strategies for brain metastasis has become a critical issue.

A recent study reported that patients with HER2/neu-positive metastatic breast cancer had a high incidence of subsequent brain metastasis than HER2/neu-negative patients [2–4]. The human epidermal growth factor

receptor family is involved in cell proliferation, differentiation, and survival. HER2/neu amplification is widely known to indicate an aggressive tumor behavior and poor clinical outcome in breast cancer patients. HER2/neu over expression occurs in approximately 20–30% of breast cancer patients [5]. Trastuzumab, a monoclonal antibody against HER2/neu, has been shown to be significantly effective in both adjuvant and metastatic settings [6, 7].

One interpretation of these results was that HER2/neu-positive breast cancer may have a biological affinity toward the development of brain metastasis or trastuzumab-containing therapy prolongs survival until the eventual development of brain metastasis. On the other hand, evidence indicated trastuzumab cannot enter cerebrospinal fluid [8]. Blood brain barrier creates a "sanctuary" for cancer cells where antitumor agents cannot penetrate in high enough concentrations to have any substantial effect. However, whether prolonged patient survival enables cancer cells that have become resistant to trastuzumab to metastasize to the brain remains uncertain. Furthermore, whether brain metastases continue to overexpress HER2/neu and to be sensitive to trastuzumab therapy also remains unknown.

On the other hand, triple negative breast carcinomas (TNBCs) are a group of primary breast tumors with aggressive clinical behavior that account for 10–15% of all breast cancers [9]. Most TNBCs possess a basal phenotype and show varying degrees of basal cytokeratin (CK) and myoepithelial marker expression. Histologically, such cancers are poorly differentiated, and most fall into the basal subgroup of breast cancers, characterized by staining for basal markers (i.e., CK5/6) [10]. A previous study, in which primary tumors were immunohistochemically analyzed, reported that patients with estrogen receptor (ER)-negative and progesterone receptor (PgR)-negative tumors either with or without HER2/neu over-expression had a high risk of brain metastasis in a case-controlled study. A multivariate analysis of a database containing 10,782 patients in another study also reported that the independent risk factors for central nervous system metastasis were ER negativity, a young age, and a histology of invasive ductal carcinoma [11].

We therefore investigated the immunohistochemical profiles, including ER, PgR, HER2/neu, and CK5/6 of brain metastases in breast cancer patients. In addition, we investigated the changes in the immunohistochemical profiles of the primary tumors and the brain metastases.

Patients and methods

Patients

Two hundred fifty-two patients with breast cancer received trastuzumab-based chemotherapy between January 1999 and January 2006 at the National Cancer Center Hospital (NCCH), in Japan. (48 patients in neo-adjuvant setting and 204 patients in metastatic or recurrent setting) Of these, 74 patients (36.3%) developed brain metastases. Twenty-nine patients with brain metastasis were retrospectively identified based on records in the hospital's surgical database. All clinical information was collected from patient chart. Trastuzumab had been initially administered at an intravenous loading dose of 4 mg/kg, followed by weekly infusions of trastuzumab (2 mg/kg) in combination with chemotherapy. The present study was approved by the Institutional Review Board of the National Cancer Center.

Tissue samples and microscopic and immunohistochemical analysis

Hematoxylin–eosin stained specimens were reviewed by two pathologists (K.T. and K.S.) and were confirmed to contain an adequate amount of cancer tissue available for use in the present study. All tumor specimens from brain metastasis resections were available for immunohistochemical analysis. Tumor specimens from primary breast cancers were available for immunohistochemical analysis of ER, PgR, and HER2/neu in 24 of the 29 patients.

The pathological and immunohistochemical examinations were conducted by the same pathologists (K.T. and K.S.), who were blinded to the clinical statuses of the patients. Formalin-fixed, paraffin-embedded tissue samples were sectioned (4-μm thick) and mounted on charged slides. Immunohistochemical staining for ER (clone 1D5; DAKO, Carpinteria, CA), PgR (clone PgR636; Dako), and CK5/6 (clone D5/16B4; Dako) were performed using the streptavidin-biotin method and were considered positive if 10% or more of the nuclei in the invasive component of the tumor were stained [12, 13]. The HER2/neu status, as assessed using Herceptest (Dako), was scored on a scale of 0 to 3+, according to the Dako scoring system. HER2/neu-positive was defined by HER2/neu 3+ or HER2/neu 2+ and fluorescence in situ hybridization-positive. Negative controls, in which the primary antibody was omitted, were also included in each run.

Statistical analysis

The comparisons were made between two groups using a Chi-square test, a Fisher exact test. All the statistical analyses were performed using SPSS 12.0J (SPSS Inc., Chicago, IL, USA), and the significance level for the results was set at 0.05 (two-sided).

Results

The present study included 29 patients with brain metastasis, and the median age at the time of the diagnosis of

Table 1 Patient characteristics

Characteristics of brain metastasis	Prior history of trastuzumab		Total
	(+)	(−)	
Median size (in mm) of brain metastasis (range)	32 (30–40)	36 (20–60)	35 (20–60)
Number of brain metastases			
1	6	18	24
2	2	2	4
3	0	1	1
Side (right/left/bilateral)	1/6/1	13/8/0	14/14/1
Site			
Frontal lobe	0	1	1
Parietal lobe	0	2	2
Temporal lobe	1	5	6
Occipital lobe	1	2[a]	3[a]
Cerebellum	6	10[a]	16[a]

[a] One patient had brain metastases in both the cerebellum and occipital lobe

brain metastasis was 53 years old (range, 39–78 years). The median time to brain metastasis from the time of breast cancer diagnosis was 2.9 years (range, 0–23.1 years).

In this study, eight patients had a prior history of receiving chemotherapy containing trastuzumab, seven of these patients had received trastuzumab-containing chemotherapy in a metastatic setting, and one had received trastuzumab-containing neo-adjuvant chemotherapy, five patients had a prior history of receiving hormone therapy, seven patients had a prior history of receiving chemotherapy, two patients had a prior history of receiving both hormone therapy and chemotherapy, and seven patients had received no systemic therapy prior to the brain tumor resection.

The patient characteristics are shown in Table 1. After the brain tumor resection, most patients (59%) received whole brain radiotherapy, eight patients received local brain radiotherapy, one patient received γ-knife radiotherapy, and three patients did not receive additional brain radiotherapy. After the completion of the local treatment for metastatic brain tumor, 14 patients received systemic chemotherapy, including three patients who received combination chemotherapy including trastuzumab, one patient who received trastuzumab therapy alone, five patients who received hormone therapy, and three patients who received intrathecal chemotherapy because of the rapid progression of meningeal carcinomatosis. Six patients received supportive care alone. The median overall survival time was 14.7 months.

The positive frequency of immunohistochemical profiles of ER, PgR, and HER2/neu, in 24 primary tumors were 12.5% (N = 3/24), 8.3% (N = 2/24), 37.5% (N = 9/24), respectively. The positive frequency of immunohistochemical profiles of ER, PgR, HER2/neu, and CK5/6, in 29 brain metastases were 13.8% (N = 4/29), 6.9% (N = 2/29), 37.9% (N = 11/29), and 24.1% (N = 5/29), respectively (Table 2). Among patients with both hormone-negative and HER2/neu-negative statuses, basal type (CK5/6 positive) breast cancer was observed in 42.9% (N = 6/14). The staining results for ER, PgR, HER2/neu and CK5/6 in the brain metastases were not statistically correlated with a prior history of receiving trastuzumab-containing chemotherapy (Table 2).

Table 2 Relationship between prior history of receiving trastuzumab and immunohistochemical profiles in specimens obtained from brain metastasis (Chi-square test and Fisher exact test)

Variables	Total (%) n = 29	Prior history of trastuzumab		*P*-value
		(+)	(−)	
ER				0.99
Negative	25	7	18	
Positive	4	1	3	
PgR				0.99
Negative	27	8	19	
Positive	2	0	2	
HER2/neu				0.402
Negative	18	2	16	
Positive	11	6	5	
CK5/6				0.99
Negative	22	6	16	
Positive	7	2	5	
Basal type[a]				0.647
No	23	7	16	
Yes	6	1	5	

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; HER2/neu, human epidermal receptor type 2; NA, not applicable

[a] Basal type in present study defined as ER negative, PgR negative, HER2/neu negative, and CK5/6 positive brain metastasis

The frequencies of immunohistochemical change in ER, PgR, and HER2/neu were 12.5% (N = 3/24), 4.2% (N = 1/24), and 12.5% (N = 3/24), respectively (Table 3). The immunohistochemical profiles for ER, PgR, and HER2/neu differed between the primary tumors and the brain metastases in seven patients (29.2%; N = 7/24, see Table 4). With regard to the systemic treatment options, the treatment options for six patients (25%) were changed based on the immunohistochemical profiles of their brain metastases. Among the patients who had been previously treated with trastuzumab, 25% of the patients (N = 2/8) had changed to a HER2/neu-negative status.

Discussion

The present study demonstrated immunohistochemical characteristics for ER, PgR, HER2 receptor, and CK5/6 of

Table 3 Changes in immunohistochemical profiles in estrogen receptor, progesterone receptor or HER2 receptors between primary tumors and brain metastases (n = 24)

Primary tumor	Brain metastasis	N
ER (−)	ER (−)	19
	ER (+)	*2*[a]
ER (+)	ER (−)	*2*
	ER (+)	1
PgR (−)	PgR (−)	22
	PgR (+)	0
PgR (+)	PgR (−)	*1*
	PgR (+)	1
HER2/neu (−)	HER2/neu (−)	14
	HER2/neu (+)	*1*
HER2/neu (+)	HER2/neu (−)	*2*[a]
	HER2/neu (+)	7

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; HER2/neu, human epidermal receptor type 2

Italic number indicated seven patients who had changes of immunohistochemical profiles

[a] One patient with an ER-negative, PgR-negative, HER2-positive primary tumor developed an ER-positive, PgR-negative, HER2/neu-negative brain metastasis

Table 4 Discordance cases of immunohistochemical profiles between primary tumor and brain metastasis (N = 7)

Case no.	ER		PgR		HER2/neu	
	Primary	Brain meta	Primary	Brain meta	Primary	Brain meta
1	+	−	−	−	−	−
2	−	−	+	−	−	−
3	+	−	+	−	−	−
4	−	+	−	−	+	+
5	−	−	−	−	−	+
6	−	−	−	−	+	−
7	−	+	−	−	+	−

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; HER2/neu, human epidermal receptor type 2

brain metastases from breast cancer patients. Although present study had small number of patients and potential selection bias, it is remarkable that the biological characteristics of brain metastasis sometimes changed from the primary tumors.

ER, PgR and HER2/neu immunohistochemical profiles of the primary tumors differed from those of the brain metastases in 29.2% of the patients in the present study; in particular, the HER2/neu status of the two specimens differed in 12.5% of the patients. Generally, marked intratumoral heterogeneity is rare than the degree of heterogeneity (as determined using immunohistochemical analyses and FISH) between primary tumors and their metastases [14–16]. Some previous reports have compared the HER2/neu status of primary tumors and metastases, including axillary lymph node metastasis, local recurrence, distant metastasis, and autopsy findings, but only one study addressed brain metastases. These previous studies, excluding the study that examined brain metastases, described discordance rates of between 0% and 37.5% [14, 16–22]. In a study comparing primary tumors and axillary or metastatic lymph nodes, 0–2% of the patients had a discordance in their HER2/neu statuses [23, 24]. Similar to the results of these studies, discordance has been reported in <5% of primary tumors and their local recurrences [14, 16]. Meanwhile, the rate of discordance in studies examining distant metastasis at various sites was between 6% and 37.5% [17–21]. Among these studies, only two included patients with brain metastasis, but less than five patients were reported and they were not described in detail [17, 18].

Only one study examining patients with brain metastasis reported that 51.5% (N = 17/33) of the patients exhibited the over-expression of HER2/neu (as determined using immunohistochemical analyses) and 25.8% (N = 8/31) exhibited HER2/neu gene amplification. The concordance rate between the immunohistochemical analyses and FISH was 86% when compared with individual metastatic lesions (43 lesions). The concordant rates between the primary tumors and the brain metastases were 58% (N = 7/12) according to the immunohistochemical analyses and 100% (N = 10/10) according to FISH [25]. Although the frequency of discordance between the primary tumors and the brain metastases differed among the studies, probably because of differences in the measurement methods and the small sample sizes, it is important to realize that the brain metastases in some patients have different HER2/neu statuses from those of the primary tumors.

Hormone positivity in the brain metastases was relatively low in this cohort. Hormone receptor discordance in locoregional recurrences or lymph node metastasis may be more frequent than HER2/neu discordance. The rates of discordance have been reported to be 10–25% for ER and 21–44% for PgR between the primary tumor and locoregional recurrence or lymph node metastasis [14, 24, 26, 27]. The rate of discordance between the primary tumor and distant metastasis was similar to that of regional lymph node metastasis or recurrence, which were reported to be 28–42% for ER and 17% for PgR [26, 27]. The discordance rate for hormone receptors in the present study was slightly lower than those reported in previous studies, but whether the hormonal characteristics of brain metastasis differ from those of other distant metastases remains uncertain.

Although the overall frequency of CK5/6 positive brain metastases was low (24.1%, N = 7/29) in present

study, most of patients (71.3%, N = 5/7) were basal type subgroup in patients with CK5/6 positive brain metastases. A previous study revealed that primary tumors which were negative for ER but that expressed basal CK5/6 and overexpressed HER1 or HER2/neu were more likely to develop brain metastasis [28]. The changes in basal type markers between the primary tumors and the brain metastases are uncertain in the present study because CK5/6 staining was not performed in the primary tumors.

There is one report that suggests chemotherapy do not modify the HER2/neu status in metastatic lesions [16]. On the other hand, Regitnig et al. reported that HER2/neu amplification and overexpression may occur de novo in distant metastasis at a late stage of disease [18]. Currently, possible mechanisms of trastuzumab-resistance include the down-regulation of p27, the activation of insulin-like growth factor receptor (IGF-1R), the loss of PTEN, pAkt, interactions between HER family members, the masking of HER2/neu by membrane-associated glycoprotein mucin-4, angiogenesis, and the induction of antibody-dependent cellular toxicity by the immune system [29, 30]. These hypotheses remain controversial, and some studies assessing IGF-1R and p53 in clinical samples have reported negative results [12, 31]. Our study suggests that some patients with HER2/neu negative primary whose brain metastasis were HER2/neu positive responded to trastuzumab therapy after diagnosis of brain metastasis [21]. However, whether the biological changes in the breast cancer cells described in the present study and in previous studies influence the mechanism of resistance to trastuzumab therapy remains unknown.

The present results suggest that all distant metastases should not be assumed to be biologically equal to locoregional metastasis, and that a re-assessment of the immunohistochemical status of the brain metastasis, if possible, may be useful to optimize treatment. Further studies are warranted to address the reason of discordance in the immunohistochemical profiles of the primary tumors and the brain metastases.

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References

1. Lin N, Bellon J, Winer E (2004) CNS metastases in breast cancer. J Clin Oncol 22:3608–3617. doi:10.1200/JCO.2004.01.175
2. Clayton AJ, Danson S, Jolly S et al (2004) Incidence of central metastasis in patients treated with trastuzumab for metastatic breast cancer. Br J Cancer 91:639–643
3. Gabos Z, Sinha R, Hanson J et al (2006) Prognostic significance of human epidermal growth factor receptor positivity for the development of brain metastasis after newly diagnosed breast cancer. J Clin Oncol 24:5658–5663. doi:10.1200/JCO.2006.07.0250
4. Matsumoto K, Shimizu C, Fujiwara Y (2006) The next step to approaching central nervous system metastasis in HER-2-positive metastatic breast patients. Asia Pac J Clin Oncol 2:6–8. doi:10.1111/j.1743-7563.2006.00044.x
5. Slamon DJ, Clark GM, Wong SG et al (1987) Human breast cancer: correlation of relapse and survival with amplification of HER2/neu oncogene. Science 235:177–182. doi:10.1126/science.3798106
6. Slamon DJ, Leyland-Jones B, Shak S et al (2001) Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783–792. doi:10.1056/NEJM200103153441101
7. Piccart-Gebhart MJ, Proctor M, Leyland-Jones B et al (2005) Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med 353:1659–1672. doi:10.1056/NEJMoa052306
8. Pestalozzi BC, Brignoli S (2000) Trastuzumab in CSF. J Clin Oncol 18:2349–2351
9. Cleator S, Heller W, Coombes RC (2007) Triple-negative breast cancer: therapeutic options. Lancet Oncol 8:235–244. doi:10.1016/S1470-2045(07)70074-8
10. Nielsen TO, Hsu FD, Jensen K et al (2004) Immunohistochemical and clinical characterization of the basal like subtype of invasive breast carcinoma. Clin Cancer Res 10:5367–5374. doi:10.1158/1078-0432.CCR-04-0220
11. Tham YL, Sexton K, Kramer R et al (2006) Primary breast cancer phenotypes associated with propensity for central nervous system metastases. Cancer 107:696–704. doi:10.1002/cncr.22041
12. Perren A, Weng L-P, Boag AH et al (1999) Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinoma of the breast. Am J Pathol 155:1254–1260
13. Harvey JM, Clark G, Ml Osborne CK et al (1999) Estrogen receptor status by immunohistochemistry is superior to the ligand-biding assay for predicting response to adjuvant endocrine therapy in breast cancer. J Clin Oncol 17:1474–1481
14. Shimizu C, Fukutomi T, Tsuda H et al (2000) c-erbB-2 Protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. J Surg Oncol 73:17–20. doi:10.1002/(SICI)1096-9098(200001)73:1<17::AID-JSO5>3.0.CO;2-2
15. Pertschuk LP, Axiotis CA, Feldman JG et al (1999) Marked intratumoral heterogeneity of the proto-oncogene Her-2/neu determined by three different detection systems. Breast J 5:369–374. doi:10.1046/j.1524-4741.1999.97088.x
16. Gong Y, Booser DJ, Sneige N (2005) Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. Cancer 103:1763–1769. doi:10.1002/cncr.20987
17. Gancberg D, Di Leo A, Cardoso F, Rouas G, Pedrocchi M, Paesmans M et al (2002) Comparison of HER2-status between primary breast cancer and corresponding distant metastatic sites. Ann Oncol 13:1036–1043. doi:10.1093/annonc/mdf252
18. Regitnig P, Schippinger W, Lindbauer M, Samonigg H, Lax SF (2004) Change of HER2/neu status in a subset of distant metastases from breast carcinomas. J Pathol 203:918–926. doi:10.1002/path.1592
19. Pectasides D, Gaglia A, Arapantoni-Dadioti P, Bobota A, Valavanis C, Kostopoulou V et al (2006) Her2/neu status of primary breast cancer and corresponding metastatic sites in patients with advanced breast cancer treated with trastuzumab-based therapy. Anticancer Res 26:647–654

20. Lower EE, Glass E, Blau R, Harman S (2008) Her-2/neu expression in primary and metastatic breast cancer. Breast Cancer Res Treat. doi:10.1007/s10549-008-9931-6
21. Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C, Hadary A (2005) Comparison of HER2/neu overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 93:552–556. doi:10.1038/sj.bjc.6602738
22. Niehans GA, Singleton TP, Dykoski D, Kiang DT (1993) Stability of HER-2/neu expression over time and at multiple metastatic sites. J Natl Cancer Inst 85:1230–1235. doi:10.1093/jnci/85.15.1230
23. Cardoso F, Di Leo A, Larsimont D, Gancberg D, Rouas G, Dolce S et al (2001) Evaluation of HER2, p53, bcl-2, topoisomerase II-alfa, heat shock proteins 27 and 70 in primary breast cancer and metastatic ipsilateral axillary lymph nodes. Ann Oncol 12:615–620. doi:10.1023/A:1011182524684
24. Tsutsui S, Ohno S, Murakami S, Kataoka A, Kinoshita J, Hachitanda Y (2002) EGFR, c-erbB2, and p53 protein in the primary lesions and paired metastatic regional lymph nodes in breast cancer. EJSO 28:383–387. doi:10.1053/ejso.2002.1259
25. Lear-Kaul KC, Yoon HR, Kleinschmidt-DeMasters BK et al (2003) HER2/neu status in breast cancer metastases to the central nervous system. Arch Pathol Lab Med 127:1451–1457
26. Kamby C, Rasmussen BB, Kristensen B (1989) Oestrogen receptor status of primary breast carcinomas and their metastases. Relation to pattern of spread and survival after recurrence. Br J Cancer 60:252–257
27. Li BD, Byskosh A, Molteni A, Duda RB (1994) Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. J Surg Oncol 57:71–77. doi:10.1002/jso.2930570202
28. Hicks DG, Short SM, Prescott NL et al (2006) Breast cancers with brain metastases are more likely to be estrogen receptor negative, express the basal cytokeratin CK5/6, and overexpress HER2 or EGFR. Am J Surg Pathol 30:1097–1104. doi:10.1097/01.pas.0000213306.05811.b9
29. Mohsin SK, Weiss HL, Gonzarrez MC et al (2005) Neoadjuvant trastuzumab induces apoptosis in primary breast cancers. J Clin Oncol 23:2460–2468. doi:10.1200/JCO.2005.00.661
30. Nagata Y, Lan LH, Zhou X et al (2004) PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell 6:117–127. doi:10.1016/j.ccr.2004.06.022
31. Nahta R, Yu D, Hung MC et al (2006) Mechanisms of disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat Clin Pract Oncol 3:269–280. doi:10.1038/ncponc0509

Image of the Month

Three Cases of Sub-scalp Tumor Presenting with Protrusion of the Head

Case 1 was an 81-year-old male who underwent right nephrectomy for renal cell carcinoma 5 years ago. When he fell down and hit his back of the head, he noticed an occipital lump (Fig. 1). The bulging was growing gradually within several months. He underwent an excisional biopsy and pathological findings showed a metastatic tumor from renal cell carcinoma. Irradiation was administered. Case 2 was a 58-year-old female who suffered head trauma 3 years ago. She noticed a protrusion on her parietal cranium while grooming her own hair (Fig. 2). She received a needle biopsy and the pathological diagnosis was meningioma. She underwent surgical resection of the tumor and cranioplastic surgery. Case 3 was an 81-year-old male who had tingling-numb on his forehead for 6 months. Because of the persistent dysesthesia accompanied by progressive swelling (Fig. 3), he consulted our hospital and was operated on for histological confirmation. Obtained tissues were composed of poorly differentiated carcinoma. He subsequently received irradiation on the frontal lesion.

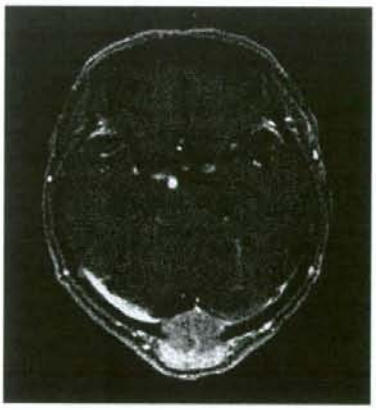

Figure 1.

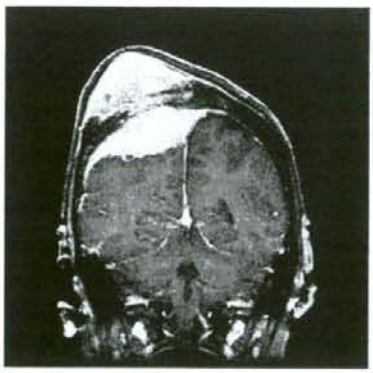

Figure 2.

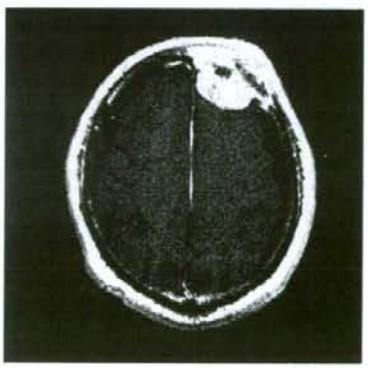

Figure 3.

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悪性神経膠腫に対する長期temozolomide投与例の検討

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"How Long Should We Give Temozolomide for Malignant Glioma Patients?" Long Time Follow Up of Malignant Glioma Patients Who Had Temozolomide for 24 Cycles.

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More than 100 patients with malignant glioma were given temozolomide (TMZ) in our hospital from December 2003. Eight patients with recurrent malignant gliomas (2 glioblastoma (GBM), 3 anaplastic astrocytoma (AA), 1 anaplastic oligodendroglioma (AO), 2 diffuse astrocytoma (DA)) have survived more than two years since they started TMZ. 7 patients had 24 cycles of TMZ for 24 months but they did not have any side effect. Six patients (2 GBM, 3 AA, 1 DA) do not have a new lesion even after they stopped TMZ. TMZ became a standard therapy for malignant glioma patients, however, the duration and quantity of TMZ for them are often discussed. We report these 8 cases with malignant gliomas and discuss the safety, efficacy and problems of the patients who had TMZ for a long time.

Key Words: glioma, glioblastoma, temozolomide, long term follow-up

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Ⅰ. はじめに

Temozolomide (TMZ) は2006年9月より国内でも発売になり，悪性神経膠腫に対する標準薬として広く使用され，優れた効果が認められている．

当院では2003年12月から，治験および個人輸入により，再発悪性神経膠腫に対するTMZ療法を開始した．2007年12月の時点で初発・再発悪性神経膠腫に対してTMZを投与した症例が100例となったが，TMZによりCR (complete response) を得て，TMZの投与を2年間行い経過観察している症例も経験している．TMZ投与後，画像上変化の見られない症例も多数あり，いつまでTMZを投与するかがしばしば問題となる．これまでTMZ開始後2年間経過した症例は8例となったが，これらの症例について報告し，TMZ長期投与の安全性と効果について検討を行った (表)．

Ⅱ. 症例報告

症例1 (Fig.1)

59歳，男性．2003年10月にけいれん発作・失語にて発症．左側頭葉の腫瘍生検術を行い，診断はanaplastic astrocytoma (AA) grade Ⅲ，MIB-1 index 26%，MGMT met (+)，ACNU (ニドラン®) による放射線化学療法 (局所照射60Gy) および維持療法を行ったが，2004年4月のMRIで腫瘍の増大を認め，失語も悪化した．

TMZを150mg/m^{2}で開始し，ほぼ4週間おきに

Table The summary of malignant glioma patients who have survived more than two years since they started TMZ

Case	Age	Initial diagnosis	Time to start TMZ	Number of TMZ	Effect of TMZ	Outcome after stopping TMZ	OS
1	59M	Lt. temporal AA	4 mo	24 x/24 mo	CR	CR (22 mo)	50 mo
2	57M	Rt. thalamic AA	4 mo	24 x/24 mo	CR	CR (20 mo)	48 mo
3	43F	Rt. frontal AA	20 mo	23 x/24 mo	SD	SD (24 mo)	68 mo
4	67F	Rt. frontal GBM	4 mo	24 x/24 mo	CR	CR (4 mo)	32 mo
5	64M	Lt. fronto-temporal GBM	5 mo	17 x/15 mo	SD	SD (8 mo)	28 mo
6	52F	Lt. frontal AO	26 mo	25 x/24 mo	Rec. after CR	Rec. 6 mo after stopping TMZ	63 mo
7	50M	Pontine DA 再発	6 mo	24 x/24 mo	Rec. after PR	Rec. just after stopping TMZ	34 mo
8	58M	Lt. temporal DA 再発	5 mo	24 x/24 mo	PR	PR (1 mo)	30 mo

200mg/m^{2}で治療した．徐々に腫瘍は縮小して失語も改善し，TMZ開始12サイクル（1年間）後に画像上CRとなった．TMZはさらに1年間継続し，2006年3月まで24サイクル（2年間）の投与を行ったが有害事象はまったく見られなかった．TMZを終了して約1年8ヵ月たったが再発はなく，初期治療より4年2ヵ月が経過し，KPS 80を維持している．

症例2（Fig.2）

57歳，男性．2003年12月に頭痛で発症．右視床腫瘍に対して第三脳室より内視鏡的生検術を行い，診断はAA grade Ⅲ．初期治療としてACNUによる放射線化学療法（局所照射60Gy）および維持療法を施行したが，2004年3月のMRIで腫瘍の増大を認めた．

2004年5月よりTMZを150または200mg/m^{2}で投与し，TMZ開始5サイクル目で画像上CRとなった．2006年2月まで24サイクル（2年間）のTMZを投与したが，grade Ⅰを含む有害事象はまったく見られなかった．けいれん等も見られず，KPSは100で職場復帰もしている．TMZ終了後1年10ヵ月たったが再発は認めず，初期治療から4年が経過している．

症例3（Fig.3）

43歳，女性．2002年3月にけいれん発作にて発症．右前頭葉腫瘍摘出術を行い，診断はAA grade Ⅲ．ACNUによる放射線化学療法（局所照射60Gy）およ

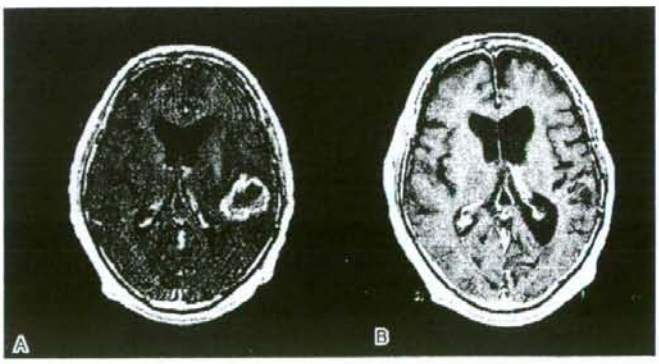

Fig.1 59M, AA grade Ⅲ. A, B: MRI before (A) and 3 years after (B) TMZ was given for a patient.

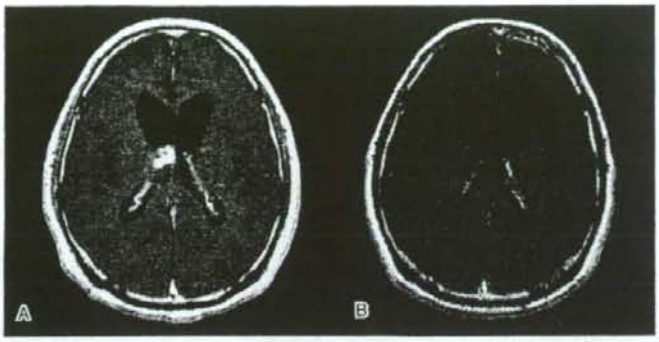

Fig.2 57M. AA grade Ⅲ. A: MRI at recurrence. B: 6 cycles of TMZ distinguished a tumor. No tumor was seen for 3 years.

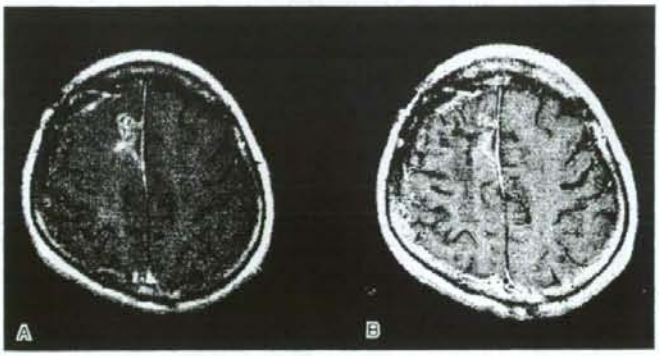

Fig.3 43F. AA grade Ⅲ. MRI before (A) and 4 years after (B) TMZ was given for a patient. No tumor progression was seen.

び維持療法を行ったが，2003年11月に左片麻痺の悪化を認め局所再発となった．

その後TMZを150または200mg/m^{2}で投与し，2005年11月まで23サイクル（2年間）のTMZを投与した．grade Ⅲ/Ⅳの有害事象は見られなかったが，TMZ内服中は食欲低下・全身倦怠感をしばしば訴えた．画像上はTMZ開始時と比べ造影病変の縮小を認めたがSD（stable disease）の範囲であった．TMZ投与は2年間で終了したが，造影病変の拡大はない．TMZ終了後2年1ヵ月，初期治療より5年10ヵ月が経過し，KPSは70で独歩可能な状態である．

症例4（Fig.4）

67歳，女性．2005年5月にけいれん発作にて発症．右前頭葉腫瘍摘出術を行い，診断はglioblastoma(GBM)．MIB-1 index 20%，MGMT met（+）．ACNUによる放射線化学療法（局所照射60Gy）を施行したが，2005年9月に局所再発となり再手術を行った．

その後TMZを150または200mg/m^{2}で投与し2006年8月まで24サイクル（2年間）のTMZを投与した．TMZ開始1年後にgrade Ⅲのリンパ球減少を認めたため，ニューモシスチス肺炎予防のためにST合剤（バクタ®）を併用した．他のgrade Ⅲ/Ⅳの有害事象は見られなかったが，TMZ内服中は食欲低下・全身倦怠感をしばしば訴えた．TMZ終了後4ヵ月経過して，リンパ球減少も回復し，再発も認められない．初期治療より2年8ヵ月となり，独歩可能で

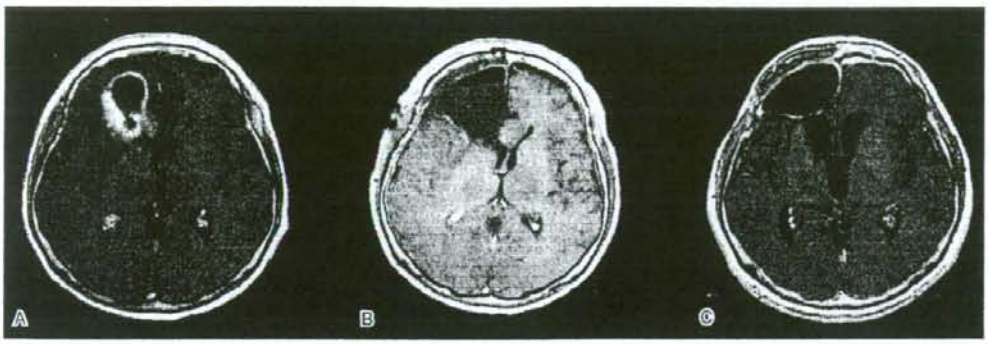

Fig.4 67F, GBM. A: MRI at recurrence. B: MRI at tumor removal. C: 24 cycles of TMZ was given for a patient and no new lesion was seen.

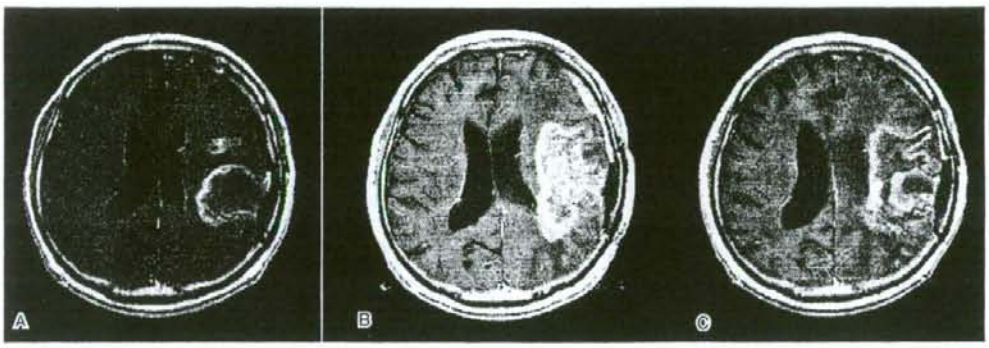

Fig.5 64M, GBM. A: MRI at recurrence. B: A tumor gradually increased until a patient had 6 cycles of TMZ. C: The tumor did not increase 8 months after he had 17 cycles of TMZ and stopped TMZ.

KPSは70を維持している.

症例5 (Fig.5)

64歳, 男性. 2005年8月に失語で発症し, 他院で左側前頭頭葉腫瘍生検術を行い, 診断はGBM. ACNUによる放射線化学療法 (局所照射60Gy) および維持療法を施行したが, 2005年12月に腫瘍の増大を認め, 当院へ転院となった.

TMZを開始し, 投与6ヵ月までに造影病変は漸増したが, その後大きさに変化がなくTMZを200mg/m^{2}で維持した. 経過中, 失語・右麻痺が悪化したため, 家族と相談のうえ2007年3月にTMZを17サイクル (1年3ヵ月間) 投与したところでTMZを中止した. その後も神経学的には変化なく, 在宅療養を行っている. 2007年11月にTMZ中止8ヵ月を経過した時点のMRI上も腫瘍の増大は認められていない.

症例6 (Fig.6)

52歳, 女性. 2002年10月に頭痛・左片麻痺で発症し, 右前頭葉腫瘍摘出術. 診断はanaplastic oligodendroglioma (AO) grade Ⅲ. ACNU + VCR (Vincristine) による放射線化学療法 (局所照射60Gy) を施行し,

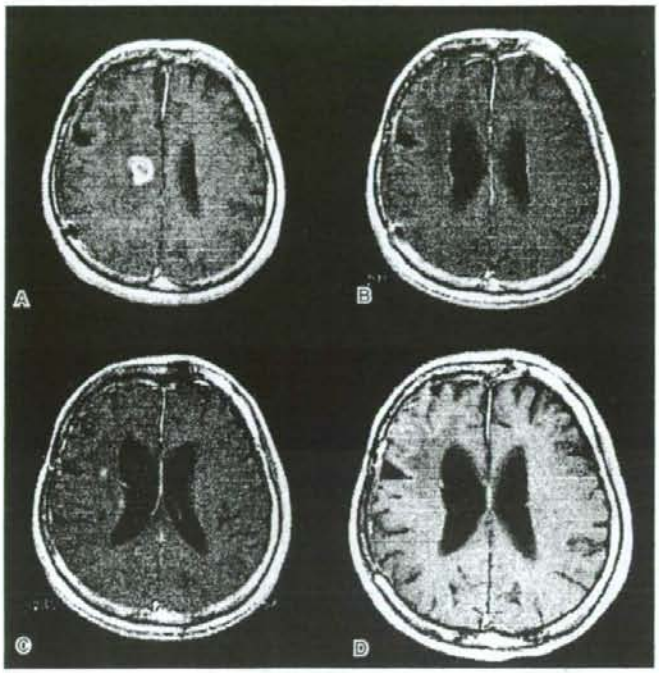

Fig.6 52F. AO grade Ⅲ. A: MRI at recurrence. B: A tumor disappeared after 24 cycles of TMZ was given to a patient. C: A new lesion appeared within T2 high intensity area 6 months after she stopped TMZ. D: CR was obtained after 6 cycles of TMZ.

PAV (Procarbazine, ACNU, VCR) 療法を2ヵ月おきに行った. Grade Ⅳの骨髄抑制により輸血やG-CSFを投与しながら2003年12月までPAV療法を6回行った.

2005年1月に腫瘍の再発を認め, CBDCA+VP-16 (Carboplatin+Etoposide) を投与したが, grade Ⅳの骨髄抑制を認め, さらに腫瘍が大きくなったため, 2005年2月よりTMZを開始した. 2006年7月, TMZ開始1年半後に造影病変はほぼ消失し, 2007年1月までTMZを24サイクル (2年間) 投与し治療を終了した. TMZ投与中, grade Ⅲ/Ⅳの有害事象は見られなかったが, TMZ内服中は食欲低下・悪心・全身倦怠感をしばしば訴えた. しかし2007年7月, TMZ投与終了6ヵ月後にT2高信号域内に再び造影病変出現し再々発の診断. 再びTMZを開始した. TMZを6サイクルまで投与し, 再々発病変は縮小し現在もTMZ継続中.

症例7 (Fig.7)

50歳, 男性. 2005年2月に歩行障害で発症し, 右背側部腫瘍生検術施行し, 診断はdiffuse astrocytoma (DA) grade Ⅱ, MIB-1 index 4.1%. ACNUによる放射線化学療法 (局所照射56Gy) および維持療法を施行した.

2005年8月に局所再発となり, その後TMZを150/200mg/m^{2}で投与し腫瘍は縮小した. 2006年8月まで24サイクル (2年間) の投与を行ったところで, 左脳室に新規病変を認めた.

症例8 (Fig.8)

58歳, 男性. 2005年9月にけいれんで発症し, 左側頭葉腫瘍生検術施行し, 診断はDA grade Ⅱ.

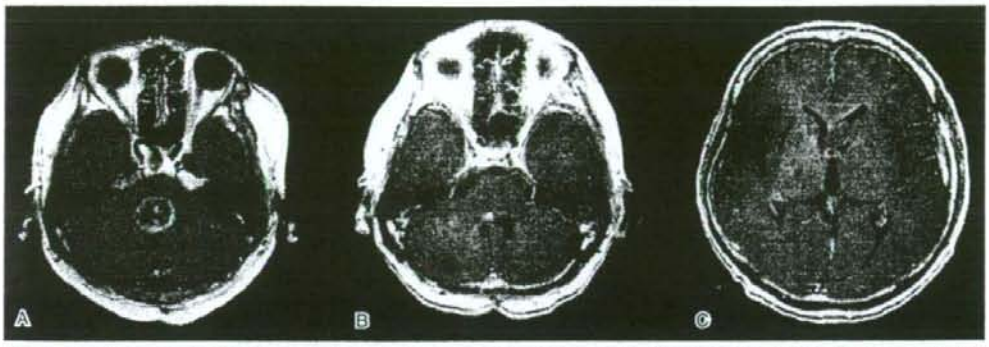

Fig.7 50M, DA grade 2. A: MRI at recurrence. B: A pontine lesion decreased after 24 cycles of TMZ. C: However, a new lesion appeared in the anterior horn of left lateral ventricle.

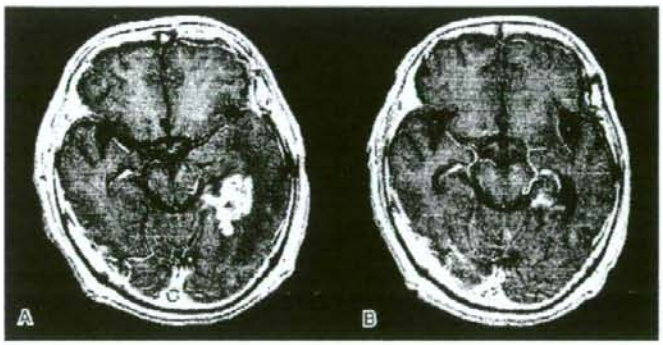

Fig.8 58M, DA grade 2. A: MRI at recurrence. B: 24 cycles of TMZ decreased the tumor markedly.

MIB-1 index 0.6%. MGMT met (−). ACNUによる放射線化学療法（局所照射54Gy）および維持療法を施行した.

2006年2月に局所再発となり，その後TMZを150/200mg/m^{2}を投与し，2008年1月まで24サイクル（2年間）の投与を行い，腫瘍は著明に縮小した．有害事象は認められず，2年間28日おきにTMZを投与した．今後残存病変はあるが経過観察とする.

Ⅲ. 結果・考察

EORTC/NCICの治療プロトコールに代表されるように[1]，欧米では悪性神経膠腫に対してTMZによる放射線化学療法併用後に，維持療法としてTMZを6サイクル（6ヵ月間）投与することが標準治療となっている．しかし実際には欧米でもTMZを6サイクル投与した後も，効果のある症例に対しては患者と相談しながらTMZを継続していることが多いようである.

TMZはこれまで用いられてきた抗悪性腫瘍薬よりもはるかに有害事象が少ないものの，後述するように二次性発癌などの重篤な副作用も報告されていること．またTMZ投与中はgrade Ⅰ / Ⅱ程度であっても悪心・全身倦怠感などを訴える患者も少なくないことから，われわれはGBMの生存期間中央値（MST：

median survival time）が1年半，AAのMSTが2年半程度であることを考え，CR/PR/SDの症例はTMZ開始2年を経過した時点で一度投与を終了し，少なくとも2ヵ月おきのMRIを撮りながら，経過観察することとしている．

今回提示した8例中4例（AA 3例，GBM 1例，AO 1例）はCRが得られたが，AOの症例6は6ヵ月後に再発し，TMZを再投与し，再々発病変は縮小した．残存病変がある4例中，AAの症例3はTMZ終了後2年を経過しても腫瘍の増大を認めていない．症例5は，広範に腫瘍が残存しているにもかかわらず，TMZ投与中に腫瘍は漸増したが，その後変化を認めなかった．ただし右片麻痺・失語によるPSが悪いため，TMZ投与17回（1年3ヵ月）で投与を中止したが，これまでのところ腫瘍の大きさに変化はない．延髄DA再発例の症例7はTMZ開始後ちょうど2年目に遠隔部再発をきたしている．症例8は24サイクル目が終了したところで，今後経過観察予定である．

症例3と5のように，TMZを中止しても腫瘍の大きさに変化が認められない例があることから，一定期間TMZを投与して経過観察可能な症例も今後多数増えてくると考えられる．実際TMZを一旦終了して腫瘍が再発増大しても，また同じ治療プロトコールを用いて腫瘍が縮小することも報告されている[3, 7]．

TMZによる治療後再発した場合，われわれはTMZの効果を上げるためにPCZを併用したり，CB-DCA + VP-16による化学療法を行っているが，現在その効果を検証中である．通常の5日間投与/28日サイクルで投与した症例が再発した場合，TMZの投与量を増やして，1週間おきにTMZを投与する方法（one week on/off），3週間の継続投与・1週間休薬（3 weeks on/one week off），あるいは連日継続投与などの効果が報告されており[7, 10]，今後日本でも臨床試験を行っていく必要がある．

Hau等はTMZを開始し1年以上経過した128例の悪性神経膠腫についてその安全性を検証した[2]．初期放射線化学療法を含むTMZの平均投与回数は13サイクル（9～40サイクル）であった．Grade Ⅲ/Ⅳ以上の血小板減少が10%，白血球減少が7%，消化器症状が5%，感染症が4%と報告し，TMZ投与の長期投与は安全であると報告している．Stupp等の膠芽腫に対する初期治療を含む6サイクルまでのTMZ投与例の有害事象の報告は，好中球減少が4%で，血小板減少が3%であったが[8]，TMZの長期投与により骨髄抑制の頻度が高くなると考えられる．

今回提示した症例では，症例5を除く7例のTMZを2年間投与した症例中，1例でgrade Ⅲのリンパ球減少を認めたのみで，他のgrade Ⅲ/Ⅳの有害事象は認められなかった．

われわれはgrade Ⅲ以上のリンパ球減少に対しては，週2回のST合剤（バクタ®）を投与しニューモシスチス肺炎を予防している[5]．TMZによる嘔吐は，制吐剤（セロトニン拮抗薬）を併用することにより抑えることが可能であったが，8例中3例は，TMZ服用期間はgrade Ⅱ程度の食欲低下・悪心・全身倦怠感をしばしば訴えており，TMZによる有害事象がまったく見られないわけではなかった．便秘に対しては投与前より酸化マグネシウムなどの緩下剤を使用することにより予防することができた[4]．

TMZを含むアルキル化剤（ACNU，BCNU，CCNU，PCZ）により，MDS/AML（melodysplastic syndrome/acute myeloid leukemia）などの二次性発癌リスクが高まることが知られており，注意が必要である．Su等はAA症例にACNU/Interferon投与し，再発後にTMZ開始4ヵ月目にMDSを発症した例を報告している[6, 9]．実際われわれもanaplastic oligoastrocyotmaの再発例で，PAV療法4サイクル・TMZ 6サイクル・PCZ + TMZ 4サイクル後にMDS/AMLを発

症した症例を経験した．

ACNU や PCZ などのアルキル化剤は，無月経や無精子症などの性腺機能障害を高率に発症することが知られており[1]，今後 TMZ を長期投与していくうえで注意を要する．

Ⅳ．結　語

再発悪性神経膠腫に対して TMZ を開始し，2 年以上経過した 8 症例について報告したが，これらの長期投与可能例では重篤な有害事象は認められなかった．今後 TMZ の長期投与例を経験するにつれて，MDS/AML などの 2 次性発癌例や重篤な骨髄抑制 などの報告も増えると考えられる．CR が得られた症例では，TMZ 投与を終了して経過観察していくことも検討する必要がある．

一方，残存病変がある症例では，悪性神経膠腫の MST が 2 年半未満であり，われわれの治療方針のように少なくとも 2 年間は TMZ を継続投与する必要があると考える．一定期間の服用後に残存病変が画像上変化しない場合，服用中の悪心や全身倦怠感，経済的負担を考慮して，TMZ を終了し経過を観察していくことも可能と考えられる．

2006 年 9 月に国内で TMZ が発売されて，初期放射線治療から TMZ を使えるようになってまだ 1 年あまりであり，今後 CR になった症例を集積して TMZ の投与期間について検討していく必要がある．

文　献

1) Clayton PE, Shalet SM, Price DA, et al: Ovarian function following chemotherapy for childhood brain tumours. Med Pediatr Oncol 17: 92-96, 1989.
2) Hau P, Koch D, Hundsberger T, et al: Safety and feasibility of long-term temozolomide treatment in patients with high grade glioma. Neurology 68: 688-690, 2007.
3) Jauch T, Hau P, Bogdahn U: Re-challenge with temozolomide (TMZ) at recurrence in high-grade gliomas (HGG). J Clin Oncol, ASCO Meeting Abstracts 25: 2034, 2007.
4) 成田善孝：脳腫瘍に対する抗腫瘍薬の基礎．脳外速報 16: 642-648, 2006.
5) 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．Clinical Neuro Science 25: 1242-1248, 2007.
6) Noronha V, Berliner N, Ballen KK, et al: Treatment-related myelodysplasia/AML in a patient with a history of breast cancer and an oligodendroglioma treated with temozolomide: case study and review of the literature. Neuro Oncol 8: 280-283, 2006.
7) Strik HM, Buhk JH, Bock HC, et al: Rechallenge of malignant gliomas with temozolomide: Can it be effective?. J Clin Oncol, ASCO Meeting Abstracts 25: 2072, 2007.
8) Stupp R, Mason WP, van den Bent MJ, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
9) Su YW, Chang MC, Chiang MF, et al: Treatment-related myelodysplastic syndrome after temozolomide for recurrent high-grade glioma. J Neurooncol 71: 315-318, 2005.
10) Wick A, Felsberg J, Steinbach JP, et al: Efficacy and tolerability of temozolomide in an alternating weekly regimen in patients with recurrent glioma. J Clin Oncol 25: 3357-3361, 2007.