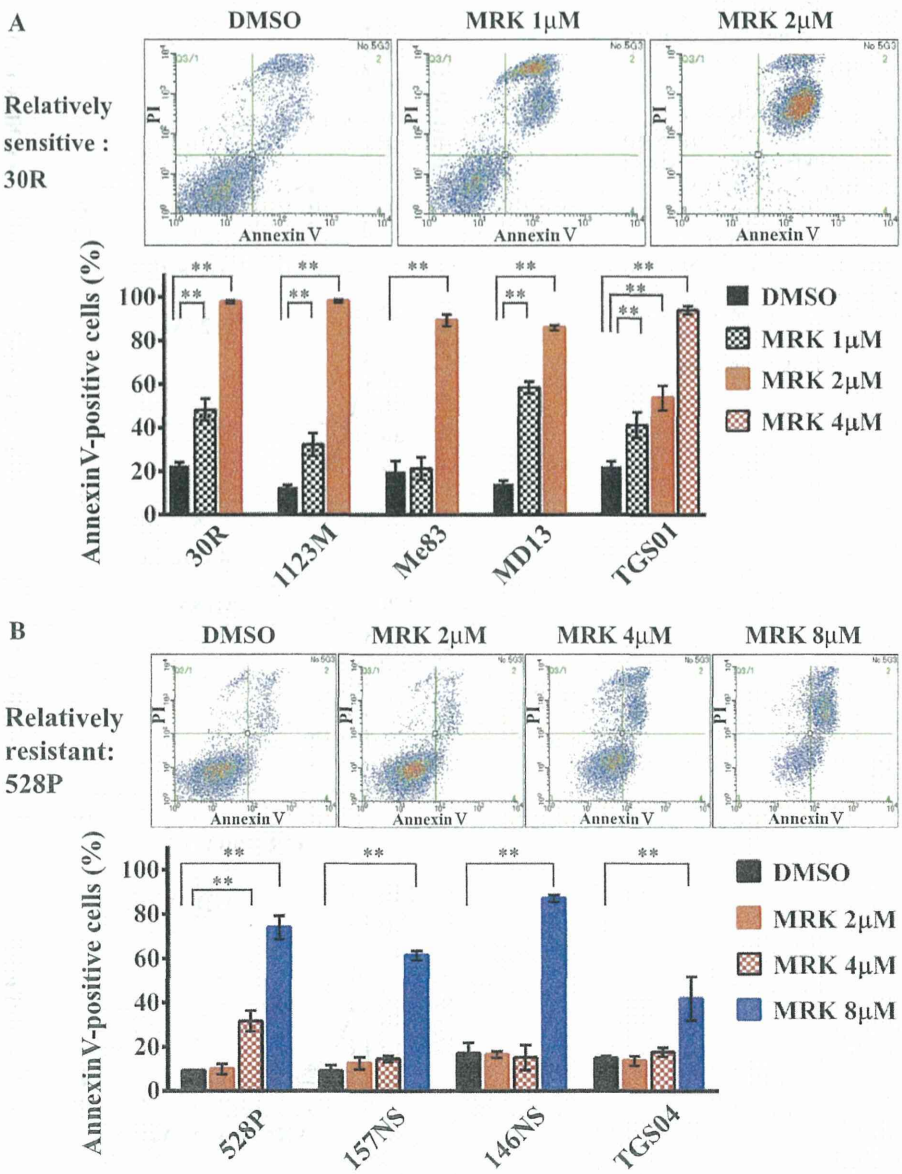


Fig. 2 MRK003 induced apoptosis in GICs. **a** MRK003 relatively sensitive GICs exhibited dramatically increased apoptosis percentage with 2 or 4 μM MRK003 compared with DMSO control. **b** 528P GIC exhibited significantly increased percentage of Annexin V-positive cells with 4 μM MRK003. In 157NS, 146NS, and TGS04 GICs, Annexin V-positive cell percentages were significantly increased in 8 μM MRK003, compared with DMSO. Representative data of flow cytometry for each GIC were shown. X-axis was indicated as Annexin V-FITC. Y-axis was indicated as PI. Experiments were conducted three times, $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ using 1-way ANOVA

GICs were treated for 72 h. Annexin V-FITC and propidium iodide (PI) double-positive cells and only Annexin V-FITC positive cells were measured by flow cytometry (JSAN; Bay bioscience Inc., Kobe, Japan).

Tumorsphere-forming assay

Sphere forming assay was performed as described previously [36, 37]. Briefly, the cultured GICs were dissociated with Stempro Accutase. The dissociated in single-cell suspension (5×10^2 cells/200 μl) were seeded in 96-well Coaster Ultra-low attachment plate in 1.0 % methylcellulose neurosphere medium with MRK003 or DMSO (control). After incubation for 7 days, tumorsphere was counted and tumorsphere-diameter was measured.

Western blot

GICs were treated with DMSO or MRK003 for 48 h and then lysed in a buffer containing 100 mM Tris (pH 6.7), 4 % SDS, phosphatase inhibitor (Thermo Fisher Scientific Inc., Waltham, MA) and protease inhibitors (complete mini) (Roche Applied Science, Indianapolis, IN). Western blotting was performed as previously [36, 37]. The following antibodies were used; total Akt, phospho-Akt (Ser473) (1:1,000 dilution, Cell Signaling Technology,

Danvers, MA) and β-actin (1:100,000 dilution, Sigma-Aldrich, St. Louis, MO).

Fluorescence activated cell sorting (FACS) for CD44/CD133 analysis

GICs were dissociated into single cells and stained with FITC-conjugated human CD44 antibody (BD Biosciences, San Jose, CA, USA) and PE-conjugated human CD133/2 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). FITC mouse IgG2b isotype (BD Biosciences) and PE mouse IgG2b isotype (Miltenyi Biotec) were used as control antibodies. The stained GICs were analyzed by flow cytometry.

Statistical analysis

All values were expressed as mean ± SD. Statistical analyses were done using Graph Pad Prism 5 software. Comparisons were drawn using 1-way ANOVA or 2-way ANOVA, followed by the Bonferroni post hoc test. The correlativity between expression of CD44-FITC or CD133-

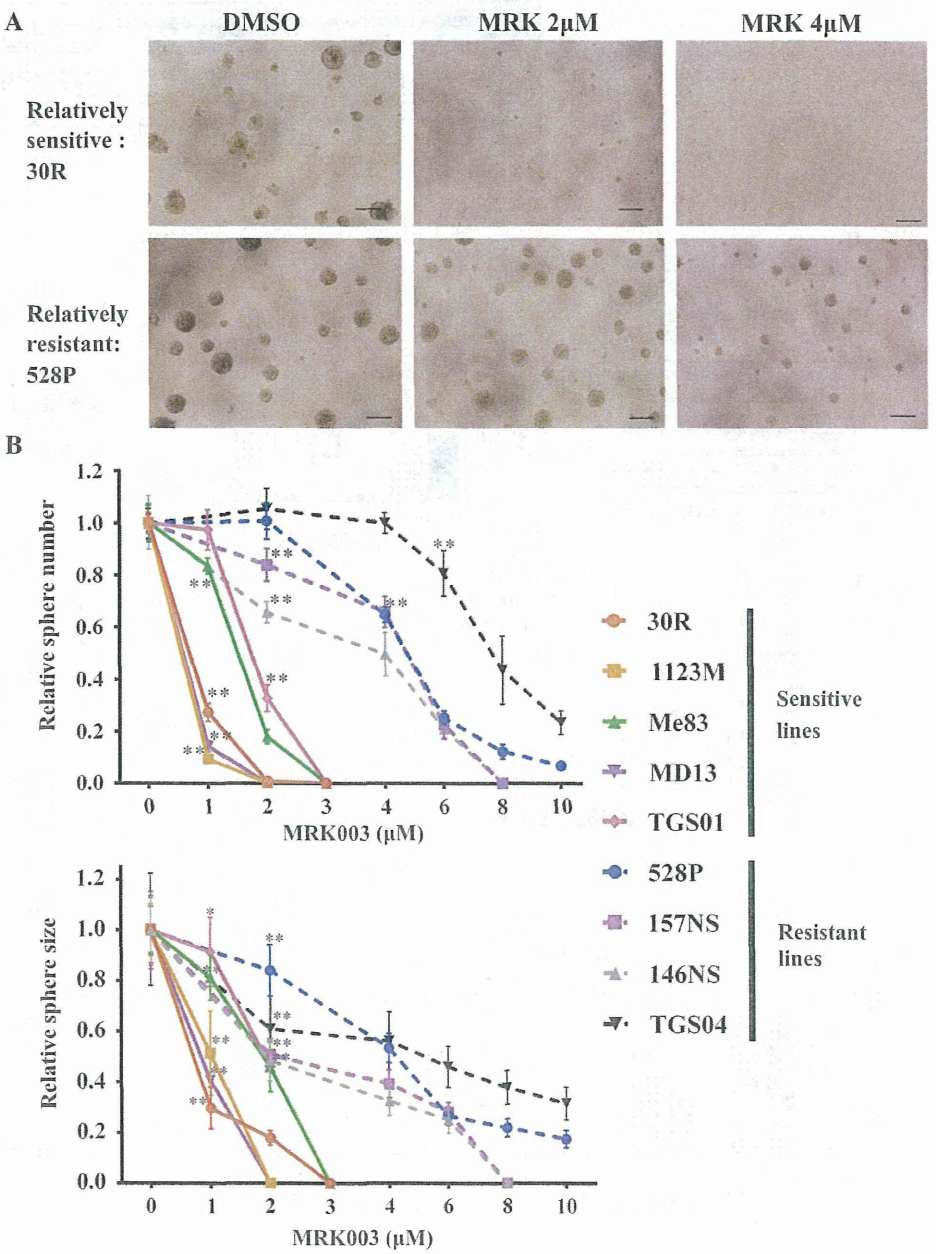
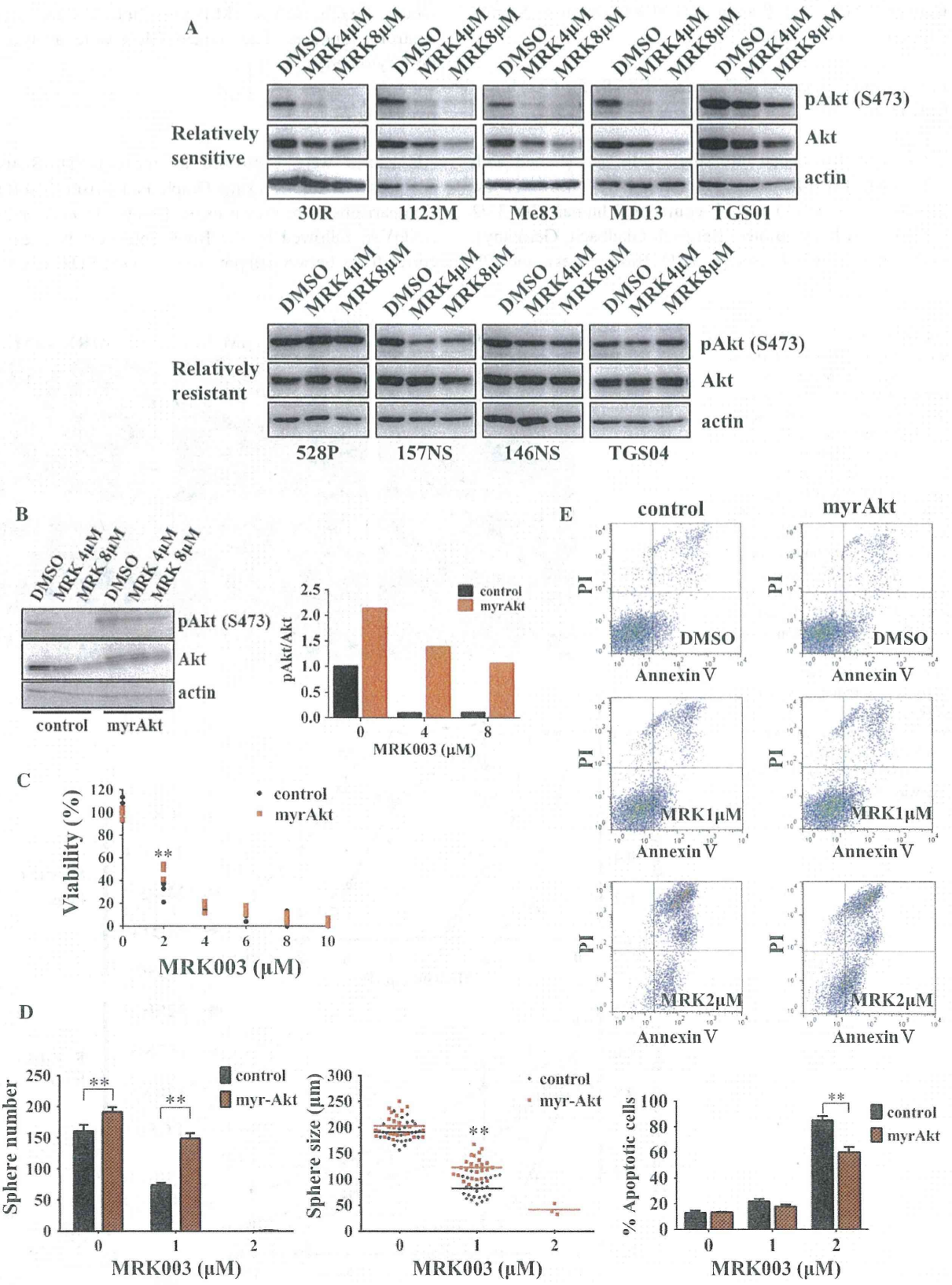


Fig. 3 MRK003 suppressed sphere-forming ability of GICs. **a** Micrographs showed the representative tumorsphere of relatively sensitive 30R and relatively resistant 528P after 7 days of MRK003 treatment. *Scale bar*, 200 μm. **b** The sphere number and size of *sensitive lines* were dramatically decreased in 1 or 2 μM MRK003. *Sphere* number of *resistant lines* was strongly decreased in more than 4 μM MRK003. Both 157NS and 146NS sphere were diminished in 8 μM MRK003. *Sphere-size* of resistant lines was significantly reduced in a dose-dependent manner from 2 μM MRK003. Experiments were conducted three times. $^{*}P < 0.05$, $^{**}P < 0.01$, using one-way ANOVA


A
DMSO
MRK4μM
MRK8μM
Relatively sensitive
pAkt (S473)
Akt
actin
30R
1123M
Me83
MD13
TGS01
Relatively resistant
528P
157NS
146NS
TGS04
B
control
myrAkt
pAkt/Akt
MRK003 (μM)
C
Viability (%)
MRK003 (μM)
D
Sphere number
Sphere size (μm)
% Apoptotic cells
myr-Akt
E
PI
Annexin V
DMSO
MRK1μM
MRK2μM

◀**Fig. 4** Relatively sensitive GICs exhibited strong inhibition of the Akt pathway by MRK003. Response of MRK003 was not completely dependent on Akt. **a** GICs treated with MRK003 for 48 h were immunoblotted with phospho-Akt (ser473) and total-Akt. Actin was used as a protein loading control. **b** Relatively sensitive GIC, 30R was transfected with empty-vector (control) or myristoylated-Akt (myrAkt) vector. Western blot analysis of phospho-Akt and total-Akt was shown. The phosphorylation levels of Akt in control-30R and myrAkt-30R were quantified using ImageJ (1.45 s, National Institutes of Health, Bethesda, MD, USA) and shown as a diagram. Actin was used as loading control. The quantified level of Akt phosphorylation in control-30R with DMSO was considered as to be 1.0. **c** Viability of myrAkt-30R was only significantly increased than control-30R in 2 μM MRK003. **d** In tumorsphere-forming assay, myrAkt-30R sphere number and sphere-size were significantly increased compared to control-30R with 1 μM MRK003. Both myrAkt-30R and control-30R exhibited negligible sphere formation in 2 μM MRK003. **e** Apoptosis assay after MRK003 treatment for 72 h was analyzed by flow cytometry of AnnexinV-FITC. In 2 μM MRK003, the AnnexinV-FITC positive percentage of myrAkt-30R was about 60 %, was significantly decreased in comparison to control-30R. All data were representative. Experiments were conducted in at least triplicate. $^{**}P < 0.01$ using two-way ANOVA

PE and IC_{50} of MRK003 was analyzed using Spearman correlation test.

Results

MRK003 decreased cell viability of patient-derived GICs

We initially investigated the chemosensitivity of the nine GICs to MRK003 by using the viability assay (Fig. 1). MRK003 decreased viability in all species of GICs in a dose dependent fashion compared with DMSO control. Based on the above results we categorized these cells into two types: the former as relatively sensitive GICs and the latter as relatively resistant GICs. Viability of five relatively sensitive GICs (30R, 1123M, MD13, Me83 and TGS01) decreased dramatically with the use of less than 2 μM MRK003 compared to DMSO control. In contrast, viability of four relatively resistant GICs (528P, 157NS, 146NS, and TGS04) was reduced to half by the addition of more than 3 μM MRK003 compared to DMSO control.

MRK003 induced apoptosis in GICs

Subsequently, we assessed whether the reduction of viability by MRK003 was associated with the induction of apoptosis since the Notch signal was related to apoptosis regulation [10, 17]. As expected, MRK003 induced apoptosis in all species of GICs. In case of the sensitive GIC lines, even less than 4 μM MRK003 induced a significant increase in the apoptosis percentage as compared to the DMSO control (Fig. 2a, Supplementary Fig. 1a). However, in case of the resistant GIC lines, more than 8 μM MRK003 was required to induce an enhanced rate of apoptosis in comparison to the control (Fig. 2b, supplementary Fig. 1b). This assay demonstrated that even low concentrations of MRK003 were capable of inducing accelerated apoptosis in GICs and reducing their viability. These findings are parallel with the results of cell viability assay, suggesting that suppression of cell viability by MRK003 might be attributable to apoptosis.

MRK003 inhibited sphere formation of GICs

We employed the tumorsphere forming assay to analyze the role of MRK003 in GICs. The basal sphere forming abilities in the 9 GICs were rich in variety. Our results demonstrated that MRK003 significantly reduced both, sphere-number and -diameter in all species of GICs in a dose dependent manner (Fig. 3a, b). In the sensitive lines, both sphere-number and -diameter were significantly decreased with the use of up to 2 μM MRK003 ($P < 0.01$), and further, sphere formation was completely blocked by 3 μM MRK003. In the resistant lines, although sphere numbers and diameters were suppressed by 2, 4 or 6 μM MRK003, dramatic suppression of sphere formation was achieved with the use of more than 8 μM MRK003, compared with DMSO control ($P < 0.01$) (Fig. 3b). These data supported the observations that MRK003 impaired self-renewal in GICs. MRK003 sensitivity of GICs in the viability and apoptosis assays corresponded well with the sensitivity of the sphere forming assay.

Akt activation modestly improved the effect of MRK003

Notch signal is known to be associated with the subsequent activation of the PI3K/Akt pathway which is related to cell proliferation and the most major pathway in GBM [11, 15, 18, 19]. Therefore, phosphorylation level of Akt was assessed in all GICs after treatment with MRK003 for 48 h. Basal level of phospho-Akt in both the sensitive and the resistant lines were approximately same. It was observed that though MRK003 effectively decreased phospho-Akt level in a dose-dependent manner in the sensitive lines, it poorly affect the phosphorylation level of Akt in the resistant lines (Fig. 4a). The phospho-Akt level in sensitive lines might be caused that MRK003 could be due to the inhibition of total Akt production. Therefore, this data led us to hypothesize that dephosphorylation of Akt by MRK003 can explain the efficacy for the sensitive lines.

We further evaluated whether Akt was responsible for the effectiveness by MRK003 in the sensitive lines. To demonstrate that Akt activation rescues cells from the

effect of MRK003, myristoylated Akt vector was constitutively anchored at the membrane for maintaining it in an activated form, and was used for the transfection of the representative relatively sensitive GIC, 30R (myrAkt-30R). MyrAkt-30R exhibited higher levels of Akt activation than 30R transfected with empty vector (control-30R) (Fig. 4b). Expression of phospho-Akt was completely suppressed in control-30R by MRK003 treatment. In contrast, phospho-Akt levels in myrAkt-30R were maintained by MRK003 treatment, although a dose dependent decrease was observed (Fig. 4b).

In cell viability assay, the decrease of viability in control-30R by treatment with 2 μM MRK003 was reduced in myrAkt-30R ($P < 0.01$). However, rescue of viability was not significant in myrAkt-30R when treated with more than 4 μM MRK003 (Fig. 4c). It suggested that transfection with myr-Akt only partially abrogated the effect of MRK003. In the tumorsphere-forming assay performed with 1 μM MRK003, number and size were significantly increased in cells transfected with myrAkt-30R as compared to cells transfected with the control-30R ($P < 0.01$). However, when the concentration of MRK003 was increased to 2 μM, Akt induction hardly rescued tumorsphere formation (Fig. 4d). In consistence with the above results, Akt induction only partially suppressed apoptosis from 85.0 ± 3.4 % (mean ± SD) to 60.0 ± 4.2 % upon treatment with 2 μM MRK003 ($P < 0.01$) (Fig. 4e). Altogether, the effect of MRK003 in 30R could not be completely recovered with transfection of the cells with myrAkt. We observed similar results in 1123 M, another population of relatively sensitive GIC used in our study (supplementary Fig. 2a–d). Accordingly, the effect of MRK003 might be only partially dependent upon the inhibition of the Akt pathway.

CD44-high and CD133-low GICs were sensitive to MRK003 treatment

CD44 and CD133 were known as cancer stem cell surface markers [5, 38–40]. We analyzed the expression of CD44 and CD133 in the nine GICs. Our data indicated that sensitive lines presented high CD44 and low CD133 expression level, and that resistant lines exhibited high level expression of CD133 (Fig. 5a, b, supplementary Fig. 3a, b). We further investigated whether the expressions of CD44 and CD133 could serve as indicators of MRK003 sensitivity and analyzed the correlation between CD44 and CD133 expression levels and IC_{50} of MRK003. IC_{50} of GICs for MRK003 correlated negatively with CD44 expression ($P = 0.005$, $r = -0.865$), and positively with CD133 expression ($P = 0.037$, $r = 0.712$) (Fig. 5c). These findings indicated that the expression level of CD44 and CD133 may be useful as biomarkers for MRK003 efficacy.

Discussion

The data in the present study have demonstrated that MRK003 suppressed cell viability and sphere formation ability, and induced apoptosis in GICs derived from multiple patients. These results support effectiveness of MRK003 for GICs properties. Interestingly, GICs were divided into relatively sensitive GICs and relatively resistant GICs against MRK003. MRK003 sensitive and resistant GICs were demonstrated to be different by CD44 and CD133 expressed patterns. This study is the first to demonstrate that MRK003 might have strong therapeutic potential for CD44-high and CD133-low expressed GICs.

Although the downstream details of Notch signaling are yet to be deciphered, the Akt pathway which was crucial pathway in GBM had been known to be associated with Notch downstream signal [10, 13, 17, 20]. Our data demonstrated that phosphorylation of Akt in MRK003 relatively sensitive GICs was remarkably decreased by MRK003 treatment. However, transfection with constitutively active Akt vector could not negate the effect of MRK003 completely, thereby suggesting that the effect of MRK003 was only partially dependent on the inhibition of the Akt pathway. Notch signal is a major regulator of survival pathway to play a prominent role in bridging various pathways. Alternate cross-talk pathways for Notch signaling besides PI3K/Akt are TGF-β, MEK/ERK and JAK/STAT3 pathways [41, 42], which are also associated with cell proliferation in GBM [17, 20, 43]. The nature of Notch cross-talk system was flexible and was depended on each cell background [41]. Therefore, it is possible that MRK003 inhibits not only Notch signaling but also other pathways. Further investigations are warranted in this regard.

CD44 is reported to be related to tumor initiations and a novel therapeutic target [40, 43]. CD133 is a cell surface marker of cancer initiating cells in several cancer types although its validity remains controversial [5, 44]. In our findings, CD44-high and CD133-low expressed GICs are relatively sensitive to MRK003, whereas CD133-high expressed GICs are relatively resistant to MRK003. One explanation for chemoresistance to MRK003 is that CD133-high GICs may have potential drug resistance gene against chemotherapy. For example, CD133-high GICs possess high level of drug resistance gene, BCRP1 [45]. CD44-enriched GICs was regulated by TGF-β pathway which is a key regulator of malignant phenotype [35, 43, 46]. It may be hypothesized that the effect of Notch inhibition is exhibited partially via the TGF-β inhibition [43, 47]. GBM was segregated into four subtypes based on gene expression signature. CD44 was enriched in the mesenchymal subtype of GBM [48]. In our findings, GICs with high CD44 expression demonstrated to be very sensitive to

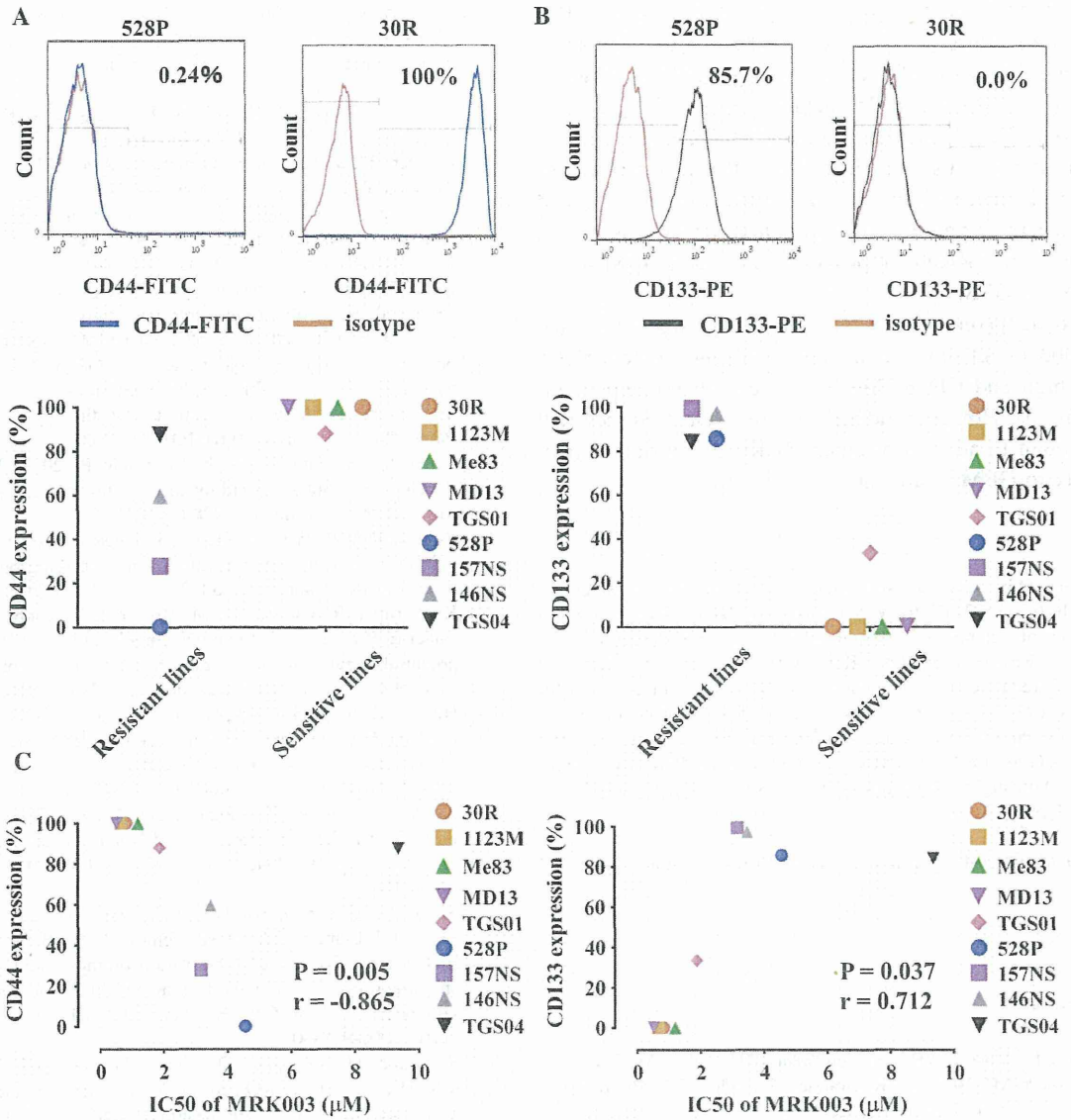


Fig. 5 MRK003 might be effective for CD44-high and CD133-low GICs. **a** *Sensitive lines* were high CD44-FITC positive percentage (range 87.9–100 %). On the other hand, *resistant lines* were broad CD44-FITC positive percentage (range 0.24–87.7 %). **b** *Sensitive lines* exhibited a low CD133-PE positive percentage (range 0–33.4 %). *Resistant lines* exhibited high CD133-PE positive percentage (range 84.2–99.4 %). **c** IC_{50} of MRK003 for GICs correlated to both CD44 and CD133 expression. Data shown was *scatter diagram* of nine GICs. X-axis was IC_{50} (μM) of MRK003. Y-axis was expression (%) of CD44 or CD133

MRK003. Therefore, MRK003 might be effective to mesenchymal subtype of GBM.

We performed sphere forming assay with other γ-secretase inhibitor, DAPT and L685,458. DAPT had the strong effect for 30R which was a relatively sensitive line to MRK003 (supplementary Fig. 4). On the other hand, L685,458 reduced sphere formation not only in 30R but also in TGS01 and TGS04 (data not shown). Generally, γ-secretase inhibitors can be classified according to the chemical structures and pharmacological modes of action. MRK003, DAPT and L685,458 are a sulfonamide-containing non-transition state analog, an azepine-containing non-transition state analog and an azepine-containing transition state analog, respectively [42, 49]. The different experimental results with three γ-secretase inhibitors might be caused by different pharmacological modes of action. These inhibitors might modulate different downstream signaling pathways. In fact, these inhibitors affect various intracellular signaling including PI3K/Akt signaling [50].

A further point of investigation in our study was to confirm the role of Notch pathway inhibition in effecting

the MRK003 response, since the possibility of MRK003 acting via off-targets cannot be ruled out. However, accumulated evidence confirmed the strong effect of MRK003 for cancer initiating cells including leukemia [22, 27], lymphoma [25], breast [24, 26], and pancreas [23] in vitro and in vivo. In this study also, MRK003 provided a therapeutic advantage against the chemo resistant population of GICs derived from the nine patients. Regardless of the specificity of MRK003, it promises to be an effective target therapy for GBM.

In conclusion, results of this study suggested that MRK003 might have significant therapeutic potential for CD44-high and CD133-low expressed GICs (supplementary Fig. 5). However, additional pre-clinical studies will be required to address whether MRK003 contributes beneficially to GBM treatment.

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Conflict of interest All authors disclosed no potential conflicts of interest.

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RADIATION ONCOLOGY

CASE REPORT Open Access

A case of radiation-induced osteosarcoma treated effectively by boron neutron capture therapy

Gen Futamura[1], Shinji Kawabata[1], Hiroyuki Siba[1], Toshihiko Kuroiwa[1], Minoru Suzuki[2], Natsuko Kondo[2], Koji Ono[2], Yoshinori Sakurai[3], Minoru Tanaka[4], Tomoki Todo[4] and Shin-Ichi Miyatake[5*]

Abstract

We treated a 54-year-old Japanese female with a recurrent radiation-induced osteosarcoma arising from left occipital skull, by reactor-based boron neutron capture therapy (BNCT). Her tumor grew rapidly with subcutaneous and epidural extension. She eventually could not walk because of cerebellar ataxia. The tumor was inoperable and radioresistant. BNCT showed a marked initial therapeutic effect: the subcutaneous/epidural tumor reduced without radiation damage of the scalp except hair loss and the patient could walk again only 3 weeks after BNCT. BNCT seems to be a safe and very effective modality in the management of radiation-induced osteosarcomas that are not eligible for operation and other treatment modalities.

Introduction

The incidence of radiation-induced sarcoma has been estimated to be between 0.03% and 0.3% of all patients who have received radiation therapy [1,2]. Radiation-induced osteosarcomas are being encountered more frequently as the use of radiation therapy becomes more common, and the number of long-term cancer survivors has increased. The original diagnostic criteria for radiation-induced osteosarcomas were proposed in 1948 by Cahan et al. [3], and a short latency period was recently accepted for these tumors [1,4,5]. The diagnosis of radiation-induced osteosarcoma must fulfill the following four criteria: (1) the sarcoma must arise in a previously irradiated field, (2) the sarcoma must be histologically distinct from the original neoplasm, (3) there was no evidence of tumor in the involved bone at the time of initial irradiation, and (4) there must be a latency period between the irradiation and the development of the sarcoma at least 3 years.

Radiation-induced osteosarcoma of the head is a devastating complication of radiation therapy. It is very rare but aggressive, with high recurrence and a poor prognosis [6]. The median overall survival time was reported to be 29 months [1]. Osteosarcoma is thought to be radioresistant [7,8]. Therefore, complete surgical resection has been described as the most important prognostic factor [9] and the first choice of treatment for radiation-induced osteosarcoma. However, if complete surgical resection is difficult (as it was in the present case), adjuvant chemotherapy and radiotherapy should be considered. These therapeutic effects have thus far been found to be insufficient, however. We report here the case of a patient with recurrent radiation-induced osteosarcoma who was treated effectively by boron neutron capture therapy (BNCT).

BNCT is based on the nuclear capture reactions that occur when non-radioactive boron-10 is irradiated with neutrons of the appropriate energy to yield high linear energy transfer (LET) alpha particles (4He) and recoiling lithium-7 (7Li) nuclei. Since these particles have short path-lengths of approximately one cell diameter, their lethality is primarily limited to boron-containing cells. Theoretically, high LET particles have the advantage to overcome radioresistance to photon radiotherapies (such as X-rays). BNCT can thus be regarded as tumor cell-selective and an intensive particle radiation modality with minimal damage to normal tissue, [10,11] even for X-ray-resistant tumors. Here we report a successfully treated a case of radiation-induced osteosarcoma by reactor-based BNCT.

Case report

A 54-year-old Japanese female was referred to our institute for treatment by BNCT of a recurrent radiation-induced

* Correspondence: neu070@poh.osaka-med.ac.jp
[5]Cancer Center, Osaka Medical College, Takatsuki, Japan
Full list of author information is available at the end of the article