Tumor Biol. (2014) 35:5593–5598
DOI 10.1007/s13277-014-1739-x

RESEARCH ARTICLE

EGFR signaling-dependent inhibition of glioblastoma growth by ginsenoside Rh2

Shaoyi Li · Yun Gao · Weining Ma · Wenchang Guo · Gang Zhou · Tianci Cheng · Yunhui Liu

Received: 22 January 2014 / Accepted: 7 February 2014 / Published online: 21 February 2014

Abstract Glioblastoma is the most common and most aggressive malignant primary brain tumor in humans, accounting for 52 % of all functional tissue brain tumor cases and 20 % of all intracranial tumors. The typical treatment involves a combination of chemotherapy, radiation, and surgery, whereas it still achieves fairly poor patient survival. Ginsenoside Rh2 has been reported to have a therapeutic effect on some tumors, but its effect on glioblastoma has not been extensively evaluated. Here, we show that ginsenoside Rh2 can substantially inhibit the growth of glioblastoma in vitro and in vivo in a mouse model. Moreover, the inhibition of the tumor growth appears to result from combined effects on decreased tumor cell proliferation and increased tumor cell apoptosis. Further analyses suggest that ginsenoside Rh2 may have its antiglioblastoma effect through inhibition of the epidermal growth factor receptor (EGFR) signaling pathway in tumor cells. In a lose-of-function experiment, recombinant EGFR was given together with ginsenoside Rh2 to the tumor cells in vitro and in vivo, which completely blocked the antitumor effects of ginsenoside Rh2. Thus, our data not only reveal an anti-glioblastoma effect of ginsenoside Rh2 but also demonstrate that this effect may function via inhibition of EGFR signaling in glioblastoma cells.

Keywords Glioblastoma · Ginsenoside Rh2 · Epidermal growth factor receptor signaling

S. Li (✉) · W. Ma · W. Guo · G. Zhou · T. Cheng · Y. Liu
Department of Neurosurgery, Shengjing Hospital of China Medical University, 36 Sanhaojie, Shenyang 110004, Liaoning Province, China
e-mail: lishaoyi2014@163.com

Y. Gao
He University, Shenyang, China

Abbreviations

EGFR	Epidermal growth factor receptor
GRh2	Ginsenoside Rh2

Introduction

Glioblastoma multiforme (GBM) is the most malignant primary human brain cancer and cannot be cured by any therapy available today. Current therapy includes complex treatments of neurosurgical resection, radiotherapy, and chemotherapy, with extreme poor outcome. The typical life expectation for GBM patient is less than 1 year. The tumor localizes in the central nervous system and appears to be very solid to prevent its impermeability to large particles [1]. A major challenge for GBM treatment is to efficiently control tumor growth by killing the cancer cells on the surface of the tumor more rapidly than replication of the inner tumor cells. Thus, high selectivity and safety of the treatment to avoid damage to the normal brain tissue, rapid and efficient cell killing, and inhibition of tumor cell replication may be all crucial for a successful treatment for GBM [1].

Most of GBM overexpressed epidermal growth factor receptor (EGFR) [2–17]. Therefore, drugs that specifically target EGFR and subsequently inhibit EGFR signaling in glioblastoma cells may have a therapeutic effect on GBM. Although approaches have been widely taken by researchers to search for ways to inhibit EGFR signaling in glioblastoma cells, no safe and efficient therapy for glioblastoma has been achieved [2–17].

Ginsenoside Rh2 is one of the characteristic components in red ginseng with potential bioactivity. Ginsenoside Rh2 and its derivatives have been reported of potentially therapeutic effects on ovarian cancers [18–21], pancreatic adenocarcinoma [22], prostate cancer [23], colorectal cancer [24], and

breast cancer [25, 26], with its antitumor effects through inhibition of tumor cell proliferation and induction of tumor cell apoptosis. Nevertheless, the exact molecular basis of the antitumor effect of ginsenoside Rh2 remains unclear. Moreover, the effect of ginsenoside Rh2 on glioblastoma has not been studied before.

Here, we show that ginsenoside Rh2 can substantially inhibit the growth of glioblastoma in a mouse model. Moreover, the inhibition of the tumor growth appears to result from combined effects on decreased tumor cell proliferation and increased tumor cell apoptosis. Further analyses suggest that ginsenoside Rh2 may have its antiglioblastoma effect through inhibition of the EGFR signaling pathways in tumor cells. In a lose-of-function experiment, recombinant EGFR was given together with ginsenoside Rh2 to the glioblastoma cells in vitro and in vivo, which completely blocked the antitumor effects of ginsenoside Rh2. Thus, our data not only reveal an antiglioblastoma effect of ginsenoside Rh2 but also demonstrate that this effect may function via inhibition of EGFR signaling in glioblastoma cells.

Materials and methods

Culture and label human glioblastoma cell line with a luciferase reporter

The human glioblastoma cell line A-172 (GC) established by Dr. Giard [27] was cultured in 1:1 mixture of DMEM and Ham's F12 medium supplemented with L-glutamine and 5 % FBS. To trace the glioblastoma cells in vivo, we infected the cells with a recombinant lentivirus expressing luciferase and green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter at MOI 100 and resulted in nearly 100 % infection efficiency based on green fluorescence. The reporter-carrying glioblastoma cells were termed GC-luc. Ginsenoside Rh2 (Weikeqi Bioscience, China) (0.1 mg/ml) alone, or with recombinant EGFR (0.2 μg/ml) (Millipore, USA), was given to the cultured GC-luc for 24 h before analysis.

Mouse manipulations

All mouse experiments were approved by the general principles contained in the Guide for the Care and Use of Laboratory Animals published by China Medical University. Ten-week-old male NOD/SCID mice were used for the experiments. Ginsenoside Rh2 (1 mg/kg body weight) was injected from the tail vein of the mice twice per week for 1 month, till the end of the experiment. Recombinant EGFR (2 μg/kg body weight) was stereotactically injected to the site of glioblastoma once per week, starting at the time of ginsenoside Rh2 administration, till the end of the experiment.

Induction of intracranial tumor

Into the brains of male NOD/SCID mice of 10 weeks of age, 10^4 GC-luc was stereotactically implanted as has been previously described [28]. One month after, the animals were examined of tumor growth by luciferase assay.

Imaging of glioblastoma by bioluminescence

Bioluminescence was measured with the IVIS imaging system (Xenogen Corp., Alameda, CA). All of the images were taken 10 min after intraperitoneal injection of luciferase (Sigma, China) of 150 mg/kg body weight, as a 60-s acquisition and 10 of binning. During image acquisition, mice were sedated continuously via inhalation of 3 % isoflurane. Image analysis and bioluminescent quantification were performed using Living Image software (Xenogen Corp.).

Histology and Western blot

Brain tissue was fixed in 4 % paraformaldehyde and embedded in paraffin and sectioned at 6 μM, before it was stained with hematoxylin and eosin. Proteins were extracted from the brain tissue by RIPA buffer (Sigma) and used for Western blot. Primary antibodies for Western blot are rabbit EGFR and beta-actin (Cell Signaling). Secondary antibody is HRP-conjugated antirabbit (Jackson Labs). Images shown in the figure were representative from five individuals.

In vitro proliferation assay and apoptosis assay

Bromodeoxyuridine (BrdU, Sigma) was given at a concentration of 1 mg/ml to the cultured cells 3 h before analysis to label proliferating glioblastoma cells. At least 1,000 cells were counted for each condition. Each condition contained at least five replicates. Five mice were used for each group. Apoptotic cells were determined by staining of propidium iodide (PI), as has been previously described [29].

Statistics

All values are depicted as mean ± standard deviation and are considered significant if $p<0.05$. All data were statistically analyzed using two-tailed student t test.

Results

Ginsenoside Rh2 efficiently inhibited the growth of glioblastoma in vivo

The human glioblastoma cell line A-172 (GC) established by Dr. Giard [27] was used to generate a glioblastoma model to examine the effect of ginsenoside Rh2. To trace these glioblastoma cells in vivo, we infected the cells with a recombinant lentivirus expressing luciferase and GFP under the control of a CMV promoter. The reporter-carrying glioblastoma cells were termed GC-luc, which were stereotactically injected into the brains of male NOD/SCID mice 10 weeks of age as described (Fig. 1a) [28]. One month after, the animals were examined of tumor growth by luciferase assay, showing establishment of the brain tumor (Fig. 1b).

Ginsenoside Rh2 was then intravenously administrated at a concentration of 1 mg/kg body weight to the mice, twice per week for another month (Fig. 1a). We chose this dose for ginsenoside Rh2, since it has been applied before in the treatment of other cancers [18–26]. The animals were again examined of tumor growth by luciferase assay. The bioluminescence levels in the mice significantly decreased 1 month after ginsenoside Rh2 injection (76.8±12.5 %) (Fig. 1b), suggesting tumor growth inhibition. Moreover, the bioluminescence levels in the mice that received saline injection increased by 6.8±1.5 % fold in 1 month (Fig. 1b), suggesting aggressive tumor growth. Histology further confirmed these findings from bioluminescence imaging, showing a significant decrease in the size of tumor by ginsenoside Rh2 treatment (Fig. 1c). These data suggest that ginsenoside Rh2 efficiently inhibited the growth of glioblastoma in vivo.

Ginsenoside Rh2 decreased proliferation, and increased cell death, of glioblastoma cells

To study the mechanism underlying the inhibitory effect of ginsenoside Rh2 on the growth of glioblastoma, we added 0.1 mg/ml ginsenoside Rh2 into the culture of glioblastoma cells. After 24 h, BrdU was added to evaluate cell proliferation and PI was used to evaluate cell death. We found that ginsenoside Rh2 significantly inhibited cell proliferation of the glioblastoma cell line (Fig. 2a) and significantly increased cell death (Fig. 2b). These data suggest that the

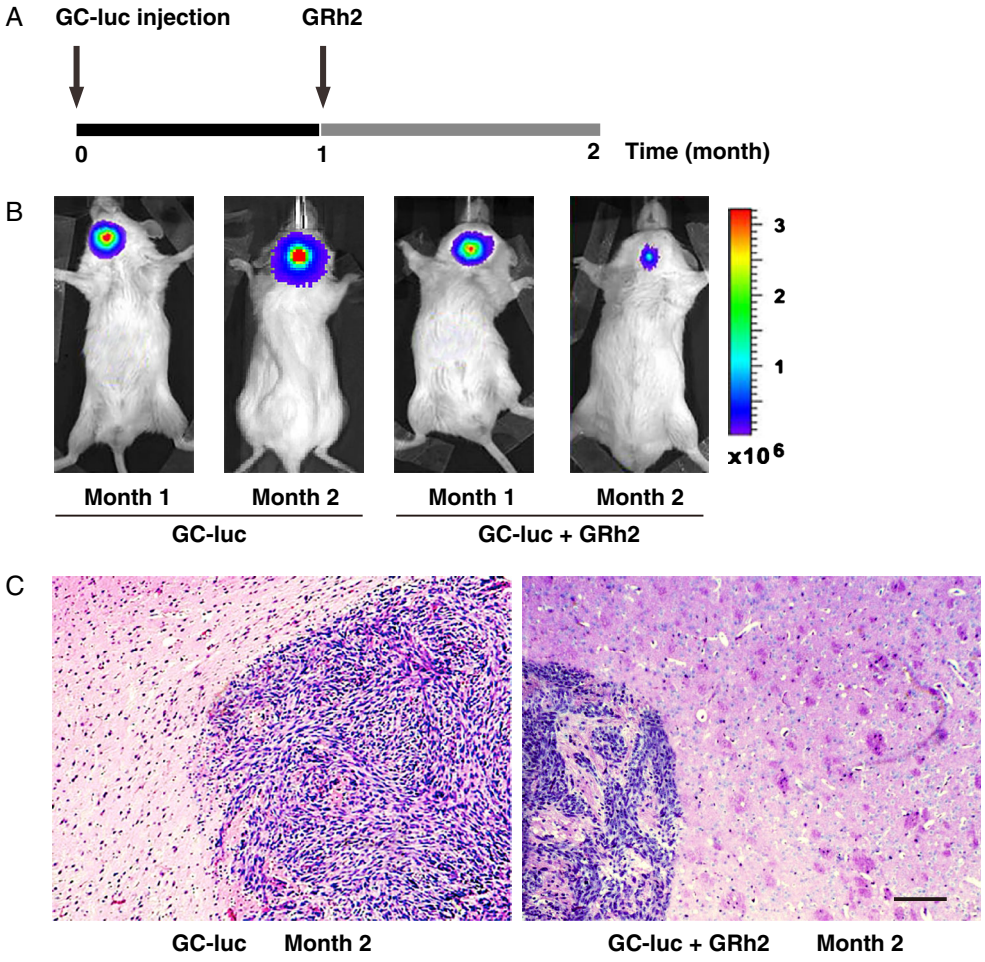

Fig. 1 Ginsenoside Rh2 efficiently inhibited the growth of glioblastoma in vivo. **a** The schematic of the experiment. The luciferase-reporter-carrying human glioblastoma cell line A-172 (GC-luc) was stereotactically injected into the brains of NOD/SCID mice. One month later (month 1), the mice received i.v. injection of ginsenoside Rh2 twice per week for another month (month 2). **b** Detection of bioluminescence of the implanted GC-luc in the same mouse at month 1 and month 2, without ginsenoside Rh2 treatment. Representative bioluminescence images were shown. **c** Histology of grafted glioblastoma in the brain. *Scale bar* is 40 μm

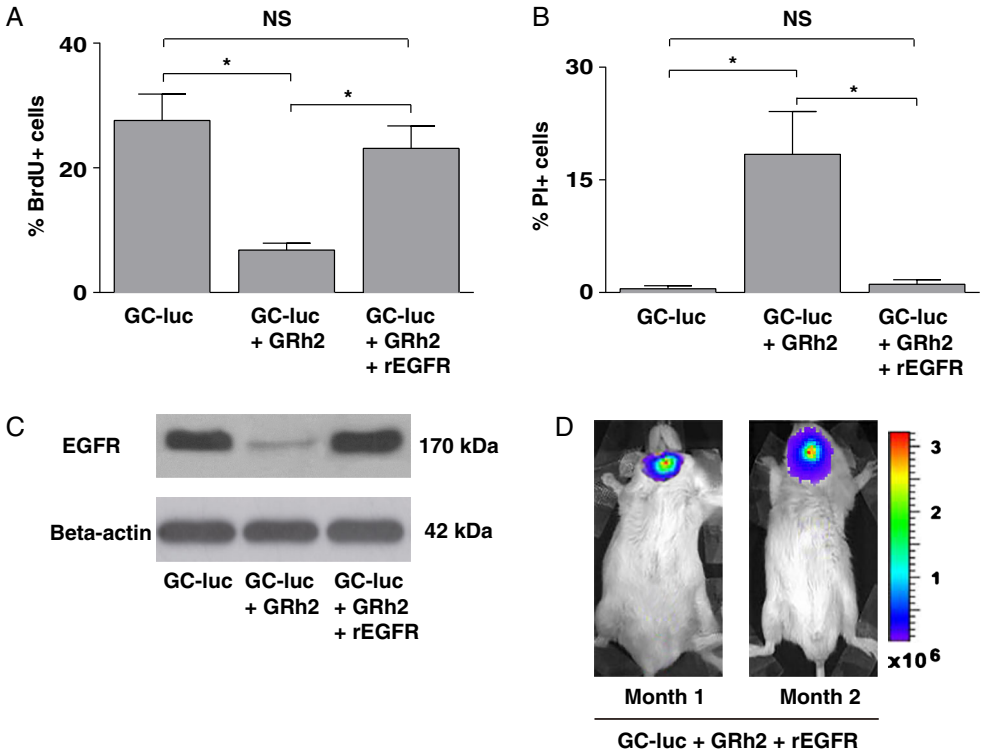

Fig. 2 Inhibition of glioblastoma by ginsenoside Rh2 is EGFR signaling dependent in vitro and in vivo. **a**, **b** Ginsenoside Rh2 without rEGFR was added into the culture of glioblastoma cell line for 24 h to evaluate the inhibitory effect of ginsenoside Rh2 on glioblastoma growth. Three hours of BrdU labeling was applied to evaluate the changes in cell proliferation (**a**). PI was used to examine cell death (**b**). **c** Representative Western Blot images for EGFR and beta-actin (loading control). Ginsenoside Rh2 treatment significantly downregulated EGFR expression in the brain glioblastoma, which could be inhibited by rEGFR administration. **d** Detection of bioluminescence of the implanted GC-luc in the same mouse that received ginsenoside Rh2 treatment, without eEGFR, at month 1 and month 2. Representative bioluminescence images were shown. *$p<0.05$

antiglioblastoma effect of ginsenoside Rh2 is from a combination of decreased cell proliferation and increased cell death.

Inhibition of glioblastoma growth in vitro by ginsenoside Rh2 is EGFR signaling dependent

Since many reports showed that inhibition of EGFR signaling in glioblastoma cells may reduce the tumor growth [2–17], we examined whether it may also account for the antiglioblastoma effect of ginsenoside Rh2. We extracted protein from the glioblastoma dissected from the mice at the end of a 1-month treatment with ginsenoside Rh2 or saline (Fig. 1a) and analyzed EGFR levels by Western blot. We found that the ginsenoside Rh2-treated glioblastoma had a significantly lower level of EGFR, compared with control saline-treated glioblastoma (Fig. 2c), suggesting that the ginsenoside Rh2 treatment significantly decreased the EGFR expression in glioblastoma.

To define whether EGFR signaling inhibition is necessary for the ginsenoside Rh2-induced inhibition of glioblastoma growth, we gave a recombinant EGFR (rEGFR), together with ginsenoside Rh2, to the cultured glioblastoma cell line, which potentially inhibited the antiproliferative and apoptosis-inductive effects of ginsenoside Rh2 (Fig. 2a, b). This loss-of-function experiment demonstrates that the inhibition of the growth of cultured glioblastoma cell line by ginsenoside Rh2 is EGFR signaling dependent.

Inhibition of glioblastoma in vivo by ginsenoside Rh2 is EGFR signaling dependent

To examine whether the in vivo antiglioblastoma effect of ginsenoside Rh2 is also EGFR dependent, as what we had found in vitro, rEGFR was stereotactically injected to the site of glioblastoma once per week, starting at the time of ginsenoside Rh2 administration for 1 month, till the end of the experiment. Strikingly, presence of rEGFR in situ completely prevented the antiglioblastoma effect by ginsenoside Rh2 administration (Fig. 2c). The bioluminescence levels in the mice that received both ginsenoside Rh2 and rEGFR increased by 6.3±1.3 % fold in 1 month (no difference from saline injection, 6.8±1.5 %, Fig. 1b).

Moreover, the protein isolated from the tumor cells of the rEGFR- and ginsenoside Rh2-treated mice had a similar

EGFR level as from the tumors without ginsenoside Rh2 treatment, suggesting that the effect of ginsenoside Rh2 on glioblastoma was completely blocked by the administration of rEGFR. Thus, inhibition of the growth of glioblastoma in vivo by ginsenoside Rh2 is EGFR signaling dependent.

Discussion

According to previous reports on GBM, EGFR was overexpressed in 62 % of brain glioblastoma [30]. EGFR activation increases brain tumor angiogenesis and promotes tumor metastasis [2–17]. Therefore, drugs that specifically target EGFR and subsequently inhibit EGFR signaling in glioblastoma cells may have a therapeutic effect on GBM. Ginsenoside Rh2 has been shown to have antitumor effects on various cancers [18–26]. Nevertheless, its effect on glioblastoma has not been examined yet.

Here, for the first time, we show that the growth of human glioblastoma can be efficiently inhibited in vivo and in vitro. Since immunodeficient NOD/SCID mice, which generate little immune responses to xenografts, were chosen to be the host for the grafted tumor cells, the potential inhibitory effect of ginsenoside Rh2 on glioblastoma growth in the current study may not essentially result from an adaptation of the immune responses to the tumor. Indeed, the inhibitory effect of ginsenoside Rh2 on glioblastoma cells was shown here to be from both decreased cell proliferation and increased cell death. Immune cells was not involved in our in vitro system, further suggesting that the effect of ginsenoside Rh2 on glioblastoma cells was direct and may work via induction of autologous changes in the glioblastoma cells themselves, which subsequently leads to inhibition of cell proliferation and increased cell death.

These notions were confirmed by our examination of EGFR signaling. We found that the ginsenoside Rh2-treated glioblastoma had a significantly decreased EGFR level, compared with control saline-treated glioblastoma. Since EGFR signaling has been shown to be essential for most glioblastoma cells, our data suggest that the ginsenoside Rh2 significantly decreased the EGFR expression in glioblastoma cells. There are two possibilities for these in vivo findings of the decreased level of EGFR in the glioblastoma by ginsenoside Rh2. First, it may result from a selective elimination of highly EGFR-expressing glioblastoma cells. Thus, only low-EGFR-expressing glioblastoma cells survived the ginsenoside Rh2 treatment, and these low-EGFR-expressing glioblastoma cells are supposed to be less proliferative and less aggressive than highly EGFR-expressing glioblastoma cells. A second possibility is that the glioblastoma cells may downregulate EGFR expression by ginsenoside Rh2 treatment, which may also decrease cell proliferation and increase cell death. By either way, ginsenoside Rh2 may substantially inhibit the growth of glioblastoma.

To examine whether EGFR signaling inhibition is necessary for the ginsenoside Rh2-induced inhibition of glioblastoma growth, we used a rEGFR together with ginsenoside Rh2, to treat glioblastoma in vitro and in vivo. Since ginsenoside Rh2 has its effect on glioblastoma via EGFR signaling, the presence of rEGFR here may act as a decoy receptor to saturate ginsenoside Rh2 and thus leave the EGFR on glioblastoma cells unaffected. Our in vitro and in vivo data both support an EGFR signaling-dependent inhibitory effect of ginsenoside Rh2 on the growth of glioblastoma.

Taken together, our findings here not only reveal ginsenoside Rh2 as an instrumental treatment for glioblastoma but also demonstrate that its antitumor effect may function via inhibition of EGFR signaling in glioblastoma cells. In future, exact substance from ginsenoside Rh2 that may directly bind and affect EGFR on glioblastoma cells should be figured out, which may substantially improve our current GBM therapy.

Acknowledgments This work was supported by the Natural Science Foundation of China (nos. 30772246 and 81072655), Liaoning Provincial Department of Education Science Research Foundation (L2010586), and Shengjing Free Researcher Project (2011).

Conflicts of interest None

References

1. Schonberg DL, Bao S, Rich JN. Genomics informs glioblastoma biology. Nat Genet. 2013;45:1105–7.
2. Tian XX, Chan JY, Pang JC, Chen J, He JH, To TS, et al. Altered expression of the suppressors **PML** and p53 in glioblastoma cells with the antisense-**EGF**-receptor. Br J Cancer. 1999;81:994–1001.
3. Tsatas D, Kanagasundaram V, Kaye A, Novak U. E**GF** receptor modifies cellular responses to hyaluronan in glioblastoma cell lines. J Clin Neurosci. 2002;9:282–8.
4. Stea B, Falsey R, Kislin K, Patel J, Glanzberg H, Carey S, et al. Time and dose-dependent radiosensitization of the glioblastoma multiforme U251 cells by the **EGF** receptor tyrosine kinase inhibitor zd1839 ('iressa'). Cancer Lett. 2003;202:43–51.
5. Carpentier C, Laigle-Donadey F, Marie Y, Auger N, Benouaich-Amiel A, Lejeune J, et al. Polymorphism in Sp1 recognition site of the **EGF** receptor gene promoter and risk of glioblastoma. Neurology. 2006;67:872–4.
6. Shir A, Ogris M, Wagner E, Levitzki A. E**GF** receptor-targeted synthetic double-stranded rna eliminates glioblastoma, breast cancer, and adenocarcinoma tumors in mice. PLoS Med. 2006;3:e6.
7. Vauleon E, Auger N, Benouaich-Amiel A, Laigle-Donadey F, Kaloshi G, Lejeune J, et al. The 61 **A/G EGF** polymorphism is functional but is neither a prognostic marker nor a risk factor for glioblastoma. Cancer Genet Cytogenet. 2007;172:33–7.
8. Stish BJ, Oh S, Vallera DA. Anti-glioblastoma effect of a recombinant bispecific cytotoxin cotargeting human **IL**-13 and **EGF** receptors in a mouse xenograft model. J Neurooncol. 2008;87:51–61.
9. Gadji M, Crous AM, Fortin D, Krcek J, Torchia M, Mai S, et al. E**GF** receptor inhibitors in the treatment of glioblastoma multiform: **o**ld

clinical allies and newly emerging therapeutic concepts. Eur J Pharmacol. 2009;625:23–30.
10. Ozer BH, Wiepz GJ, Bertics PJ. Activity and cellular localization of an oncogenic glioblastoma multiforme-associated egf receptor mutant possessing a duplicated kinase domain. Oncogene. 2010;29:855–64.
11. Sjostrom S, Andersson U, Liu Y, Brannstrom T, Broholm H, Johansen C, et al. Genetic variations in **EGF** and **EGFR** and glioblastoma outcome. Neuro Oncol. 2010;12:815–21.
12. Hu J, Jo M, Cavenee WK, Furnari F, VandenBerg SR, Gonias SL. Crosstalk between the urokinase-type plasminogen activator receptor and **EGF** receptor variant **III** supports survival and growth of glioblastoma cells. Proc Natl Acad Sci U S A. 2011;108:15984–9.
13. Fenton TR, Nathanson D, Ponte de Albuquerque C, Kuga D, Iwanami A, Dang J, et al. Resistance to egf receptor inhibitors in glioblastoma mediated by phosphorylation of the pten tumor suppressor at tyrosine 240. Proc Natl Acad Sci U S A. 2012;109:14164–9.
14. Prabhu A, Sarcar B, Kahali S, Shan Y, Chinnaiyan P. Targeting the unfolded protein response in glioblastoma cells with the fusion protein **EGF**-**S**ub**A**. PLoS One. 2012;7:e52265.
15. Cornez I, Joel M, Tasken K, Langmoen IA, Glover JC, Berge T. **EGF** signalling and rapamycin-mediated mtor inhibition in glioblastoma multiforme evaluated by phospho-specific flow cytometry. J Neurooncol. 2013;112:49–57.
16. Emlet DR, Gupta P, Holgado-Madruga M, Del Vecchio CA, Mitra SS, Han SY, et al. Targeting a glioblastoma cancer stem cell population defined by EGF receptor variant III. Cancer Res. 2013. doi:10.1158/0008-5472.CAN-13-1407.
17. Zheng Y, Yang W, Aldape K, He J, Lu Z. Epidermal growth factor (**EGF**)-enhanced vascular cell adhesion molecule-1 (**VCAM**-1) expression promotes macrophage and glioblastoma cell interaction and tumor cell invasion. J Biol Chem. 2013;288:31488–95.
18. Kikuchi Y, Sasa H, Kita T, Hirata J, Tode T, Nagata I. Inhibition of human ovarian cancer cell proliferation in vitro by ginsenoside **R**h2 and adjuvant effects to cisplatin in vivo. Anticancer Drugs. 1991;2:63–7.
19. Tode T, Kikuchi Y, Hirata J, Kita T, Imaizumi E. Nagata I: [inhibitory effects of oral administration of ginsenoside **R**h2 on tumor growth in nude mice bearing serous cyst adenocarcinoma of the human ovary]. Nihon Sanka Fujinka Gakkai zasshi. 1993;45:1275–82.
20. Tode T, Kikuchi Y, Kita T, Hirata J, Imaizumi E, Nagata I. Inhibitory effects by oral administration of ginsenoside **R**h2 on the growth of human ovarian cancer cells in nude mice. J Cancer Res Clin Oncol. 1993;120:24–6.
21. Nakata H, Kikuchi Y, Tode T, Hirata J, Kita T, Ishii K, et al. Inhibitory effects of ginsenoside **R**h2 on tumor growth in nude mice bearing human ovarian cancer cells. Jpn J Cancer Res. 1998;89:733–40.
22. Tang XP, Tang GD, Fang CY, Liang ZH, Zhang LY. Effects of ginsenoside **R**h2 on growth and migration of pancreatic cancer cells. World J Gastroenterol. 2013;19:1582–92.
23. Liu J, Shimizu K, Yu H, Zhang C, Jin F, Kondo R. Stereospecificity of hydroxyl group at **C**-20 in antiproliferative action of ginsenoside **R**h2 on prostate cancer cells. Fitoterapia. 2010;81:902–5.
24. Li B, Zhao J, Wang CZ, Searle J, He TC, Yuan CS, et al. Ginsenoside **R**h2 induces apoptosis and paraptosis-like cell death in colorectal cancer cells through activation of p53. Cancer Lett. 2011;301:185–92.
25. Oh M, Choi YH, Choi S, Chung H, Kim K, Kim SI, et al. Antiproliferating effects of ginsenoside **R**h2 on **MCF**-7 human breast cancer cells. Int J Oncol. 1999;14:869–75.
26. Choi S, Kim TW, Singh SV. Ginsenoside **R**h2-mediated **G**1 phase cell cycle arrest in human breast cancer cells is caused by p15 **I**nk4**B** and p27 **K**ip1-dependent inhibition of cyclin-dependent kinases. Pharm Res. 2009;26:2280–8.
27. Giard DJ, Aaronson SA, Todaro GJ, Arnstein P, Kersey JH, Dosik H, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J Natl Cancer Inst. 1973;51:1417–23.
28. Shir A, Levitzki A. Inhibition of glioma growth by tumor-specific activation of double-stranded **RNA**-dependent protein kinase **PKR**. Nat Biotechnol. 2002;20:895–900.
29. Moore A, Donahue CJ, Bauer KD, Mather JP. Simultaneous measurement of cell cycle and apoptotic cell death. Methods Cell Biol. 1998;57:265–78.
30. Schlegel J, Merdes A, Stumm G, Albert FK, Forsting M, Hynes N, et al. Amplification of the epidermal-growth-factor-receptor gene correlates with different growth behaviour in human glioblastoma. Int J Cancer. 1994;56:72–7.