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CLINICAL ARTICLE

Development of intratumoral cyst or extratumoral arachnoid cyst in intracranial schwannomas following gamma knife radiosurgery

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Abstract
Purpose Intracranial schwannomas presenting with cyst formation following gamma knife radiosurgery (GKRS) were investigated to clarify their clinicopathological characteristics.
Methods Between 1994 and 2006, 492 patients presenting with intracranial schwannomas underwent GKRS. Among them, seven cases demonstrated either new formation of cysts or enlargement of preexisting cysts, which were treated with microsurgical intervention. These cases were retrospectively reviewed with regard to neuroradiological findings and histopathology.
Results These seven cases included five vestibular and two trigeminal schwannomas. Preexisting cysts were enlarged following GKRS in three cases, while they were newly formed in four cases. Salvage microsurgery was carried out at 7–167 months after the GKRS, and subtotal resection was achieved in three, partial resection with or without cyst fenestration in four. Neurological symptoms were improved in all six symptomatic cases. Preoperative MRI demonstrated two characteristic types of cyst. One was the intratumoral type, indicating hemorrhagic change on the MRI. Histopathological analysis demonstrated a cavernous angioma within the solid compartment of tumor. These two cases demonstrated enlargement of residual tumor with new cyst formation after resection of only the cyst. The other type was extratumoral cyst, which had a structure with a thin cyst wall without contrast enhancement, and the cyst was composed of arachnoid cells without tumor cells. Extratumoral cysts enlarged despite effective control of the tumor itself, which may be caused by osmotic gradient induced by tumor degeneration following GKRS.
Conclusions There were two types of cysts, intratumoral cyst and extratumoral arachnoid cyst, which developed following GKRS in intracranial schwannomas. Resection of the solid compartment as well as the cyst is required in schwannomas with expanding intratumoral cyst. Conversely, fenestration of the cyst alone might be effective in extratumoral arachnoid cysts.

Keywords Intracranial schwannoma · Gamma knife radiosurgery · Cyst formation · Cavernous angioma

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Introduction

Schwannomas are common benign intracranial tumors that can be cured by complete resection. Stereotactic radiosurgery, including gamma knife radiosurgery (GKRS), has achieved long-term tumor control with a low morbidity rate, and is an important treatment modality for intracranial schwannomas as an alternative to microsurgical resection. However, although it is rare, microsurgical salvage is sometimes required for the tumors demonstrating continuous expansion of the tumor after failed GKRS [10, 22, 25].

Recently delayed cyst formation following GKRS has been reported in a variety of intracranial lesions, including

arteriovenous malformations, meningiomas, brain metastases, and schwannomas [9, 12, 19, 24]. Intracranial schwannomas, vestibular schwannomas in particular, are one of the types of benign tumors most commonly treated by GKRS, but reports regarding cyst formation associated with GKRS are still rare.

The present study was conducted to analyze the intracranial schwannomas developing cysts after GKRS to address optimal treatments based on clinicopathological characteristics.

Material and methods

Between 1994 and 2006, 492 intracranial schwannomas, including 449 vestibular, 41 trigeminal, and two other schwannomas, were treated by GKRS at Furukawa-Seiryo Hospital. Among them, 11 cases (2.2%), including eight vestibular, two trigeminal schwannomas, and one lower cranial nerve schwannoma, required craniotomy because of neurological deterioration due to continuous tumor enlargement. Seven of these 11 cases demonstrated cyst development, such as formation of new cysts or enlargement of preexisting cysts on serial follow-up MRI, rather than expansion of solid compartment, and underwent microsurgical salvage because of continuous enlargement of cysts and neurological deterioration. The seven cases included five vestibular and two trigeminal schwannomas (Table 1). We retrospectively reviewed patient records, neuroimaging studies, and histopathology of the tumors.

Results

Patient background, gamma knife protocol, and clinical courses

The MRI at the time of GKRS was reviewed in 316 cases, including 291 vestibular, 19 trigeminal, and six other schwannomas, who had undergone MRI follow-up once or more after GKRS. Among 316 cases, tumors were cystic in 62 (19.6%), including 56 vestibular (19.2%), four trigeminal (21.1%), and two other schwannomas (33.3%). All but one (case 6) demonstrated intratumoral cysts.

In seven of 492 intracranial schwannomas (1.4%), microsurgical salvage was required for cyst development following GKRS. The patients consisted of three females and four males, and their ages ranged from 25–78 years old at the time of salvage surgery (mean age: 45.7 years old) (Table 1). In three of seven cases, cystic compartments were demonstrated on MRI at GKRS. GKRS was performed as the primary treatment in four, and adjunctive to incomplete resection in three patients. The target volume of GKRS was from 3.2 to 19.0 ml, and the marginal dose was 12.0 Gy on average. The cysts preexisting at GKRS were covered by radiation field in case 3 and 5, but not in case 6, which showed extratumoral cyst.

Duration from GKRS to salvage microsurgery widely ranged from 7–167 months (Table 1). The delay of failure in other four intracranial schwannomas without cyst development ranged from 20–69 months, and there was no difference in the delay of failure between intracranial schwannomas with cyst development and those without cyst development. Five cases demonstrated cyst development within 4 years after GKRS, causing neurological deterioration, while two patients showed cyst formation after tumor control longer than 10 years. Neurological symptoms progressively deteriorated as the cysts developed within 1 year after the cysts were identified on follow-up MRI. Cranial nerve symptoms, including those of trigeminal, abducens, facial, and vestibulocochlear nerves, progressed, and two patients showed long-tract signs, such as contralateral motor hemiparesis or sensory disturbance due to compression of the brainstem (Table 1).

Characteristic MRI findings of intratumoral cyst and extratumoral arachnoid cyst

The cysts were preexisting at GKRS in three cases, but newly formed in four cases (Table 1). The cysts developed within the target area of GKRS in six patients, with the exception of an extratumoral cyst in case 6. The size of five vestibular schwannomas was categorized into Koos class IV. Two trigeminal schwannomas also displaced the brain stem.

According to MRI findings, cysts were categorized into two types, intratumoral cysts and extratumoral cysts (Table 1). Five cases showed intratumoral cysts, which were composed of a thick wall with contrast enhancement (Figs. 1a and 2a). This type of cyst develops within the target area of GKRS, and demonstrated intratumoral hemorrhage in four of five cases. A fluid–fluid level was exhibited within the cysts in cases 3 and 5 (Fig. 1b). T2*-weighted images demonstrated low-intensity spots in the solid compartment adjacent to the cysts in cases 1 and 4 (Fig. 2b and c). On the other hand, two patients, cases 6 and 7, demonstrated extratumoral cysts, which had epicenter between the tumor and brain (Fig. 3a and b). These cysts were composed of a thin membrane, which was not enhanced by contrast medium. The content of cysts indicated higher signal intensity than cerebrospinal fluid on T2-weighted images.

Salvage microsurgery for schwannoma developing cysts after GKRS and outcomes

The extent of resection was a subtotal resection in three cases and a partial resection in four cases (Table 1). Intratumoral cysts contained hemorrhage or necrotic debris

Table 1 Summary of seven intracranial schwannomas developing cysts after GKRS

No.	Age, sex	Origin of schwannoma/ preexisted cyst	Preceding surgery	GKRS protocol		Neurological status		Duration between GKRS and salvage surgery (month)	MRI findings				Pathologic findings	Extent of resection	Follow-up period (month)/ outcome	Additional treatment / final outcome
				Target Volume (ml)	Marginal dose (Gy)	At GKRS	At salvage surgery		Cyst distribution	Cyst wall	Cyst content on T2	Solid component on T2*				
1	39, F	VIII/–	–	19.0	11.3	VIII	VIII	167	Intratumoral	Enhanced		Low SI spots	CA	PR	12/prog	STR/no rec for 12 months
2	78, M	VIII/–	–	4.8	12.0	VIII	V*, VIII	143	Intratumoral	Enhanced		NA		STR	36/no rec	–
3	37, M	VIII/–	–	3.2	12.0	VIII	V*, VIII, trunk ataxia*	10	Intratumoral	Enhanced	FFL	NA		STR	60/no rec	–
4	33, F	V/–	–	5.3	12.0	V	V, VI* cont sens dist*	24	Intratumoral	Enhanced		Low SI spots	CA	PR	12/prog	STR/no rec for 12 months
5	25, F	V/–	–	13.0	12.0	V	V, VII*, trunk ataxia*;	7	Intratumoral	Enhanced	FFL	NA		STR	48/no rec	–
6	49, M	VIII/–	–	13.0	12.0	VIII	VIII, cont hemiparesis & sens dist*	13	Extratumoral	Not enhanced	Higher SI	NA	AC	PR w/ cyst fenestration	36/stable	–
7	59, M	VIII/–	–	16.7	12.0	VIII	VIII, trunk ataxia*	44	Extratumoral	Not enhanced	Higher SI	–	AC	PR w/ cyst fenestration	107/stable	–

M male, *F* female, *GKRS* gamma knife radiosurgery, *cont* contralateral, *sens dist* sensory disturbance, * symptoms improved by salvage surgery, *FFL* fluid-fluid level, *NA* not assessed, *SI* signal intensity, *CA* cavernous angioma, *AC* arachnoid cyst, *PR* partial resection, *STR* subtotal resection, *prog* progression, *rec* recurrence

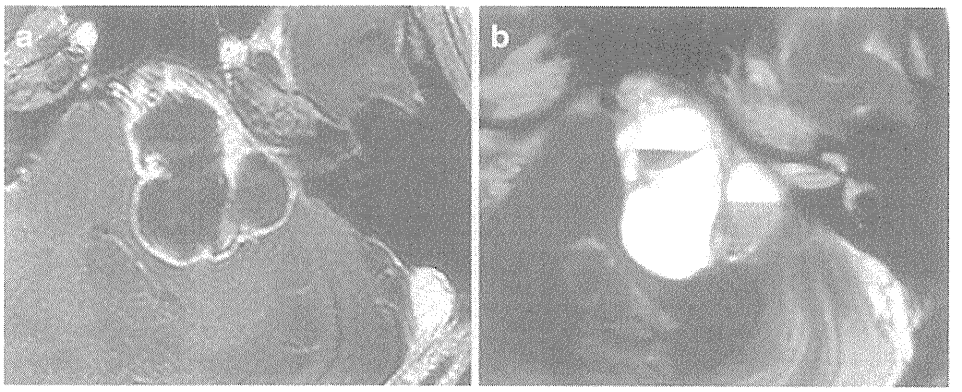

Fig. 1 Intratumoral cysts developed following GKRS, presenting with hemorrhage within the cysts in case 5. The trigeminal schwannoma, which underwent GKRS after partial removal, presented expansion of cysts following GKRS. MRI at the salvage surgery indicated enlarged cysts, which were composed of thick walls with contrast enhancement (a), and contained a fluid–fluid level (b)

in all five cases. Extratumoral cyst was composed of thin and semitransparent membrane, and included xanthochromic fluid.

Neurological status was improved immediately after the surgery. Postoperative courses were followed-up from 24 to 107 months after the salvage microsurgery (Table 1). Two patients who underwent subtotal resection demonstrated no recurrence. Cases 1 and 4, in whom only the cysts were removed, demonstrated enlargement of residual tumor with new cyst formation. The residual tumors were subtotally removed, and have not recurred. In two cases demonstrating extratumoral cyst, fenestration of the cyst with partial resection effectively improved neurological symptoms, and the solid residual tumors have been well controlled during the follow-up period.

Histopathological analysis

The tumor tissue demonstrated non-specific histology for post-GKRS. In two cases presenting with intratumoral cysts

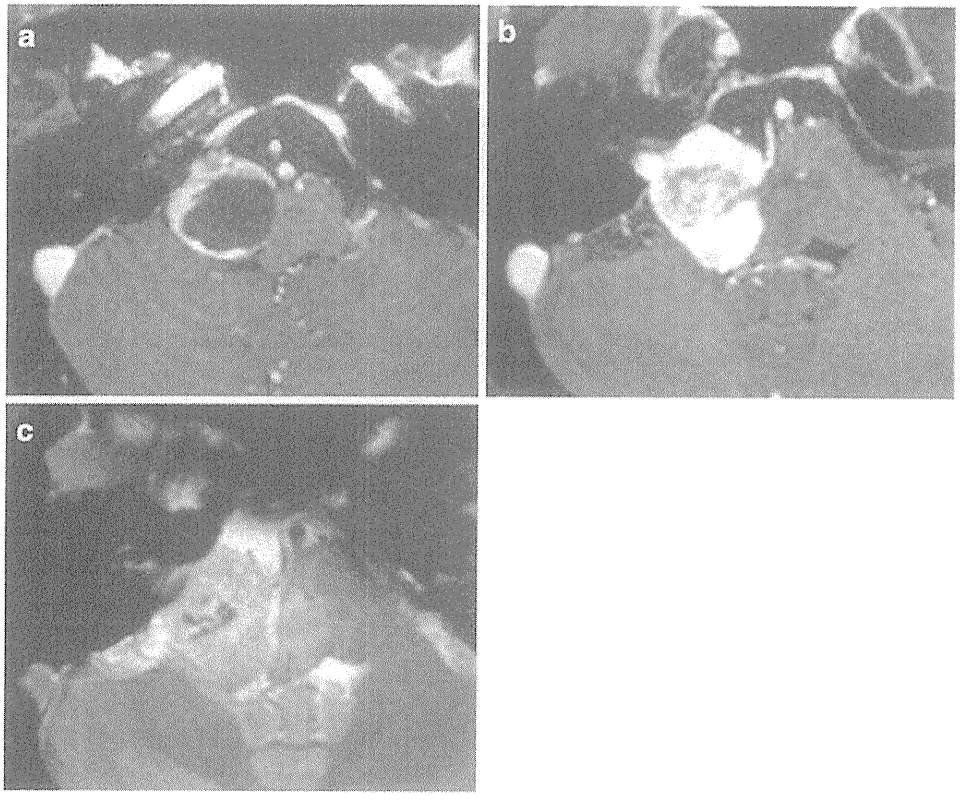

Fig. 2 Intratumoral hemorrhage was demonstrated within the solid compartment adjacent to the developed cyst in case 1. A solid vestibular schwannoma demonstrated cyst formation 13 years after GKRS, although the solid compartment was controlled effectively (a and b). T2-weighted imagery showed low-intensity signal within the solid compartment, suggesting intratumoral hemorrhage (c)

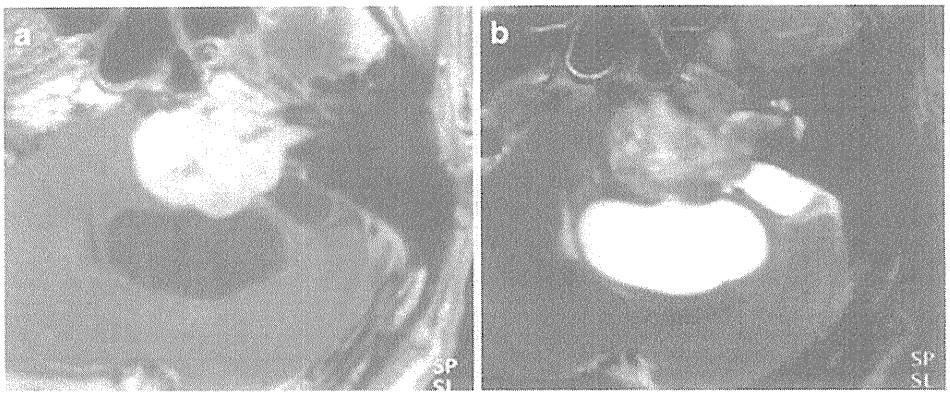

Fig. 3 Delayed formation of extratumoral arachnoid cyst following GKRS in case 7. A solid vestibular schwannoma gradually developed extratumoral cyst after GKRS despite effective control of the solid compartment. The cyst was located between the tumor and cerebellar peduncle, lacking thick enhanced cyst wall (**a**). The cyst content revealed higher signal intensity than cerebrospinal fluid on T2-weighted image (**b**)

(cases 1 and 4), cavernous angioma was identified within the solid compartment of the tumor (Fig. 4a). Thin-walled vascular channels lacking smooth muscle and internal elastic lamina were observed with intratumoral hemorrhage and hemosiderin deposition. On the other hand, extratumoral cysts had a structure with arachnoid membrane but without tumor cells (Fig. 4b). Considering the operative findings together, these were arachnoid cysts associated with schwannomas, which were enlarged after GKRS.

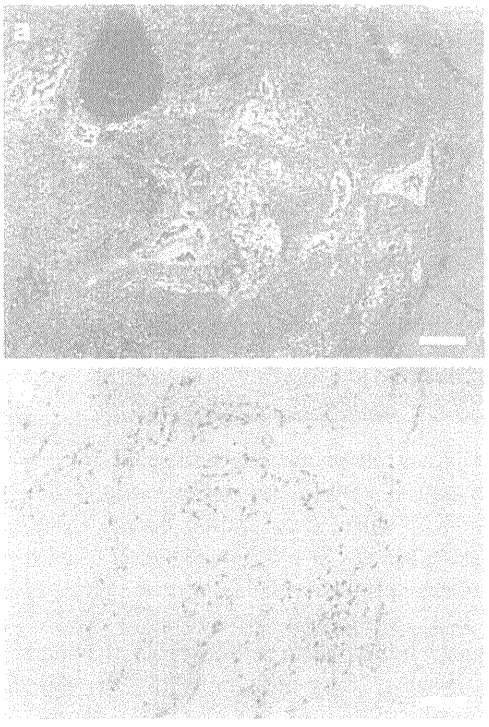

Fig. 4 Photomicrographs showing histopathology of intratumoral and extratumoral cysts by hematoxylin and eosin (H&E) staining. A cavernous angioma was identified in the solid compartment of trigeminal schwannomas with cyst formation in case 4 (**a**, H&E staining, *scale bar*: 500 μm). The cyst wall of the extratumoral cyst was composed of arachnoid membrane without schwannoma cells (**b**, H&E stain, *scale bar*: 250 μm)

Illustrative cases

Case 4

In case 4, the patient was a 33-year-old woman suffering from right facial dysesthesia due to trigeminal schwannoma (Fig. 5a). GKRS, with 12 Gy at the margin, was carried out following partial resection. A cyst had gradually developed in the prepontine cistern, causing left sensory disturbance and right abducens palsy at 24 months after the GKRS (Fig. 5b). The newly developed cyst was removed by second surgery, and the preoperative symptom improved (Fig. 5c). However, at 6 months after the second surgery, another cyst developed within the tumor located in the cavernous portion, causing right abducens palsy, again (Fig. 5d). The residual tumor was completely removed by the third surgery (Fig. 5e).

Case 6

Case 6 involved a 49-year-old man who had presented with a hearing disturbance for 10 years. An MRI revealed a solid tumor in the left cerebellopontine angle with an extratumoral cyst, which was located between the tumor and pons (Fig. 6a). The patient underwent GKRS with 12 Gy as a marginal dose. The cyst showed gradual enlargement, causing right hemiparesis after 13 months (Fig. 6b and c). Salvage surgery was carried out to open the cyst (Fig. 6d). The neurological symptom improved immediately after the surgery. The residual tumor was reduced further in size, and has been controlled for 36 months after the surgery (Fig. 6e).

Discussion

GKRS has been widely accepted as an important treatment modality for various intracranial lesions, such as arteriovenous malformations, metastatic brain tumors, meningiomas, and schwannomas. However, despite its effectiveness in the

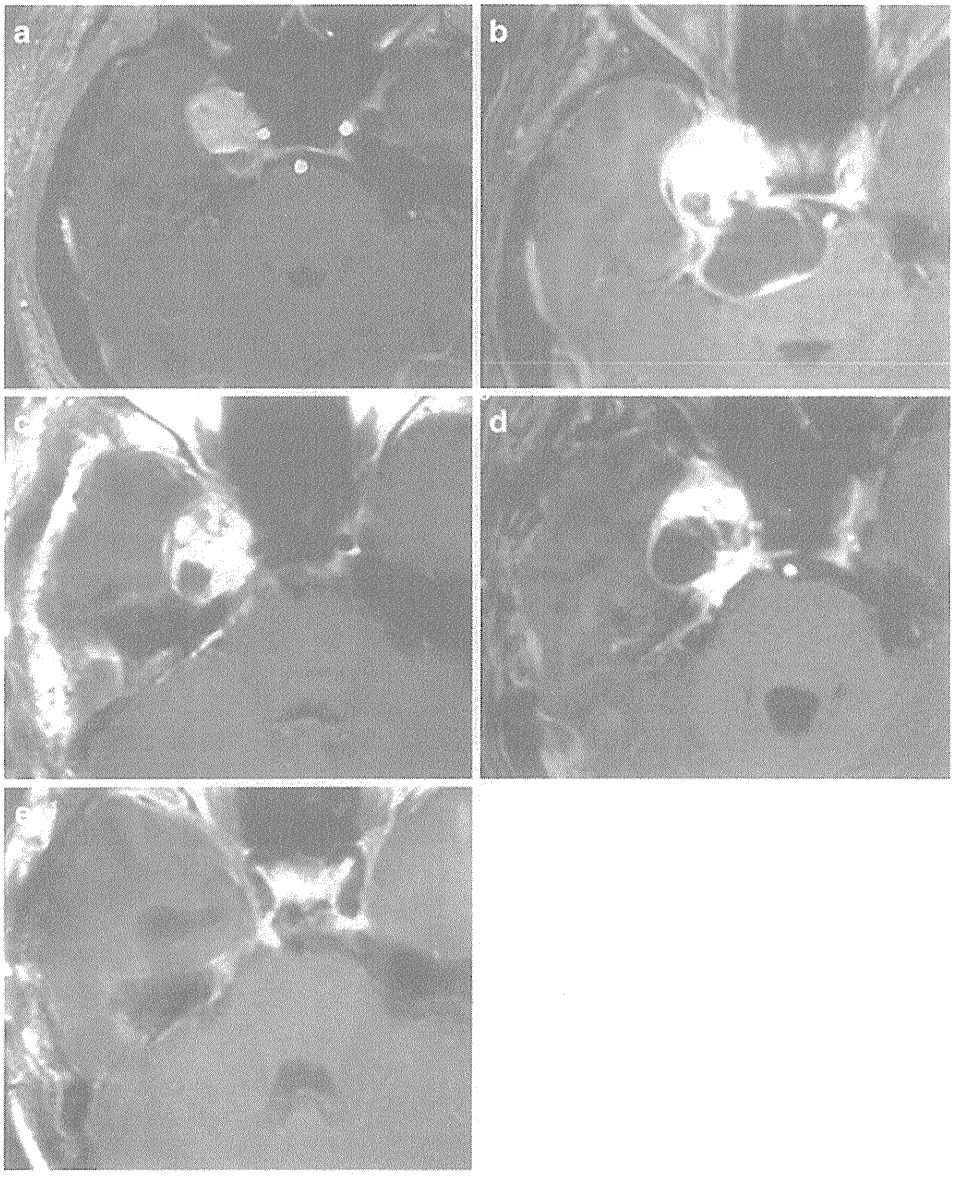

Fig. 5 Case 4. The tumor was localized within the middle cranial fossa adjacent to the cavernous sinus at initial presentation (**a**). Cysts were newly formed after GKRS, and expanded in the prepontine cistern (**b**). The cysts were removed by the first salvage surgery (**c**), but new cysts were developed within the residual tumor in the middle fossa (**d**). Enlarged residual tumor was subtotally removed by the second salvage surgery (**e**)

treatment of lesions themselves, cyst formation in the target area has been reported as a clinically important complication on long-term follow-up after GKRS [9, 12, 19, 24]. Schwannomas sometimes demonstrate cystic change with temporary expansion as the tumor degenerating after GKRS, but most of them finally shrink. Recent literatures reported some cases with intracranial schwannomas demonstrating cyst formation and enlargement after radiosurgery, which required craniotomy. However, clinicopathological mechanisms of cyst development following GKRS remain unclear [6, 8, 21].

In the present study, we demonstrated seven cases of intracranial schwannoma demonstrating development of cysts among 492 patients treated by GKRS. These cases demonstrated progressive enlargement of cysts, causing neurological deterioration, and consequently required microsurgical salvage. In seven cases, case 1 did not show neurological deterioration despite cysts formation. However, since cysts developed after stable control of the tumor for over 10 years, surgical resection is indicated considering possibility of recurrence with malignant transformation. The original tumors varied in size, ranging from 3.2 to 19.0 ml. All the cysts developed within the radiation field except one cases presenting with extratumoral cyst.

These cysts were categorized into two distinct types, intratumoral cysts and extratumoral arachnoid cysts. Intratumoral cysts were newly formed, or were preexistent and enlarged after GKRS. This type of cyst was associated with hemorrhage in four out of five cases.

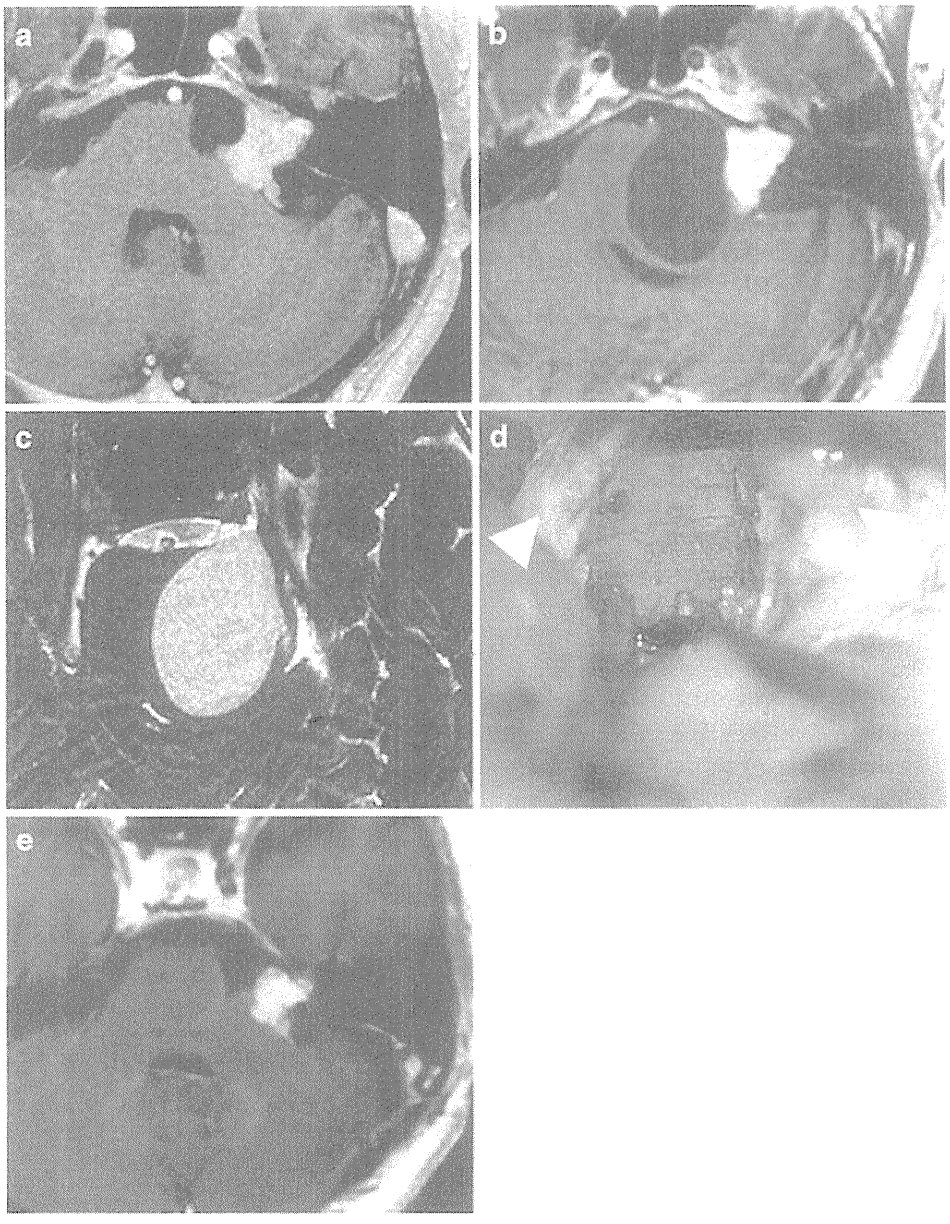

Fig. 6 Case 6. Extratumoral cyst coexisted with vestibular schwannoma at the time of GKRS (a). The cyst was found expanding on periodic follow-up MRI, and compressed the brain stem (b). The wall of the cyst was not enhanced by contrast medium, and markedly thin (b and c). The cyst was transparent and contained xanthochromic fluid (d). Fenestration of the cyst was achieved without aggressive resection of the tumor, and the cyst remained small with the tumor controlled effectively (e). The *arrowhead* indicates lower cranial nerves and the *arrow* shows the solid tumor

Intratumoral hemorrhage was identified on follow-up MRI after GKRS as a low-intensity signal on T2*-weighted image or a fluid fluid level. None of the cases showed a sudden onset of symptoms. It has been reported that intratumoral hemorrhage is involved in pathogenesis of cystic vestibular schwannomas [5, 20]. Furthermore, intratumoral hemorrhage following GKRS has been described in vestibular schwannomas [11]. Although it is unknown whether these hemorrhagic changes is directly induced by GKRS or not, schwannomas demonstrating intratumoral hemorrhage after GKRS might be at risk of symptomatic cyst development.

In cases 1 and 4, presenting with intratumoral cysts, cavernous angioma were identified within the solid compartment adjacent to the developed cyst by histological analysis. Cavernous angioma is known to be induced, in a de novo fashion, following radiation therapy or radiosurgery [16, 18]. On the other hand, cavernous angiomas located within intracranial schwannomas have been also reported [1, 2, 4, 13–16, 23]. Although a direct causal relationship between cavernous angioma and GKRS is unclear in these cases, it is likely that cavernous angioma is associated with intratumoral hemorrhage and cyst formation. Furthermore, considering that both of these two affected cases recurred with further cyst formation after the incomplete resection of the cysts, radical resection, including that of the solid compartment containing cavernous angioma, would be recommended in

salvage microsurgery for schwannomas with this type of cyst.

Extratumoral cysts originated from entrapped arachnoid membrane in two cases. A cyst was preexistent at GKRS in case 6, but newly formed after GKRS in case 7. Since both of these cases were treated by GKRS without preceding microsurgery, extratumoral cyst arose by deformation of arachnoid membrane by enlarging the tumor itself or by arachnoid adhesion by GKRS. Arachnoid cysts associated with schwannomas demonstrate higher intensity on MRI than cerebrospinal fluid, indicating higher protein content secreted by tumor which causes cyst enlargement by osmotic mechanism [7, 17, 26]. These cysts enlarged following the GKRS, while the solid tumor effectively regressed. Furthermore, the residual tumors continue to decrease in size even after partial resection and cyst fenestration. This means that enlargement of an arachnoid cyst does not reflect tumor progression due to treatment failures. Increased permeability of tumor vessels and tumor degeneration by GKRS may accelerate cyst enlargement by osmotic gradient. Fenestration of the cysts is a reasonable strategy to resolve neurological deterioration with less risk of morbidity, when MRI shows characteristic findings of arachnoid cysts associated with schwannomas and growth of solid tumor was controlled by GKRS.

It is well known that cystic vestibular schwannomas demonstrate rapid enlargement by expansion of cyst itself but not by an actual increase of tumor cells in their natural course, causing rapid symptomatic worsening, and that complete removal with preserving facial nerve function is more difficult in cystic tumors than in solid tumors [3]. We believe that GKRS is indicated for small to medium-sized schwannomas, even though containing cystic compartments. However, newly formation of cysts and/or enlargement of preexisted cysts may be unpredictably accelerated following GKRS, although infrequent. Periodic MRI follow-up is mandatory in the schwannomas involving a cyst.

Conclusions

We demonstrated seven cases presenting with cyst development among 492 intracranial schwannomas treated by GKRS. These cysts included both intratumoral cysts and extratumoral arachnoid cysts. Although cystic change is a fairly well-known side-effect of GKRS, it is an important issue in clinical settings that some of the cysts progressively enlarge and cause neurological symptom. Our management strategy for cyst formation/enlargement is to wait and scan unless neurological status deteriorates. Salvage microsurgery is indicated in the cases demonstrating continuous enlargement of the cyst, causing neurological deterioration, and it is required to plan surgical strategy on the basis of the pathogenesis of intratumoral cyst and extratumoral arachnoid cyst.

Conflicts of interest None.

References

1. Asari S, Tsuchida S, Fujiwara A, Yabuno N, Furuta T, Ohmoto T (1992) Trigeminal neurinoma presenting with intratumoral hemorrhage: report of two cases. Clin Neurol Neurosurg 94:219–224
2. Bojsen-Moller M, Spaun E (1978) Peripheral nerve tumour composed of neurilemmoma and haemangioma elements. Acta Neurochir (Wien) 40:299–305
3. Charabi S, Klinken L, Tos M, Thomsen J (1994) Histopathological and growth pattern of cystic acoustic neuromas. Laryngoscope 104:1348–11352
4. Feiz-Erfan I, Zabramski JM, Herrmann LL, Coons SW (2006) Cavernous malformation within a schwannoma: review of the literature and hypothesis of a common etiology. Acta Neurochir (Wien) 148:647–652
5. Gomez-Broushcet A, Delisle MB, Cognard C, Bonafe A, Charlet JP, Deguine O, Fraysse B (2001) Vestibular schwannomas: correlations between magnetic resonance imaging and histopathologic appearance. Otol Neurotol 22:79–86
6. Hasegawa T, Kida Y, Yoshimoto M, Koike J, Goto K (2006) Evaluation of tumor expansion after stereotactic radiosurgery in patients harboring vestibular schwannomas. Neurosurgery 58:1119–1128
7. Hayhurst C, Dhir J, Dias PS (2005) Stereotactic radiosurgery and vestibular schwannoma: hydrocephalus associated with the development of a secondary arachnoid cyst. A report of two cases and review of the literature. Br J Neurosurg 19:178–181
8. de Ipolyi AR, Yang I, Buckley A, Barbaro NM, Chenung SW, Parsa AT (2008) Fluctuating response of a cystic vestibular schwannoma to radiosurgery: case report. Neurosurgery 62: E1164–E1165
9. Ishikawa E, Yamamoto M, Saito A, Kujiraoka Y, Iijima T, Akutsu H, Matsumura A (2009) Delayed cyst formation after gamma knife radiosurgery for brain metastases. Neurosurgery 65:689–695
10. Iwai Y, Yamada K, Yamagata K, Yasui T (2007) Surgery after radiosurgery for acoustic neurinomas: surgical strategy and histological findings. Neurosurgery 60(ONS Suppl 1):ONS-75–ONS-82
11. Iwai Y, Yamanaka K, Shiotani M, Uyama T (2003) Radiosurgery for acoustic neuromas: results of low-dose treatment. Neurosurgery 53:282–288
12. Izawa M, Chernov M, Hayashi M, Nakaya K, Kamikawa S, Kato K, Higa T, Ujiie H, Kasuya H, Kawamata T, Okada Y, Kubo O, Iseki H, Hori T, Takakura K (2007) Management and prognosis of cysts developed on long-term follow-up after gamma knife radiosurgery for intracranial arteriovenous malformations. Surg Neurol 68:400–406
13. Kasantikul V, Brown J, Netsky MG (1982) Mesenchymal differentiation in trigeminal neurilemmoma. Cancer 50:1568–1571
14. Kasantikul V, Netsky MG (1979) Combined neurilemmoma and angioma. Tumor of ectomesenchyme and a source of bleeding. J Neurosurg 50:81–87

15. Kasantikul V, ShuangshotiS PP, Wangsuphachart S (1987) A combined neurilemmoma and adgioma of the parasellar region. Case report. J Neurosurg 67:307–311
16. Motegi H, Kuroda S, Ishii N, Aoyama H, Terae S, Shirato H, Iwasaki Y (2008) De novo formation of cavernoma after radiosurgery for adult cerebral arteriovenous malformation—case report. Neurol Med -Chir (Tokyo) 48:397–400
17. Muzumdar DP, Chagla AS, Goel A (2000) Acoustic schwannoma and arachnoid cyst collocated in the cerebellopontine angle—case report. Neurol Med -Chir (Tokyo) 40:230–233
18. Nimjee SM, Powers CJ, Bulsara KR (2006) Review of the literature on de novo formation of cavernous malformations of the central nervous system after radiation therapy. Neurosurg Focus 15:e4
19. Pan HC, Sheehan J, Stroila M, Steiner M, Steiner L (2005) Late cyst formation following gamma knife surgery of arteriovenous malformations. J Neurosurg (Suppl) 102:124–127
20. Park CK, Kim DC, Park SH, Kim JE, Paek SH, Kim DG, Jung HW (2006) Microhemorrhage, a possible mechanism for cyst formation in vestibular schwannomas. J Neurosurg 105:576–580
21. Pendl G, Ganz JS, Kitz K, Eustacchio S (1996) Acoustic neurinomas with macrocysts treated with gamma knife radiosurgery. Stereotact Funct Neurosurg 66(Suppl 1):103–111
22. Pollock BE (2006) Management of vestibular schwannomas that enlarge after stereotactic radiosurgery: treatment recommendations based on a 15-year experience. Neurosurgery 58:241–248
23. Sakata H, Fujimura M, Watanabe M, Tominaga T (2007) Association of cavernous malformation within vestibular schwannoma: immunohistochemical analysis of matrix metalloproteinase-2 and -9. Neurol Med -Chir (Tokyo) 47:509–512
24. Shuto T, Inomori S, Fujino H, Nagano H, Hasegawa N, Kakuta Y (2005) Cyst formation following gamma knife surgery for intracranial meningioma. J Neurosurg (Suppl) 102:134–139
25. Shuto T, Inomori S, Matsunaga H, Fujino H (2008) Microsurgery for vestibular schwannoma after gamma knife radiosurgery. Acta Neurochir (Wien) 150:229–234
26. Tali ET, Yuh WT, Nguven HD, Feng G, Koci TM, Jinkin JR, Robinson RA, Hasso AN (1993) Cystic acoustic schwannomas: MR characteristics. AJNR Am J Neuroradiol 14:1241–1247

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Radiosurgery alone for 5 or more brain metastases: expert opinion survey

Clinical article

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Object. Oligometastatic brain metastases may be treated with stereotactic radiosurgery (SRS) alone, but no consensus exists as to when SRS alone would be appropriate. A survey was conducted at 2 radiosurgery meetings to determine which factors SRS practitioners emphasize in recommending SRS alone, and what physician characteristics are associated with recommending SRS alone for ≥ 5 metastases.

Methods. All physicians attending the 8th Biennial Congress and Exhibition of the International Stereotactic Radiosurgery Society in June 2007 and the 18th Annual Meeting of the Japanese Society of Stereotactic Radiosurgery in July 2009 were asked to complete a questionnaire ranking 14 clinical factors on a 5-point Likert-type scale (ranging from 1 = not important to 5 = very important) to determine how much each factor might influence a decision to recommend SRS alone for brain metastases. Results were condensed into a single dichotomous outcome variable of "influential" (4–5) versus "not influential" (1–3). Respondents were also asked to complete the statement: "In general, a reasonable number of brain metastases treatable by SRS alone would be, at most, ___." The characteristics of physicians willing to recommend SRS alone for ≥ 5 metastases were assessed. Chi-square was used for univariate analysis, and logistic regression for multivariate analysis.

Results. The final study sample included 95 Gamma Knife and LINAC-using respondents (54% Gamma Knife users) in San Francisco and 54 in Sendai (48% Gamma Knife users). More than 70% at each meeting had ≥ 5 years experience with SRS. Sixty-five percent in San Francisco and 83% in Sendai treated ≥ 30 cases annually with SRS. The highest number of metastases considered reasonable to treat with SRS alone in both surveys was 50. In San Francisco, the mean and median numbers of metastases considered reasonable to treat with SRS alone were 6.7 and 5, while in Sendai they were 11 and 10. In the San Francisco sample, the clinical factors identified to be most influential in decision making were Karnofsky Performance Scale score (78%), presence/absence of mass effect (76%), and systemic disease control (63%). In Sendai, the most influential factors were the size of the metastases (78%), the Karnofsky Performance Scale score (70%), and metastasis location (68%). In San Francisco, 55% of respondents considered treating ≥ 5 metastases and 22% considered treating ≥ 10 metastases "reasonable." In Sendai, 83% of respondents considered treating ≥ 5 metastases and 57% considered treating ≥ 10 metastases "reasonable." In both groups, private practitioners, neurosurgeons, and Gamma Knife users were statistically significantly more likely to treat ≥ 5 metastases with SRS alone.

Conclusions. Although there is no clear consensus for how many metastases are reasonable to treat with SRS alone, more than half of the radiosurgeons at 2 international meetings were willing to extend the use of SRS as an initial treatment for ≥ 5 brain metastases. Given the substantial variation in clinicians' approaches to SRS use, further research is required to identify patient characteristics associated with optimal SRS outcomes.
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KEY WORDS • **brain metastasis** • **oligometastasis** • **stereotactic radiosurgery** • **survey**

It has been increasingly recognized over the last decade that patients with multiple brain metastases can be treated effectively with SRS alone with survival outcomes similar to WBRT.[7,9,19] The twin goals of SRS treatment—control of brain metastases and avoidance of normal tissue injury—have led to an increased use of initial SRS for brain metastases and a deferral of WBRT, even for patients whose anticipated survival is expected to be brief.

Since the landmark study by Patchell et al.[17] published 2 decades ago that documented the importance of focal treatments for patients with a single brain metastasis, SRS for brain metastases grew in popularity because this minimally invasive approach could be used to control or eradicate more than 1 metastasis in a single

Abbreviations used in this paper: KPS = Karnofsky Performance Scale; SRS = stereotactic radiosurgery; WBRT = whole brain radiation therapy.

session, and because it could also be used in conjunction with WBRT.[24] A randomized clinical trial sponsored by the National Cancer Institute (RTOG 95–08) enrolling patients with 1–3 brain metastases showed a survival advantage and improved local control for consolidative SRS in patients with a single brain metastasis as compared with WBRT alone.[1] Statistically significant improvements in functional autonomy at 6 months follow-up were documented in the cohort randomized to WBRT and SRS, no matter whether 1, 2, or 3 metastases were treated with consolidative SRS.

A clinical trial begun by the Japanese Radiation Oncology Study Group (JROSG 99–1) turned this management strategy on its head. Aoyama et al.[2] asked if the addition of WBRT to upfront SRS for patients with 1–4 brain metastases showed any benefit for survival or neurological function compared with SRS alone. No survival advantage was observed with the use of WBRT, and there was no difference in neurological outcomes after 1 year. Editorial commentaries and letters regarding this study highlighted divergent opinions among neurosurgeons, neurooncologists, and radiation oncologists about the appropriateness of deferring WBRT in patients with 1–4 brain metastases treated with SRS.[11,14,15,18]

The publication of these 2 important studies and the increasing interest in SRS for brain metastases as an initial, definitive treatment raises the question of whether practice patterns for patients with newly diagnosed brain metastases might be becoming divergent. No data-driven, universally accepted management standards for brain metastasis management exist today for patients with 1–4 metastases, and even less data exist for patients with ≥ 5 brain metastases managed with anything more than WBRT. Despite the absence of data, patients are seeking SRS alone, and physicians have started to provide SRS as an initial definitive therapy for patients with far more numerous brain metastases than is supported by published randomized, controlled clinical trials.

Because of the lack of guidance from clinical trials for the physicians who would be offering this novel treatment, we conducted a survey of radiosurgery practitioners to help determine what patient, physician, and equipment-related factors may be associated with offering SRS as the first management step for brain metastases. Furthermore, we assessed physician characteristics associated with recommending first-line SRS for ≥ 5 metastases.

Methods

Study Design

We conducted a cross-sectional survey of physicians attending the 8th Biennial Congress and Exhibition of the International Stereotactic Radiosurgery Society in San Francisco in June 2007. The Yale University Human Investigation Committee provided approval for the collection of data on this subject. A second survey using the same instrument (translated into Japanese) was conducted at the 18th Annual Meeting of the Japanese Society of Stereotactic Radiosurgery in Sendai in July 2009.

Survey Instrument

The survey instrument collected information on survey participant demographics, experience, and SRS equipment used and was distributed to all physician attendees at a single session in both meetings. The survey asked physicians to use a 5-point Likert-type scale to score 14 clinical factors that were believed to possibly be important in making a decision about offering SRS as an initial, definitive therapy to patients with multiple brain metastases (ranging from 1 = not important to 5 = very important).

Factors that physicians were asked to rank in the survey included patient age; sex; KPS score; social situation; the presence of metastases-related neurological symptoms; location, size, and radiographic characteristics of the brain metastases (cystic vs solid, nonhemorrhagic vs hemorrhagic); presence or absence of mass effect; histopathology of the brain metastases; status of the patients' systemic disease control; and the availability of additional useful chemotherapy for the malignancy undergoing treatment. Self-assessed physician workload was also to be scored. Respondents were also given an opportunity to write in any additional factors they might consider important. Lastly, physicians were asked to write in a number to answer the question: "In general, a reasonable number of brain metastases treatable by SRS alone would be, at most, _____."

Statistical Analysis

Results for each survey item addressing factors affecting the use of SRS were condensed into a single dichotomous outcome variable of "influential" (4–5) versus "not influential" (1–3). The characteristics of physicians willing to recommend SRS alone for ≥ 5 metastases were then assessed using bivariate chi-square tests, as well as multivariate logistic regression analysis (outcome: recommending SRS for ≥ 5 metastases). Statistical analyses were performed using STATA version 10.0.

Results

In San Francisco, 95 completed surveys were collected, and in Sendai, 54 were collected. The results of the demographic portions of these 2 surveys are summarized in Table 1. The response to the query about what would be a reasonable number of brain metastases to be treated with SRS alone ranged from 2–50 in San Francisco and 3–50 in Sendai. In San Francisco, the median number of metastases considered reasonable to treat with SRS alone was 5 and the mean was 6.7; in Sendai, the median was 10 and the mean was 11. In San Francisco, 55% of physicians considered treating ≥ 5 metastases with SRS alone reasonable, and 22% of physicians considered it reasonable to treat ≥ 10 metastases with SRS alone. In Sendai, 83% of the physicians surveyed believed that treating ≥ 5 metastases with SRS alone was reasonable, and 57% of physicians believed that treating ≥ 10 metastases with SRS alone was reasonable.

Bivariate analysis (Table 2) showed that private practitioners were significantly more likely than academic

TABLE 1: Stereotactic radiosurgery survey demographic data

Variable	San Francisco	Sendai
no. of completed surveys	95	54
academicians	61%	31%
private practice physicians	39%	69%
neurosurgeons	54%	80%
radiation oncologists	46%	21%
>5 years experience performing SRS	75%	70%
treat >30 patients w/ brain metastases w/ SRS annually	65%	83%
use Gamma Knife	54%	48%
use LINAC	46%	52%

physicians to consider SRS alone an appropriate initial definitive therapy for ≥ 5 brain metastases (77% vs 44%, respectively, in San Francisco, p = 0.002; 88% vs 69%, respectively, in Sendai, p = 0.017). Neurosurgeons were significantly more likely to recommend SRS for patients with ≥ 5 metastases than radiation oncologists (69% vs 39%, respectively, in San Francisco, p = 0.003; 93% vs 54%, respectively, in Sendai, p = 0.004), and physicians who use a Gamma Knife to perform SRS were also significantly more likely to recommend SRS alone than LINAC users (72% vs 35%, respectively, in San Francisco, p = 0.002; 100% vs 68%, respectively, in Sendai, p = 0.005).

A multivariate analysis performed on data collected in San Francisco confirmed that neurosurgeons (p = 0.033) and Gamma Knife users (p = 0.002) were independently significantly more likely to treat ≥ 5 metastases with SRS alone, whereas the impact of private practice versus academic practice was no longer significant. The OR of recommending treating ≥ 5 metastases with SRS alone was 4.2 for Gamma Knife users versus LINAC users (p = 0.002). The OR of recommending SRS alone for patients with ≥ 5 metastases was 2.7 for neurosurgeons compared with radiation oncologists (p = 0.033).

Neurosurgeons participating in the Japanese survey were more likely to be in community practice, and this factor correlated strongly with the opinion that SRS alone for ≥ 5 metastases was reasonable. Because 100% of Gamma Knife users at the Japanese meeting indicated that they would treat ≥ 5 metastases, a multivariate analysis was unable to provide stable parameter estimates (Gamma Knife use predicted the treatment recommendation for ≥ 5 metastases perfectly).

From the survey in San Francisco (Table 3), the most influential decision-making factors were KPS score (78%), presence/absence of mass effect (76%), and systemic disease control (63%). Less important were metastases-related neurological symptoms (56%) or unrelated neurological disease (51%), metastases location (55%), patient age (50%), size of the metastases (45%), tumor histopathology (40%), and availability of additional potentially effective chemotherapy (38%). Radiographic appearance (20%), social situation (21%), and physician workload (5%) were mostly believed to not be influential factors. Nobody considered patient sex to be an influential factor.

At the Sendai meeting (Table 3), the most influential factors were the size of the metastases (78%), KPS score (70%), and metastasis location (68%). Although less influential, the presence or absence of neurological symptoms from the metastases (57%), mass effect (57%), other neurological disease (48%), systemic diseases control (44%), stability of the patient's social situation (40%), patient age (35%), and tumor histopathology (39%) were all considered more important than the availability of additional potentially effective chemotherapy (26%) or the radiographic appearance of the metastatic disease (25%). As in San Francisco, only a small percentage (6%) of the physicians considered their own workload a significant factor when advising patients about SRS alone for brain metastases, and again, no one considered patient sex an influential factor.

Discussion

The key finding of this study is the previously undocumented willingness of more than half of the surveyed physicians who are experienced in radiosurgical treatment of brain metastases to offer and recommend SRS alone to patients with numerous brain metastases. The range of number of metastases considered reasonable to treat with first-line SRS was essentially the same at both survey locations (2–50 and 3–50). While the median and the mean number of metastases considered reasonable to treat with SRS alone were significantly different between San Francisco (mean 6.7, median 5) and Sendai (mean 11, median 10), both medians and means fall outside the 1–4

TABLE 2: Bivariate analysis of physician and equipment characteristics associated with recommending SRS for ≥ 5 metastases

Variable	San Francisco		Sendai	
	No. of Patients (%)	p Value	No. of Patients (%)	p Value
no. recommending SRS for ≥5 metastases	52/95 (55)		45/54 (83)	
private practice physicians	27/35 (77)	0.002	23/26 (88)	0.017
academic physicians	24/54 (44)		11/16 (69)	
neurosurgeons	35/51 (69)	0.003	38/41 (93)	0.004
radiation oncologists	17/44 (39)		7/13 (54)	
Gamma Knife users	36/50 (72)	0.002	26/26 (100)	0.005
LINAC users	15/43 (35)		19/28 (68)	

TABLE 3: Percentage of respondents who scored each factor as influential (score 4 or 5) in deciding to perform or not perform SRS alone for oligometastatic brain metastases

Assessed Factors	Percentage of Respondents Considering This Factor Important	
	San Francisco	Sendai
KPS score	78	71
presence/absence of mass effect	76	57
systemic disease control	63	44
neurological symptoms from metastases	56	57
other neurological disease	51	48
metastases location	55	68
metastases size	45	78
metastases histopathology	40	39
radiographic appearance	20	25
patient age	50	35
patient social situation	21	40
availability of additional potentially effective chemotherapy	38	26
patient sex	0	0
physician workload	5	6

range set by the randomized controlled clinical trial data supported by the literature.[2] Stated conversely, the majority (55%) of physicians were willing to offer SRS alone to patients with ≥ 5 brain metastases at the 2007 meeting in San Francisco, and the vast majority (83%) were willing at a 2009 meeting in Sendai.

Although a minority (22%) of the clinicians surveyed at the 2007 meeting in San Francisco believed treating > 10 metastases was reasonable, a majority (57%) of those surveyed in Sendai in 2009 believed that using SRS alone for > 10 metastases was reasonable. It should be clearly recognized that this survey's overall response rate is unknown and that there is no knowledge about whether respondents differed from nonrespondents with regard to professional characteristics or inclination to use SRS, and that the generalizability of any analysis must be regarded as limited.

Based on the current literature, clinicians who strive to practice evidence-based medicine might not consider referring a patient with > 4 brain metastases for a consultative opinion regarding definitive SRS. Historical practice would certainly deem this acceptable, and such a patient might be offered WBRT without a discussion of SRS alone as a possible therapeutic option.

Given that the treatment of brain metastases with SRS is a labor-intensive process that requires the meticulous identification of each metastasis to be treated and the development and subsequent delivery of individualized, highly customized treatment plans, the escalating threshold of the number of "oligo" metastases that may "reasonably" be considered for SRS alone by radiosurgery practitioners is a remarkable finding. The more metastases that are treated, the more work (and more time) is involved, but our study shows that only 5% of physicians in San Francisco and 6% in Sendai considered their workload important in making a recommendation for SRS alone.

Our study further shows that neurosurgeons are more willing than radiation oncologists both in San Francisco and in Sendai to declare this management practice reasonable. This likely reflects differences in professional training and experience. Neurosurgeons implement focal treatments for intracranial pathology for mass effect or diagnosis and rarely see the benefits of WBRT, but only deal with its focal failures. In comparison, for a radiation oncologist, the use of a regional treatment such as WBRT is common, easy to institute, can prevent the progression of subclinical disease into clinically evident metastases, and can help control larger metastases that receive a focal treatment such as resection or SRS.[1,2,5,16] For a patient with multiple metastases in which staged craniotomies and resection of multiple metastases are rarely employed, the noninvasive focal alternative of SRS can be an attractive alternative that continues to involve the neurosurgeon.

The equipment used for SRS was also identified to be a significant variable in assessing whether individual physicians would treat a patient with SRS. Gamma Knife users in both surveys were statistically much more likely than LINAC users to offer SRS to a patient with ≥ 5 brain metastases. Why might opinions vary with the technology used? Treatment recommendations are guided by individual experience. The Gamma Knife machine is solely dedicated to the treatment of intracranial lesions accompanied by its own dedicated staff and the several hours required to provide comprehensive radiosurgical treatment can be accomplished during regular working hours. However, because LINACs are commonly used for treating many patients daily with fractionated radiotherapy, single-fraction SRS may only be started when the full daily roster of patients receiving fractionated radiotherapy is completed. Radiosurgical treatment for patients with multiple brain metastases with such equipment might often extend into the evening hours, and perhaps could lead to a greater likelihood to decline to offer SRS to a patient with multiple brain metastases by a busy radiation oncologist, particularly because of the higher potential of requiring additional short-term salvage treatment if WBRT is not part of the management plan. The absence of Class I data supporting the use of SRS alone for patients with > 4 brain metastases for important outcomes such as neurocognitive function provides good justification for such a management practice.

The recently published Phase III study by Chang et al.,[5] however, may change this justification.[10] This study showed that short-term neurocognitive outcomes for patients who were treated with SRS alone for 1–3 metastases was significantly better than those receiving SRS and WBRT. A survival advantage was also observed for deferring WBRT. Because this study's primary end point was not survival, this observation may be due to an imbalance of treatment arms and asymmetrical use of aggressive salvage treatment rather than a true treatment effect. In addition to the 2 randomized controlled trials that have been published on the use of SRS with or without WBRT for up to 4 brain metastases, a third trial, also randomizing patients with 1–3 brain metastases between

SRS alone and SRS with WBRT, is underway under the aegis of the National Cancer Institute as an Intergroup study (NCCTG-N0574) and is powered to evaluate neurocognitive outcomes in the two arms.[20]

Controversy is almost certain concerning whether this new study's (NCCTG-N0574) results may be extrapolated to patients with 4 or more brain metastases. In addition, appropriate studies still need to be conducted to determine what other factors may be critical in determining which patients may be appropriate for initial, definitive SRS. Important factors might include age, comorbidities, tumor site of origin, and histology, and a host of other variables.[13] Rational patient management recommendations ideally derive from high quality studies performed on patient populations that appropriately reflect disease stage, severity, comorbidities, and available therapeutic options, but recommendations for populations in general often differ significantly from recommendations for individual patients. Treatment individualization is a hallmark of modern oncological and medical practice.

It is far from certain that even well-designed oncological clinical trials and meta-analyses will affect patient management patterns if their results run counter to the perceived benefit of the treatment. University of Toronto investigators surveyed American oncologists about management recommendations for 5 hypothetical patients with breast cancer.[4] One scenario—offering systemic chemotherapy to postmenopausal women with early stage, estrogen receptor–negative, axillary node–negative breast cancer—was noted by the authors as counter to both large randomized controlled trials and a meta-analysis of available trial data, as neither evaluation had shown a survival benefit for the use of adjuvant chemotherapy in that setting. It took additional trials and a meta-analysis with 10- and 15-year follow-up to document a survival advantage for women up to 70 years of age with early stage, node-negative breast cancer who received adjuvant systemic chemotherapy in Phase III trials testing the value of that intervention.[6] Expert opinion preceded confirmatory trial data in this oncological situation, but it is unlikely that clinical trials will ever adequately resolve all management questions regarding patients with brain metastases because of 2 major issues: the heterogeneity of patients presenting with brain metastases, and the short expected lifespan for patients with metastatic cancer.

Stereotactic radiosurgery as a single, definitive treatment for brain metastases has been used with increasing frequency in North America, Europe, Asia, and around the world.[2,3,5,8,12,21–24] This approach defers the use of WBRT to a supplementary or salvage role. Stereotactic radiosurgery has been documented to provide excellent control for targeted lesions, and the focal nature of SRS permits retreatment if additional oligometastatic brain metastases are identified on surveillance images after SRS. Whole brain radiation therapy can be used for salvaging of patients who develop metastatic involvement of the leptomeninges or miliary parenchymal metastases. This management approach represents a paradigm shift for the management of brain metastases. Whole brain radiation therapy has been the standard of care for approximately 50 years because of its ease of application and palliative benefit for patients with symptomatic metastases. Whole brain radiation therapy has also been proven to delay or prevent the growth of clinically inapparent metastases. Stereotactic radiosurgery is not ubiquitous, however, and not all radiation oncologists or neurosurgeons may offer this treatment to patients with brain metastases because of a lack of specialized equipment or of appropriate training.

The decision to survey physician attendees of the 8th Biennial Congress and Exhibition of the International Stereotactic Radiosurgery Society in San Francisco was undertaken to assess the factors that physicians (quite familiar with SRS) consider as significant when evaluating patients referred for an opinion regarding brain metastasis management. In the absence of guidelines derived strictly from evidence-based medicine, it was believed that value might be provided by determining what types of practices are common and what might be deemed acceptable in light of a changing paradigm for management of brain metastases. The survey instrument asked specific questions about the physician and the nature of their practice and equipment, and deliberately posed very general questions about patient and tumor-specific factors to avoid portraying clinical scenarios that might influence responses. A follow-up survey was performed at the 18th Annual Meeting of the Japanese Society for Stereotactic Radiosurgery in 2009 to try to confirm and extend the survey findings.

There are numerous limitations to interpretation of the survey responses obtained. No data were collected to allow an assessment of response rate of the physicians surveyed at the 2 meetings. The 2 surveys were conducted more than a year apart at radiosurgery meetings on 2 different continents. The San Francisco meeting was an international meeting, with physician attendance from around the globe, and the Sendai meeting was a national society's meeting. The different practice environments that these respondents practice in will confound simple comparisons over time or across practice environments. No data were collected about physician reimbursement schemes (single payer vs private health insurance, and others) or how reimbursement might affect recommendations for first-line SRS for multiple brain metastases. No data were collected about what LINAC platform or platforms might be associated with a greater willingness to perform SRS on ≥ 5 metastases. Finally, this analysis focused on the respondents' reported treatment patterns, rather than assessing actual patient care. Future work should explore actual patterns of patient care, as well as outcomes associated with different management options.

Conclusions

Stereotactic radiosurgery as a definitive initial management strategy for ≥ 5 brain metastases was considered reasonable by the majority of physicians attending radiosurgery conferences in 2007 and 2009, and at the second conference, the majority of physicians were willing to offer radiosurgery to patients with ≥ 10 metastases. Neurosurgeons and Gamma Knife users were more likely to recommend SRS alone for such patients. No clear consen-

sus exists for how many metastases are reasonable to treat with SRS alone or what factors should be used to assess candidate patients. Given the early neurocognitive results from Chang et al.,[5] there appears to be an advantage to a focal philosophy and the use of SRS alone for multiple brain metastases should be standard in first-line discussions regarding management of multiple metastases, even for patients with 5 or more metastases.

Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Knisely, Chiang. Acquisition of data: Knisely, Yamamoto, Castrucci, Jokura, Chiang. Analysis and interpretation of data: Knisely, Yamamoto, Gross, Castrucci, Chiang. Drafting the article: Knisely. Critically revising the article: Knisely, Castrucci, Jokura, Chiang. Reviewed final version of the manuscript and approved it for submission: Knisely, Yamamoto, Gross, Castrucci, Jokura. Statistical analysis: Gross. Administrative/technical/material support: Knisely. Study supervision: Knisely.

References

1. Andrews DW, Scott CB, Sperduto PW, Flanders AE, Gaspar LE, Schell MC, et al: Whole brain radiation therapy with or without stereotactic radiosurgery boost for patients with one to three brain metastases: phase III results of the RTOG 9508 randomised trial. **Lancet 363:**1665–1672, 2004
2. Aoyama H, Shirato H, Tago M, Nakagawa K, Toyoda T, Hatano K, et al: Stereotactic radiosurgery plus whole-brain radiation therapy vs stereotactic radiosurgery alone for treatment of brain metastases: a randomized controlled trial. **JAMA 295:**2483–2491, 2006
3. Aoyama H, Tago M, Kato N, Toyoda T, Kenjyo M, Hirota S, et al: Neurocognitive function of patients with brain metastasis who received either whole brain radiotherapy plus stereotactic radiosurgery or radiosurgery alone. **Int J Radiat Oncol Biol Phys 68:**1388–1395, 2007
4. Belanger D, Moore M, Tannock I: How American oncologists treat breast cancer: an assessment of the influence of clinical trials. **J Clin Oncol 9:**7–16, 1991
5. Chang EL, Wefel JS, Hess KR, Allen PK, Lang FF, Kornguth DG, et al: Neurocognition in patients with brain metastases treated with radiosurgery or radiosurgery plus whole-brain irradiation: a randomised controlled trial. **Lancet Oncol 10:** 1037–1044, 2009
6. Early Breast Cancer Trialists' Collaborative Group (EBCTCG), Clarke M, Coates AS, Darby SC, Davies C, Gelber RD, et al: Adjuvant chemotherapy in oestrogen-receptor-poor breast cancer: patient-level meta-analysis of randomised trials. **Lancet 371:**29–40, 2008
7. Flickinger JC, Kondziolka D, Lunsford LD, Coffey RJ, Goodman ML, Shaw EG, et al: A multi-institutional experience with stereotactic radiosurgery for solitary brain metastasis. **Int J Radiat Oncol Biol Phys 28:**797–802, 1994
8. Fox J, Kleinberg L: Evolving management of newly diagnosed brain metastases: expanding role of radiosurgery in lieu of whole brain radiation. **Future Oncol 3:**285–293, 2007
9. Kihlström L, Karlsson B, Lindquist C: Gamma Knife surgery for cerebral metastases. Implications for survival based on 16 years experience. **Stereotact Funct Neurosurg 61 (Suppl 1):** 45–50, 1993
10. Knisely JP: Focused attention on brain metastases. **Lancet Oncol 10:**1024, 2009
11. Lunsford LD, Flickinger JC: Radiosurgery plus or minus whole brain radiation therapy for the treatment of brain metastases. An editorial comment. **Surg Neurol 66:**461–462, 2006
12. Lutterbach J, Cyron D, Henne K, Ostertag CB: Radiosurgery followed by planned observation in patients with one to three brain metastases. **Neurosurgery 52:**1066–1074, 2003
13. National Cancer Institute: **Phase III Randomized Trial of the Role of Whole Brain Radiation Therapy in Addition to Radiosurgery in Patients with One to Three Cerebral Metastases.** (http://www.cancer.gov/clinicaltrials/NCCTG-N0574) [Accessed August 26, 2010]
14. Patchell RA, Regine WF, Loeffler JS, Sawaya R, Andrews DW, Chin LS: Radiosurgery plus whole-brain radiation therapy for brain metastases. **JAMA 296:**2089–2091, 2006
15. Patchell RA, Regine WF, Renschler M, Loeffler JS, Sawaya R, Chin LS, et al: Comments about the prospective randomized trial by Aoyama et al. **Surg Neurol 66:**459–460, 2006
16. Patchell RA, Tibbs PA, Regine WF, Dempsey RJ, Mohiuddin M, Kryscio RJ, et al: Postoperative radiotherapy in the treatment of single metastases to the brain: a randomized trial. **JAMA 280:**1485–1489, 1998
17. Patchell RA, Tibbs PA, Walsh JW, Dempsey RJ, Maruyama Y, Kryscio RJ, et al: A randomized trial of surgery in the treatment of single metastases to the brain. **N Engl J Med 322:** 494–500, 1990
18. Raizer J: Radiosurgery and whole-brain radiation therapy for brain metastases: either or both as the optimal treatment. **JAMA 295:**2535–2536, 2006
19. Shu HK, Sneed PK, Shiau CY, McDermott MW, Lamborn KR, Park E, et al: Factors influencing survival after gamma knife radiosurgery for patients with single and multiple brain metastases. **Cancer J Sci Am 2:**335–342, 1996
20. Sneed PK, Lamborn KR, Forstner JM, McDermott MW, Chang S, Park E, et al: Radiosurgery for brain metastases: is whole brain radiotherapy necessary? **Int J Radiat Oncol Biol Phys 43:**549–558, 1999
21. Sneed PK, Suh JH, Goetsch SJ, Sanghavi SN, Chappell R, Buatti JM, et al: A multi-institutional review of radiosurgery alone vs. radiosurgery with whole brain radiotherapy as the initial management of brain metastases. **Int J Radiat Oncol Biol Phys 53:**519–526, 2002
22. Sperduto PW, Chao ST, Sneed PK, Luo X, Suh J, Roberge D, et al: Diagnosis-specific prognostic factors, indexes, and treatment outcomes for patients with newly diagnosed brain metastases: a multi-institutional analysis of 4,259 patients. **Int J Radiat Oncol Biol Phys 77:**655–661, 2010
23. Yamamoto M: Radiosurgery for metastatic brain tumors. **Prog Neurol Surg 20:**106–128, 2007
24. Yamamoto M, Ide M, Nishio S, Urakawa Y: Gamma Knife radiosurgery for numerous brain metastases: is this a safe treatment? **Int J Radiat Oncol Biol Phys 53:**1279–1283, 2002

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Cancer Research

NKX2.2 Suppresses Self-Renewal of Glioma-Initiating Cells

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Abstract

Glioblastoma (GBM) is the most aggressive and destructive form of brain cancer. Animal models that can unravel the mechanisms underlying its progression are needed to develop rational and effective molecular therapeutic approaches. In this study, we report the development of mouse models for spontaneous gliomas representing distinct progressive stages of disease that are governed by defined genetic alterations. Neural stem/progenitor cell (NPC)-specific constitutive Ras activation *in vivo* plus *p53* deficiency led to development of primarily anaplastic astrocytoma (grade III), whereas combined loss of *p53* plus $p16^{Ink4a}/p19^{Arf}$ led to development of GBM (grade IV) at 100% penetrance within 6 weeks. These glioma models showed enhanced stem cell properties (stemness) accompanied by malignant progression. Notably, we determined that, in our models and in human specimens, downregulation of the homeodomain transcription factor *NKX2.2*, which is essential for oligodendroglial differentiation, was correlated with increased tumor malignancy. *NKX2.2* overexpression by GBM-derived glioma-initiating cells (GIC) induced oligodendroglial differentiation and suppressed self-renewal capacity. By contrast, Nkx2.2 downregulation in mouse NPCs accelerated GBM formation. Importantly, the inhibitory effects of NXK2.2 on GIC self-renewal were conserved in human cells. Thus, our mouse models offer pathobiologically significant advantages to investigate the nature of brain tumors, with improved opportunities to develop novel mechanism-based therapeutic approaches. *Cancer Res; 71(3); 1135–45. ©2010 AACR.*

Introduction

Glioblastoma (GBM) is the most common high-grade malignant glioma in humans and is categorized as a WHO grade IV glioma, a highly aggressive, invasive, and destructive brain tumor (1). There are 2 GBM subtypes, primary and secondary, which are distinguished by clinical characteristics. Primary GBM arises *de novo* in the absence of a preexisting low-grade lesion, whereas secondary GBM develops progressively (over 5–10 years) from lower grade gliomas such as anaplastic astrocytoma (AA, grade III). Alterations in several signaling cascades are known to affect gliomagenesis. These pathways include the receptor tyrosine kinase (RTK)/RAS/PI3K pathway (including EGFR, PDGFR, NF1, and PTEN); the p53 pathway (including TP53, CDKN2A/$p14^{ARF}$, and MDM2); and the RB pathway (including RB1, CDKN2A/$p16^{INK4A}$, CDKN2B and CDKN2C; refs. 1, 2).

Several investigators have developed mouse GBM models by genetically engineering glioma mutations. Reilly and colleagues (3) report a mouse model carrying heterozygous *cis*-germline mutations in the gene encoding a Ras GTPase-activating protein, Nf1, an effector of RTK signaling, in combination with *p53* deficiency. These mice develop malignant gliomas, including GBM and AA, with varying penetrance depending on genetic background (3). Mouse models harboring a heterozygous germline or conditional somatic *p53* mutation combined with conditional somatic *Nf1* heterozygosity develop low- to high-grade astrocytomas (4). Tumor formation is accelerated into high-grade astrocytomas similar to primary GBM by additional loss of *Pten* (5). Concomitant central nervous system (CNS)-specific deletion of *p53* and *Pten* generates a high-grade malignant glioma phenotype ranging from grade III to grade IV, with notable clinical, pathologic, and molecular resemblance to human malignant gliomas (6). Furthermore, Alcantara Llaguno and colleagues (7) have shown that inactivation of Nf1 combined with loss of other tumor suppressors (*p53* and *Pten*) in neural stem/progenitor cells (NPC), but not in non-NPCs, was both necessary and sufficient to induce glioma formation, indicating critical roles

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for Ras activation in NPCs on gliomagenesis *in vivo*. Thus, these mouse models have provided critical information regarding molecular mechanisms underlying gliomagenesis. However, in these models, phenotypic variations in malignant progression are observed even in the presence of the same mutations (5, 7). To better understand mechanisms underlying malignant glioma progression, mouse models that reliably control stages of malignant progression are needed.

Although *RAS* mutations are uncommon in human malignant gliomas, the impact of *NF1* inactivation on human and mouse glioma suggested critical roles of RAS activation in gliomagenesis (8). Proliferation of these tumors requires RAS activation and many of these tumors exhibit elevated RAS signaling, which seems to be central to their pathology (9). Consistent with this notion, several mouse GBM models have been generated by inducing constitutive Ras activation (10–12). In this study, we developed mouse models of gliomagenesis by engineering NPCs to express a constitutively active form of K-Ras via the tamoxifen-induced Cre-loxP system. Interestingly, in combination with loss of the tumor suppressors *p53* and $p16^{Ink4a}/p19^{Arf}$, mutant mice developed glioma at 100% penetrance with short latency (within 10 weeks), a clear alteration in malignant progression status. We also observed a correlation between enhanced stem cell properties (stemness) and malignant progression stages, consistent with human samples. Furthermore, we report that Nkx2.2 is a critical factor controlling self-renewal of glioma-initiating cells (GIC), an activity conserved in human GICs. Our mouse brain tumor models could be used to gain important insights into new therapeutic approaches.

Mice

LSL-K-Ras^{G12D} and $p16^{Ink4a-/-}/p19^{Arf-/-}$ mice were obtained from the Mouse Models of Human Cancers Consortium of NCI-Frederick (13, 14). *p53*$^{-/-}$ and *Nestin-CreER*^{T2} mice were previously described (15, 16). Mice were maintained on a mixed 129SvJ/C57BL/6 background. All animal experiments were approved by the Committee on Animal Experimentation of Kanazawa University and performed in compliance with the University's Guidelines for the Care and Use of Laboratory Animals.

Human brain tumor samples

Tumors from patients with glioma were surgically removed and diagnosed at the Department of Neurosurgery, Kanazawa University and at the Department of Neurosurgery, Kumamoto University. All histologic analyses of NKX2.2 expression levels were performed at low-power magnification, and the entire tissue section was evaluated rather than specific foci or selected high-power fields. NKX2.2 expression levels were scored from negative/weak to positive (≥30% of tumor cells), depending on the percentage of NKX2.2$^+$ cells in a given tumor. Human GBM patient–derived GICs, termed TGS-01 and TGS-04, were established as described previously (17). All human materials and protocols used in this study were approved by ethics committees of Kanazawa University, Kumamoto University, and the University of Tokyo. Informed consent was obtained from all patients.

Tamoxifen induction

To activate CreER^{T2}*in vivo*, 1 mg tamoxifen (Sigma) in corn oil (Sigma) was administered intraperitoneally to 8-week-old mice once daily for 5 consecutive days. Immunohistochemistry and immunofluorescence analyses were performed on coronal sections of forebrains obtained at 12 weeks of age.

Tissue preparation and histology

Sacrificed mice were perfused with 4% paraformaldehyde (PFA), and brains were dissected and postfixed overnight in 4% PFA at 4°C. Serial sections were prepared at 5 μm for paraffin sections or 10 μm for cryostat sections. Paraffin-embedded mouse brains, mouse gliomas, or human gliomas were deparaffinized and rehydrated prior to immunohistochemical analysis. Tumors were graded according to the WHO grading system for malignant astrocytomas (1).

Antibodies

Immunohistochemistry and immunofluorescence analyses were performed as described (18). Sections were examined using optical, fluorescence, and confocal microscopy (Keyence BZ-9000, Olympus FV1000, and Carl Zeiss Axio Imager A1 microscopes, respectively). Primary antibodies (Abs) recognizing the following proteins were used for immunostaining assays: Nkx2.2 (Developmental Studies Hybridoma Bank; Sigma), Ki-67 (BD), Nestin (BD and Chemicon), Sox2 (Chemicon), CD133 (eBioscience), GFAP (DAKO), Olig2 (Chemicon), βIII-tubulin (Tuj-1, Covance), NG2 (Chemicon), O4 (Chemicon), cleaved caspase-3 (Cell Signaling), HP1γ (Chemicon), γ-H2AX (Upstate), phospho-Akt^{Ser473} (Cell Signaling), CD34 (Chemicon), VEGF (Santa Cruz), PDGFRα (Cell Signaling), myelin basic protein (Mbp; Abcam), and NeuN (Chemicon). Primary Abs were detected using Alexa Fluor-conjugated secondary Abs (Invitrogen), and peroxidase-conjugated secondary Ab (GE Healthcare) plus the DAB Peroxidase Substrate Kit (VECTOR).

Tumor neurospheres

For mouse tumor neurosphere (TNS) formation, mouse glioma cells were isolated from brains of *p53*$^{-/-}$:NR^{+tamo} or $p53^{-/-}$:$p16^{Ink4a-/-}/p19^{Arf-/-}$:NR^{+tamo} mice. To assay TNS numbers, dissociated cells (2×10^3 cells/200 μL) were seeded into 96-well plates and cultured for 7 days in medium containing 20 ng/mL fibroblast growth factor-2 (FGF-2; Peprotech) and 20 ng/mL epidermal growth factor (EGF; Peprotech) as described (18). TNSs derived from TGS-01 and TGS-04 cells were cultured as described (17). Dissociated cells from sphere preparations were transfected with pLXSB-human *NKX2.2* and cultured as adherent monolayers on poly-L-ornithine–coated coverslips, and then selected in blasticidine-S (8 μg/mL) for 4 days. Transfection was performed using Fugene6 transfection reagent (Roche) according to the manufacturer's instructions. The number of spheres of diameter greater than 50 μm was determined using phase-contrast microscopy.

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Retrovirus-mediated Nkx2.2 overexpression

cDNA encoding full-length mouse *Nkx2.2* was cloned into the retroviral vector pLXSB (19). Retroviral packaging cells (Phoenix-E) were transiently transfected as above with pLXSB-*Nkx2.2*. Viral titers were estimated by observing increased drug resistance in infected NIH3T3 cells. TNSs derived from *p53*$^{-/-}$; p16$^{Ink4a-/-}$/*p19*$^{Arf-/-}$;NR^{+tamo} mice were mixed with *Nkx2.2*–expressing viral suspensions and maintained in culture for 7 days, or cultured as adherent monolayers on coverslips with a *Nkx2.2*–expressing virus suspension and then selected in blasticidine-S (8 μg/mL) for 4 days.

Retrovirus-mediated RNA interference

For short hairpin RNA (shRNA)-mediated mRNA knockdown, a retroviral vector (pSM2c) expressing shRNAs under control of the U6 promoter (Open Biosystems) was used. *Nkx2.2* shRNAs were *Nkx2.2* shRNA1 (oligoID: V2HS61850) targeting GGTCAAGATCTGGTTCCAGAA, and *Nkx2.2* shRNA2 (oligoID: V2HS152272) targeting CCAGAACCACCGCTACAAG. Control shRNA was GFP shRNA targeting GCACAAGCTGGAGTACAACTA. NPCs derived from neonatal (P3–5) p16$^{Ink4a-/-}$/*p19*$^{Arf-/-}$ mice were cultured as adherent monolayers with a control GFP shRNA or an *Nkx2.2*-shRNA-expressing retrovirus suspension, and then selected with puromycin (2 μg/mL) for 4 days, infected with *EGFRvIII*-expressing retrovirus (pLERNL), and cultured for an additional 7 days. Levels of *Nkx2.2* mRNAs before infection with *EGFRvIII*-expressing retrovirus were determined by quantitative real-time RT-PCR as described in the following section.

Quantitative RT-PCR

RNAs were purified from cultured NPCs using the RNeasy kit (QIAGEN) and reverse-transcribed using the Advantage RT-for-PCR kit (Takara-Clontech). Real-time quantitative PCR was performed using SYBR green Premix EX Taq (Takara) on an Mx3000P real-time PCR system (Aligent Technology). Sense and antisense primers are listed in Supplementary Table S1 online. The following cycle parameters were used: denaturation at 95°C for 10 seconds, and annealing and elongation for 30 seconds at 57°C for *β-actin* and at 60°C for *Nkx2.2*.

Orthotopic transplants

For intracranial injections, TNSs cultured as adherent monolayers were dissociated and resuspended in Hanks Buffered Salt Solution at a concentration of 100,000 viable cells/μL. Female NOD/SCID mice (Charles River) ages 6 to 8 weeks were anesthetized and placed into a stereotactic apparatus equipped with a *z*-axis (Stoelting). A small hole was bored into the skull 0.5 mm anterior and 3.0 mm lateral to bregma using a dental drill. Cell suspensions (2 μL) were injected into the right striatum 3 mm below the surface of the brain using a 10 μL Hamilton syringe with a 26 gauge needle. The scalp was closed using an Autoclip Applier. Animals were monitored daily for neurologic deficits.

Western blotting

Western blotting was performed as described (18). The primary Abs used recognized NKX2.2 (Developmental Studies Hybridoma Bank) or α-tubulin (Sigma).

Statistics

P values were calculated using the unpaired Student's *t* test. Survival curves were plotted using the Kaplan–Meier method, and differences were analyzed using the log-rank test. The significance of the association between NKX2.2 expression and malignancy was determined by the Fisher's exact test (right tail).

Establishment of mouse glioma models that exhibit malignant progression by *in vivo* NPC-specific Ras activation

Because Ras signaling is a major pathway upregulated in gliomagenesis, we asked whether Ras activation in NPCs could induce gliomagenesis *in vivo*. To do so, we established a mouse model in which constitutively activated Ras (K-Ras^{G12D}) could be specifically and temporally induced in NPCs by binding of tamoxifen to a Cre recombinase-modified estrogen receptor ligand-binding domain fusion protein (Cre-ER^{T2}) expressed under control of the *Nestin* promoter/enhancer (13, 15). For these experiments, we designated untreated control *Nestin-CreER*^{T2};*LSL-K-Ras*^{G12D} mice as "NR" mice, tamoxifen-treated NR mice as "NR^{+tamo}" mice, *Nestin-CreER*^{T2} mice treated with tamoxifen as "control^{+tamo}" mice, and NR mice treated with vehicle only as "NR^{-tamo}" mice. First, we treated all 4 groups of mice (at 8 weeks of age) with vehicle or tamoxifen and analyzed their brains at 12 weeks of age. Although histologic analysis revealed that the lateral ventricles were slightly enlarged in NR^{+tamo} mice compared with control^{+tamo} and NR^{-tamo} mice, the gross appearance of the brain in all animals was normal (Supplementary Fig. S1). Further analysis indicated that both expression of the proliferation marker Ki-67 antigen and 5-bromodeoxyuridine incorporation were reduced in NR^{+tamo} subventricular zone (SVZ) compared with control^{+tamo} SVZ (Supplementary Fig. S2A–C). Moreover, immunohistochemical analyses showed no differences between NR^{+tamo} and control^{+tamo} SVZ in numbers of TUNEL^{+} apoptotic cells (Supplementary Fig. S3A) or cleaved caspase-3^{+} cells (Supplementary Fig. S3B). Although it has been shown that oncogenic signaling induces cellular senescence *in vitro* and *in vivo*, we observed no significant differences between control^{+tamo} and NR^{+tamo} SVZ cells in expression of the senescence-associated markers HP1γ or γ-H2AX (Supplementary Fig. S3C), or in SA-β-gal activity (data not shown; ref. 20). Overall we conclude that enlargement of the lateral ventricles observed in NR^{+tamo} mice was largely because of Ras-induced inhibition of NPC proliferation.

NR^{+tamo} mice did not develop tumors, even after long-term observation (Fig. 1A). Therefore, because inactivation of p53 and p16^{INK4A}/p14^{ARF} is commonly seen in human gliomas, we evaluated the impact of loss of these genes on our NR model mice *in vivo*. First, we crossed our NR model mice with *p53* mutant mice. Following administration of tamoxifen, *p53*$^{-/-}$;NR^{+tamo} mice began dying of brain tumors approximately 6 weeks later, and 100% of the mice were dead by approximately 10 weeks. Histologic analyses of brain tumors revealed that most (9 of 10) were AA, with only 1 of 10 showing classic features of GBM, such as necrosis, microvascular proliferation,

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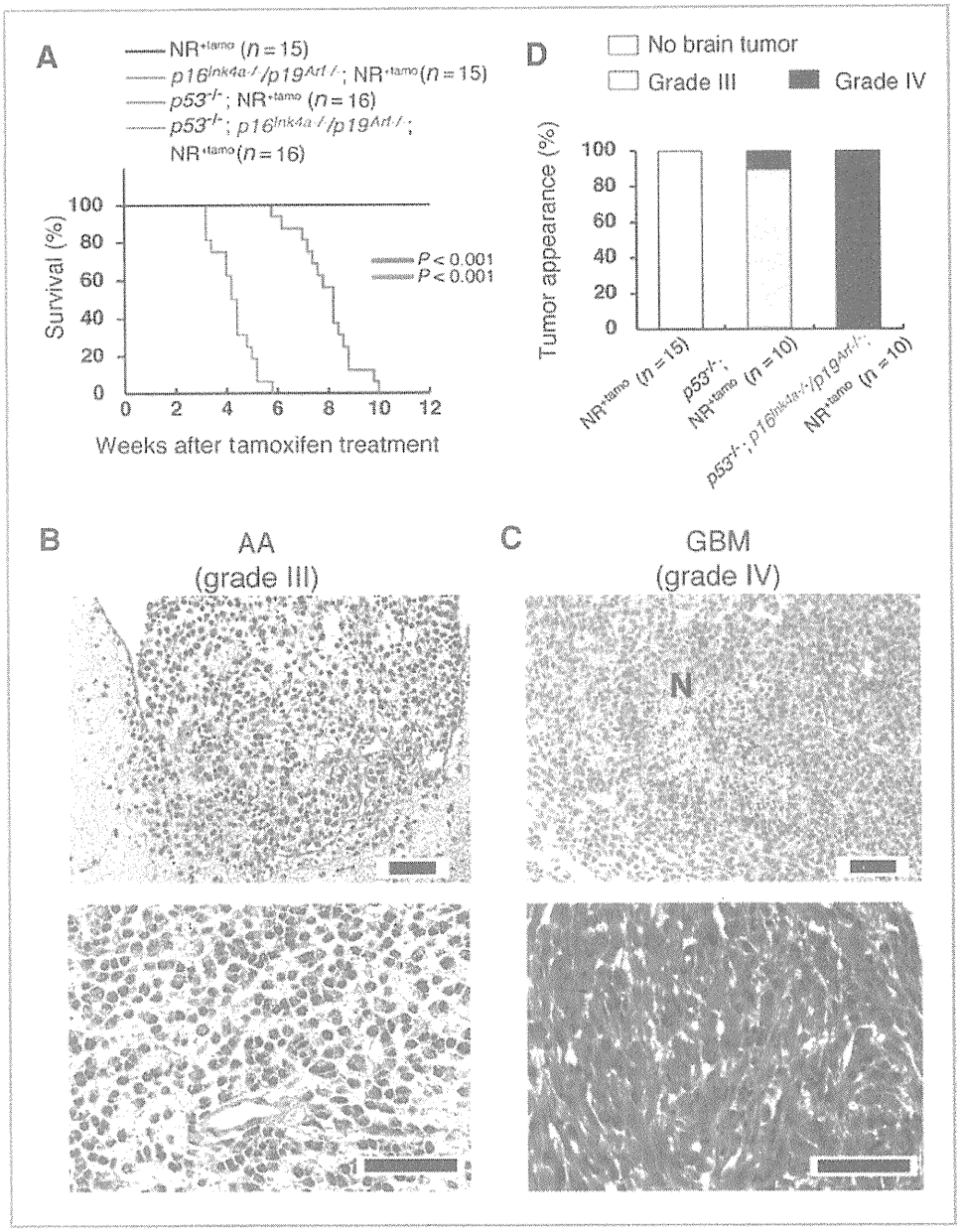

Figure 1. *p53* plus $p16^{Ink4a}/p19^{Arf}$ deficiencies combined with Ras activation drive GBM formation *in vivo*. A, altered survival. Mice of indicated genotypes (8 weeks old) were treated with tamoxifen for 5 days and monitored for tumor development. Kaplan–Meier tumor-free survival curves are shown. B and C, tumor histology. Coronal sections of forebrains of mice in (A) were prepared and stained with H&E. Tumors were graded for severity, as either AA (grade III; B) or GBM (grade IV; C). Scale bars, 50 μm. "N" indicates an area of "palisading" with regional necrosis. Data shown in (B) and (C) are representative of 10 mice/group. D, quantification of (B) and (C). Data shown are the percentage of mice in each group that developed no tumors, or grade III AA, or grade IV GBM.

marked cellular pleomorphism, and highly infiltrative spread to the cerebral cortex (Fig. 1B–D; Supplementary Fig. S4). $p53^{-/-}$;NR^{+tamo} mice did not develop any brain tumors over the period analyzed (data not shown). Next, we crossed our NR model mice with $p16^{Ink4a}/p19^{Arf}$ mutant mice. NR^{+tamo} mice on a $p16^{Ink4a}/p19^{Arf}$ null background also did not develop tumors over the 12-week period analyzed. These findings differ from previous studies showing that cultured NPCs derived from neonatal $p16^{Ink4a-/-}/p19^{Arf-/-}$ mice and infected with retrovirus carrying $K\text{-}Ras^{G12V}$ develop GBMs (10, 21). It was reported that GBM also arises when $p16^{Ink4a-/-}$ or $p19^{Arf-/-}$ mice are engineered to overexpress mutant *K-Ras* gene *in vivo* (10). By contrast to those studies, in our model the mutant *K-Ras* allele is expressed at physiologic levels. Therefore, discrepancies are likely due to differences in the amplitude of oncogenic signaling, the age of the mice used, or the cellular context (transformation *in vitro* or *in vivo*).

To investigate the effect of loss of multiple tumor suppressors in our model, we crossed NR mice with *p53* and $p16^{Ink4a}/p19^{Arf}$ mutant mice. Simultaneous deletion of $p16^{Ink4a}/p19^{Arf}$ plus *p53* significantly shortened the survival time of NR^{+tamo} mice (Fig. 1A). Interestingly, in contrast with $p53^{-/-}$;NR^{+tamo} mice, histologic analyses revealed that 100% of the tumors in $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were GBMs (Fig. 1D). As shown in Figure 2A and C, GBMs derived from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were phenotypically similar to human GBMs. Those mouse tumors showed markedly increased numbers of $Ki\text{-}67^{+}$ mitotic cells, and expressed the classic human glioma markers glial fibrillary acidic protein (Gfap) and Nestin, and high levels of vascular endothelial

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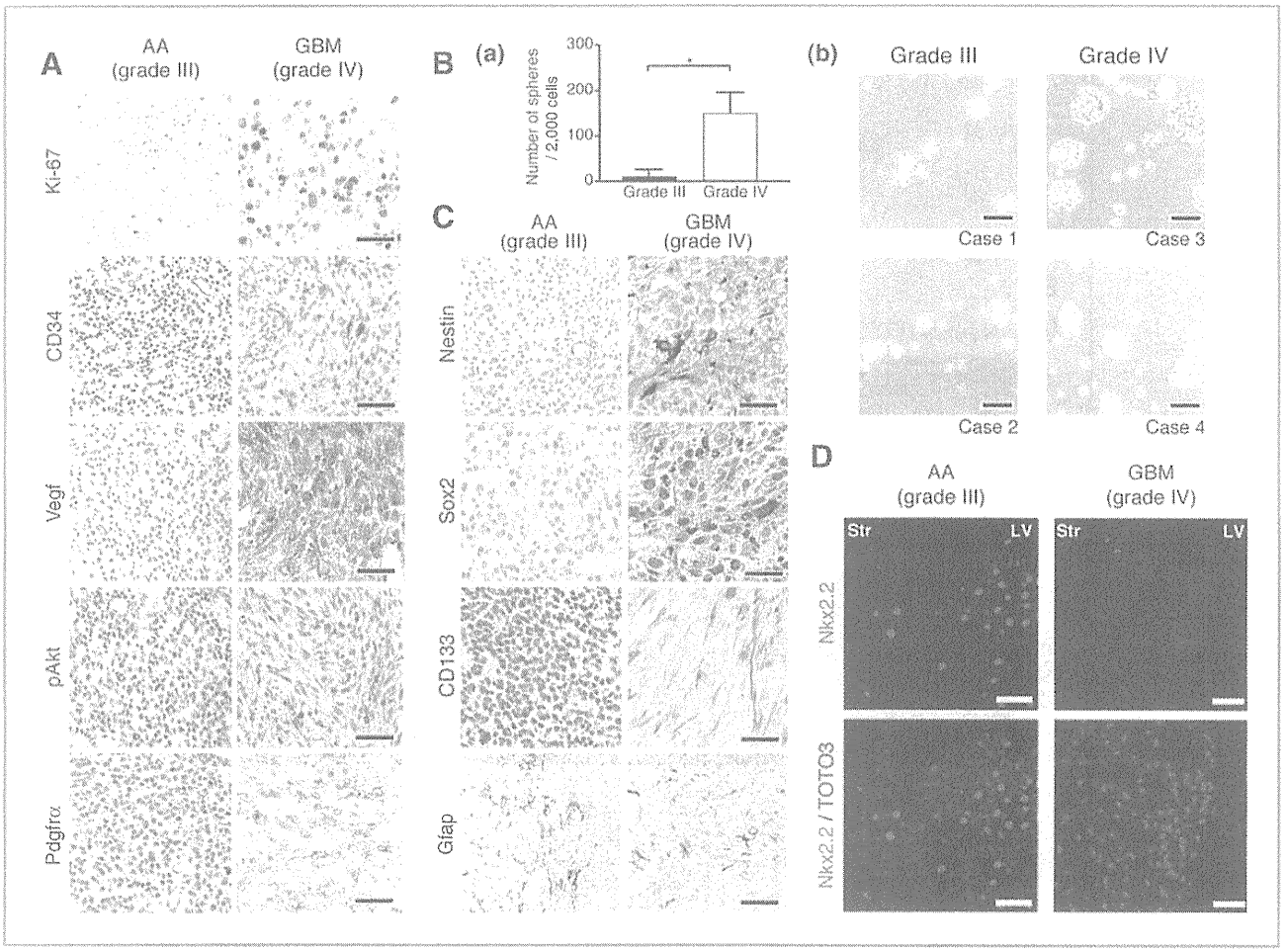

Figure 2. Enhancement of stem cell properties follows malignant glioma progression. A, sections of AAs (grade III) from $p53^{-/-}$;NR^{+tamo} mice and GBMs (grade IV) from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were immunostained to detect indicated proteins. The resulting staining patterns are highly similar to those seen in human malignant astrocytomas. Scale bars, 100 μm. B, a, increased number of TNSs derived from dissociated mouse GBM cells. b, representative images of the TNSs derived from AAs (grade III, cases 1 and 2) and GBMs (grade IV, cases 3 and 4) are shown. Scale bars, 100 μm. Data shown are the mean number ± SD of TNSs generated per 2,000 cells ($n = 5$/group). *, $P < 0.001$. C, expression of stem cell and glial differentiation markers. Sections of AA (grade III) from $p53^{-/-}$;NR^{+tamo} mice and GBMs (grade IV) from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice were immunostained to detect indicated proteins. The resulting staining patterns are reminiscent of human malignant gliomas. Scale bars, 100 μm. D, expression of oligodendroglial differentiation markers. Forebrain sections from mice in (A) were stained with anti-Nkx2.2 (red) and TOTO3. Str, striatum; LV, lateral ventricle. Scale bars, 25 μm. A, B, and D, results shown are representative of 5 mice/group and 5 experiments.

growth factor (Vegf) and phosphorylated Akt (p-Akt). Coactivation of multiple RTKs, which is often seen in human primary GBMs, was also evident in our murine GBMs, in which we observed markedly upregulated Pdgfrα expression. By contrast, AA seen in $p53^{-/-}$;NR^{+tamo} mice showed only low levels of Ki-67, Nestin, VEGF, p-Akt, and Pdgfrα (Fig. 2A and C). Microvascular formation in mouse GBMs was confirmed by detection of the endothelial marker CD34 (Fig. 2A).

Enhanced stem cell properties (stemness) accompanies malignant progression in mouse glioma models

Previous reports of human GBMs indicate that several stem cell markers, including Nestin, Sox2, and CD133 are upregulated in these malignancies (22–24). Likewise, we found that Nestin, Sox2, and CD133 were upregulated in GBMs from $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice, but not in the AA of $p53^{-/-}$;NR^{+tamo} mice (Fig. 2C). Because it has been shown that population of undifferentiated tumor cells known as tumor stem cells, or tumor-initiating cells, can undergo sphere formation *in vitro* (21), we evaluated the capacity of tumor cells in our model to form TNSs in culture by dissociating the entire tumor. A large number of TNSs was generated from cultures of cells derived from dissociated GBM, whereas AA-derived cells produced very few (Fig. 2B), indicating that mouse GBMs, like human GBMs, contain cells exhibiting stem cell properties (stemness).

Given the established inverse relationship between malignant progression and cellular differentiation, we compared expression levels of several differentiation markers in mouse GBM and AA. Consistent with evidence derived from human studies, tumor cells in either $p53^{-/-}$;NR^{+tamo} or $p53^{-/-}$;$p16^{Ink4a-/-}/p19^{Arf-/-}$;$NR^{+tamo}$ mice did not express markers