MiRNA-7 enhances erlotinib sensitivity of glioblastoma cells by blocking the IRS-1 and IRS-2 expression
Vahab Alamdari-Palangi, Razieh Amini and Hadi Karami

Keywords
erlotinib; glioblastoma; insulin receptor substrate; miRNA-7; sensitivity

Correspondence
Hadi Karami, Department of Molecular Medicine and Biotechnology, Faculty of Medicine, Arak University of Medical Sciences, Sardasht Street, Arak 3848176941, Iran. E-mail: h.karami@arakmu.ac.ir

Abstract

Objectives
Down-regulation of miRNA-7 is correlated with over-expression of IRS-1 and IRS-2 proteins, the upstream regulators of IGF-1R/Akt pathway, in glioblastoma cells. In this study, the effect of miRNA-7 on expression of IRS-1 and IRS-2 and sensitivity of the U373-MG glioblastoma cells to erlotinib was explored.

Methods
After miRNA-7 transfection, the expression of IRS-1 and IRS-2 mRNAs was measured by RT-qPCR. Trypan blue assay was used to assess the effect of miRNA-7 on cell proliferation. The effects of miRNA-7 and erlotinib, alone and in combination, on cell survival and apoptosis were measured using MTT assay and ELISA cell death assay, respectively.

Key findings
Our data showed that miRNA-7 markedly inhibited the expression of IRS-1 and IRS-2 in a time-dependent manner, inhibited the proliferation of glioblastoma cells and enhanced apoptosis ($P < 0.05$, relative to control). Pre-treatment with miRNA-7 synergistically inhibited the cell survival rate and decreased the IC$_{50}$ of erlotinib. Furthermore, miRNA-7 significantly augmented the apoptotic effect of erlotinib.

Conclusions
Our data propose that inhibition of IRS-1 and IRS-2 by miRNA-7 can effectively induce apoptosis and sensitize glioblastoma cell to EGFR-TKIs. Therefore, miRNA-7 may be a potential therapeutic target in patients with glioblastoma.

Introduction

Glioblastoma (GBM), or high-grade astrocytoma, is the most malignant brain tumour and represents more than 50% of primary brain tumours.$^{[1–3]}$ Despite progress in treatment modalities, the disease remains one of the most lethal malignancies, and the median survival rate remains 1 year.$^{[4,5]}$ Therefore, development of novel therapies against the disease is urgently needed.

The epidermal growth factor receptor (EGFR), also known as ErbB-1, is a transmembrane glycoprotein belongs to the ErbB family of receptor tyrosine kinases (RTKs) that is over-expressed in different types of human malignancies.$^{[6,7]}$ Amplification and over-expression of EGFR gene were found in more than 60% of primary glioblastomas and are believed to be linked to the proliferation, growth, angiogenesis and invasiveness in glioma cells.$^{[4,8]}$ Ligand-dependent EGFR activation leads to the excitation of two primary downstream signalling pathways: both of PI3-kinase (PI3K)/Akt and Ras/Raf/mitogen-activated protein kinase (MAPK), which are involved in the proliferation, migration and drug resistance of cancer cells.$^{[7,9,10]}$ Therefore, the EGFR is considered as a major target for molecular-targeting therapy. Erlotinib, a small-molecule inhibitor of EGFR, has been developed as a therapeutic agent for glioblastoma therapy. While clinical trials using erlotinib showed effective activity in some glioblastoma patients, other studies reported poor outcomes of this agent in glioblastoma patients. Some of the potential mechanisms causing resistance to EGFR tyrosine kinase inhibitors (EGFR-TKIs) include EGFR copy number, the EGFR point mutations and Akt activation (phosphorylation). Indeed, co-activation of other RTKs, such as insulin-like growth factor-1 receptor (IGF-1R), may confer resistance to EGFR-TKIs.$^{[11–14]}$ Understanding the other molecular pathways responsible for poor clinical
response to EGFR tyrosine kinase inhibitors is therefore essential.[2,15]

The insulin-like growth factor (IGF) pathway is involved in cell proliferation, differentiation and apoptosis.[16,17] Binding of insulin-like growth factor-1 (IGF-1) to its receptor, IGF-1R, leads to receptor autophosphorylation and subsequent phosphorylation of intracellular insulin receptor substrate-1 and -2 (IRS-1 and IRS-2).[16–19] These events lead to the activation of numerous signalling cascades, including MAPK, PI3K/Akt and mammalian target of rapamycin (mTOR).[17,19] Deregulations of IGF pathway are implicated in the development, invasion and metastasis of tumour cells.[17] For example, various studies have demonstrated the expression of both IRS-1 and IRS-2 is elevated in breast, hepatocellular, pancreatic, ovarian and prostate cancer relative to normal tissues, which is associated with tumour cell proliferation, invasion and survival.[18] Other studies have shown that Akt activity is controlled through a combination of IGF and EGF pathways, and inhibition of EGFR using specific inhibitors promotes activation of the IGF-1R pathway. Moreover, increasing the expression of IRS proteins can result in the activation of Akt and subsequently resistance to EGFR-TKIs. Therefore, combined targeting of both EGFR and IGF-1R may abrogate this resistance.[5,20,21]

MicroRNAs (miRNAs) are short non-coding RNA approximately 19–25 nucleotides in length, which directly bind to the 3'-UTR of target mRNAs to regulate gene expression, either through degradation of the RNA or translation inhibition.[22,23] MiRNAs participate in diverse molecular and cellular processes, such as cellular migration/invasion, cell growth, apoptosis and signal transduction. MiRNAs dysregulation is a hallmark of many cancers, including glioblastoma, and they can be classified into tumour-suppressive miRNAs and onco-miRNAs.[24–28] For example, miRNA-744 expression is strongly repressed in glioblastoma causing elevated transforming growth factor 1 beta (TGFβ1) and dishevelled 2 (DVL2) expression and increased tumour cell migration.[29] In contrast, up-regulation of miRNA-7 in glioblastoma is associated with suppression of phosphatase and tensin homolog (PTEN), as well as programmed cell death 4 (PDCD4) expression, causing enhancement of cell survival and inhibition of apoptosis.[30] Thus, miRNAs are potential novel targets for more effective treatment of glioblastoma.

MiRNA-7 is a known tumour suppressor that represses the expression of O-linked β-N-acetylglucosamine transferase (OGT), EGFR and upstream regulators of the Akt pathway, such as IRS-1 and IRS-2, leading to inhibition of proliferation, invasion and migration of glioblastoma cells. Moreover, down-regulation of miRNA-7 has been observed in glioblastoma. The role of the miRNA-7/IRS/Akt signalling pathway in erlotinib resistance of glioblastoma cells has not been studied so far. We hypothesized that miRNA-7 could enhance the antitumour effects of EGFR-TKIs in glioblastoma cells via the blockage of IRS/Akt pathway. Therefore, we explored the effect of miRNA-7 on cell growth, apoptosis and sensitivity of the glioblastoma cells to erlotinib.[1,31,32]

**Materials and Methods**

**Cell culture**

The U373-MG glioblastoma cell line was purchased from Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) containing 15% FBS (Sigma-Aldrich) at 37°C in a humidified atmosphere containing 5% CO₂. The cells from exponentially growing cultures were used in total experiments.

**Cell transfection**

The miRNA-7 mimic with the sense strand sequence 5'-UGGAAAGACUAGUGAUUUUGUGUUGU-3' and the negative control (NC) miRNA sense strand sequence 5'-UUUCUGAAGCGUUGACGUUTT-3' were designed and synthesized by Dharmacon (Lafayette, CO, USA). Transient transfection of lung cancer cell line at a final concentration of 50 nM was accomplished with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Total RNA from transfected cells was extracted by YTzol reagent (2020), pp. 531–538

© 2020 Royal Pharmaceutical Society, Journal of Pharmacy and Pharmacology, 72 (2020), pp. 531–538
of mRNA expression was calculated using the $2^{-\Delta \Delta C_t}$ method and β-actin as an internal control.[33]

**Cell proliferation assay**

To explore the antiproliferative effects of miRNA-7 and erlotinib on cell growth, trypan blue exclusion assay was performed. The cells (1 × 10^5 cells per well) were treated with miRNA-7 and erlotinib in 6-well plates and then incubated for 24–120 h. At indicated time points, cells were collected and then stained with 0.4% trypan blue dye (Merck KGaA, Darmstadt, Germany) for 2 min. Following on, viable cells (N, unstained cells) were counted by using a haemocytometer under an inverted microscope (Nikon Instrument Inc., Melville, NY, USA). The percentage of viable cells was determined using the following formula:

\[
\text{Cell viability(%) = } \left( \frac{N_{\text{cell}}}{N_{\text{blank control}}} \right) \times 100.
\]

**MTT assay**

The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay was used to determine the effects of miRNA-7 and erlotinib, alone and in combination, on survival of glioblastoma cell line. The experiment groups were erlotinib, NC miRNA, miRNA-7, NC miRNA and erlotinib, miRNA-7 and erlotinib blank control, miRNA blank control and combination blank control. Cells treated with only lipofectamine (solvent of miRNA), 1% DMSO (solvent of erlotinib) and a mixture of lipofectamine and DMSO were considered as miRNA, erlotinib and combination blank controls, respectively. In brief, 4 × 10^3 cells were plated in each well of 96-well plates and then, transfected with 50 nM of each miRNA. After 6 h of incubation, the cells were treated with several concentrations of erlotinib (0, 2.5, 5, 10, 20, 40, 80 and 160 μM) and incubated for 24 and 48 h. In each well, 10 μl of a 5 mg/ml solution of MTT (Sigma-Aldrich) was added and incubated at 37°C. Next, the supernatants were discarded and 150 μl of DMSO was added to each well. The absorbance (A) was measured spectrophotometrically at 450 nm with a microplate reader (Awareness Technology, Palm City, FL, USA). The survival rate (SR) was calculated from the following formula:

\[
\text{SR(%) = } \left( \frac{A_{\text{cell}}}{A_{\text{control}}} \right) \times 100\%.
\]

**Analysis of combined drug effects**

The effects of miRNA-7 and erlotinib combination treatments on cells survival were determined with the combination index (CI) analysis based upon the Chou-Talalay method.[34] The results of MTT assay were converted to fraction affected (Fa; range 0–1; where Fa = 0 is 100% cell survival and Fa = 1 is 0% cell survival) and analysed with the CompuSyn program (ComboSyn Inc., Paramus, NJ, USA). A CI value less than 1, equal to 1 or greater than 1 indicates synergistic, additive or antagonistic interaction, respectively.

**Apoptosis assay**

The tumour cells were seeded at a density of 4 × 10^4 cells/well in 24-well culture plates and treated with miRNA-7, erlotinib (IC_{50} dose) or a combination of the two, as described in MTT assay. Twenty-four and 48 h after transfection, apoptosis was determined using an ELISA Cell Death Detection Kit (Roche Diagnostics GmbH), which measures mononucleosomes and oligonucleosomes produced during apoptotic DNA fragmentation. Cell apoptosis detection was done under the supplier’s recommendations and monitored using an ELISA reader (Awareness Technology, Palm City, FL, USA) at 405 nm.

**Statistical analysis**

All data in this study were presented as mean ± standard deviation (SD) of at least three independent experiments. Differences between groups were assessed by using analysis of variance (ANOVA) and Bonferroni’s test. The statistical analyses were performed using GraphPad Prism software. P values less than or equal to 0.05 were considered significant.

**Results**

**Down-regulation of IRS-1 and IRS-2 expressions by miRNA-7 in glioblastoma cells**

To assess the effect of miRNA-7 on expression of IRS-1 and IRS-2 genes, the tumour cells were transfected with 50 nM miRNA-7 and NC miRNA for 24 and 48 h. Subsequently, RT-qPCR was performed to measure the expression levels of mRNA. As shown in Figure 1a,b, the cells transfected with miRNA-7 exhibited a time-dependent decrease in the expression levels of IRS-1 and IRS-2 genes, the tumour cells were transfected with 50 nM miRNA-7 and NC miRNA for 24 and 48 h. Subsequently, RT-qPCR was performed to measure the expression levels of mRNA. As shown in Figure 1a,b, the cells transfected with miRNA-7 exhibited a time-dependent decrease in the expression levels of IRS-1 and IRS-2 mRNA ($P < 0.05$; relative to the blank control group or NC miRNA-treated cells). After the cells were transfected by miRNA-7 for 24 and 48 h, the relative mRNA expressions were 79.33% and 64.73% for IRS-1 and 69.05% and 53.80% for IRS-2, respectively ($P < 0.05$). Notably, NC miRNA did not affect the expression levels of IRS-1 and IRS-2 mRNA compared with the blank control ($P > 0.05$).
Proliferation inhibition of U373-MG glioblastoma cells with miRNA-7

As elevated expression of IRS-1 and IRS-2 is associated with tumour progression; we therefore sought to test whether miRNA-7 could arrest the proliferation of glioblastoma cells. The U373-MG cells were transfected with NC miRNA and miRNA-7, and cell viability was measured by trypan blue assay. Results demonstrated that compared with the blank control group, miRNA-7 significantly suppressed the proliferation of tumour cells over a period of 5 days ($P < 0.05$; Figure 2). Twenty-four hours after miRNA-7 transfection, the cell viability dropped to 91.38% and to a further 58.69% on day 5. However, no significant differences in cell proliferation were found between the NC miRNA and the blank control groups ($P > 0.05$; Figure 2).

MiRNA-7 increased the cytotoxic effect of erlotinib in glioblastoma cells

To explore whether miRNA-7 could enhance the sensitivity of the glioblastoma cells to erlotinib, a combination treatment of miRNA-7 and erlotinib on U373-MG cells was investigated. As shown in Figure 3a,c, single treatment with erlotinib induced cytotoxicity in a dose-dependent manner. The results of MTT assay showed that 24- and 48-h transfection of miRNA-7 significantly decreased the cell survival rate to 87.01% and 81.16%, respectively, compared with the blank control ($P < 0.05$). Moreover, miRNA-7 in combination with erlotinib further reduced the cell survival rate relative to miRNA-7 or erlotinib alone ($P < 0.05$). Surprisingly, transfection of miRNA-7 led to a marked reduction in the IC50 values of erlotinib from 44.80 to 19.43 µM and 30.40 to 14.08 µM after 24 and 48 h, respectively (Table 1). Notably, transfection with NC miRNA had a minimal effect on the sensitivity of the U373-MG cells relative to the miRNA-7-transfected cells ($P > 0.05$; Figure 3a,c).

Combination of the miRNA-7 with erlotinib synergistically enhances the cytotoxicity of U373-MG cells

Combination index analysis based on the non-constant method of Chou-Talalay was performed to evaluate the drug interaction. The CI–Fa curves showed that combination treatment was synergistic ($CI < 1$) when miRNA-7 (50 nM) combined with erlotinib (0, 2.5, 5, 10, 20, 40, 80 and 160 µM; Figure 3b,d). Our results demonstrated that the best mean CI value of 24 h was observed at 160 µM erlotinib in combination with miRNA-7 ($CI = 0.78$) with Fa level of 0.99 (Figure 3b). In addition, strongest synergistic effect of 48 h ($CI = 0.72$, $Fa = 1$) occurred at 160 µM of erlotinib (Figure 3d). Though, significant synergism was observed at lower erlotinib concentrations (Table 2).
MiRNA-7 enhances erlotinib-mediated apoptosis

To investigate whether the apoptotic effects of erlotinib and miRNA-7 are partially related to the enhancement of apoptosis, the effects of treatments on apoptosis were examined using an ELISA-based cell death assay. The results demonstrate that 24-h administration of miRNA-7 or erlotinib alone markedly enhanced the induction of apoptosis by 3.22- and 6.83-fold respectively, compared with the blank control (P < 0.05; Figure 4). Also, miRNA-7 in combination with erlotinib at 24 h augmented the rate of apoptosis 10.10-fold relative to control and significantly increased apoptosis. Twenty-four-hour treatment of the cells with miRNA-7 and erlotinib alone increased the extent of apoptotic cells by 4.19- and 7.25-fold, respectively (relative to control; Figure 4). The combination of miRNA-7 and erlotinib for 24 h further promoted the rate of apoptosis by 11.30-fold relative to control (P < 0.05). Notably, NC miRNA alone or in combination with erlotinib had no significant effect on cellular apoptosis relative to blank control or erlotinib-treated cells, respectively (Figure 4). Therefore, we concluded that the chemosensitization effect of miRNA-7 is partially due to induction the apoptosis.

Discussion

Glioblastoma, the most aggressive grade of brain tumours, accounts for more than 50% of all primary glioma. Despite recent developments in the treatment of glioblastoma, clinical outcome is still poor. Thus, new clinical investigations are urgently needed.

Erlotinib is a HER1/EGFR TKI that binds to the catalytic tyrosine kinase domain of the receptor, thereby inducing apoptosis, inhibiting cell cycle progression and decreasing growth of glioblastoma-derived cell lines and human glioblastoma xenografts in animal models. Although erlotinib was well tolerated and showed promising antitumour activity in glioblastoma patients, clinical response to
erlotinib was limited due to recurrent problem of resistance.\[14,35\] Cooperation between TKIs and inhibitors of alternative compensatory pathways may improve the therapeutic responses to EGFR inhibitors. In this study, we explored the effect of miRNA-7 on expression of IRS-1 and IRS-2 in the U373 glioblastoma cells, and its functional involvement in response of glioblastoma cells to erlotinib.

MiRNAs are short regulatory non-coding RNAs that play pivotal roles in many cellular processes.\[5\] Experimental and clinical scenarios demonstrate that up-regulation and down-regulation of miRNAs are related to the development of malignant phenotype of glioblastoma, including enhanced cell proliferation, survival, abrogated apoptosis and promotion of angiogenesis.\[4,31\] MiRNA-7 is a tumour suppressor that inhibits the cell proliferation and survival and reduces therapeutic resistance in tumour cells through various mechanisms.\[1,3,5\] Here, we showed that monotherapy with miRNA-7 significantly reduced the proliferation of U373-MG cells, demonstrating its critical role in the growth of glioblastoma cells. Moreover, introduction of miRNA-7 inhibited the cell survival rate and enhanced apoptosis. These findings further support the tumour-suppressive effects of miRNA-7 in glioblastoma cells. Previous studies have demonstrated that miRNA-7 inhibits the expression of EGFR and other molecules involved in EGFR signalling as well as downstream Akt and ERK1/2 activity in glioblastoma, lung, breast and cervical cancer, which resulted in a reduction in colony formation, cell proliferation and survival.\[3,31,36\] Furthermore, miRNA-7 has been demonstrated to target anti-apoptotic genes in cervical and lung cancer cells, where miRNA-7 over-expression enhanced apoptotic rate.\[3\] Our data are in agreement with these observations and further confirmed the pivotal role of miRNA-7 in progression of glioblastoma.

The EGFR, a transmembrane glycoprotein belonging to the ErbB family of RTKs, is over-expressed in different types of malignant tumours.\[6\] EGFR amplification and over-expression were found in more than 60% of primary glioblastomas, which leads to excitation of several signalling pathways, including PI3K/Akt and Ras/Raf/MAPK and subsequently proliferation, invasion and survival of tumour cells.\[4,7–10\] Some reports have shown that expression of IRS-1 and IRS-2, two upstream modulators of IGF-1R/Akt pathway, are elevated in various types of cancer cells.\[17–19\] Other reports have demonstrated that Akt activity is regulated through a combination of EGFR and IGF-1R pathways, and inhibition of EGFR causes the activation of the IGF-1R pathway. Moreover, up-regulation of IRS proteins can lead to the activation of Akt and finally resistance to EGFR-TKIs.\[5,20,21\] In this study, we showed that exposure of U373-MG cells to erlotinib significantly reduced the cell survival rate and enhanced the apoptosis rate. Transfection of miRNA-7 markedly suppressed both IRS-1 and IRS-2 mRNA expression levels and enhanced the cell toxicity of erlotinib in a synergistic manner. Furthermore, miRNA-7 in combination with erlotinib enhanced apoptosis rate compared with the monotherapy. These data suggest that EGFR-TKIs resistance is partly related to decreased expression of the IRS-1 and IRS-2 and up-regulation of miRNA-7 could sensitize glioblastoma cells to EGFR-TKIs through inhibition of IRS-1 and IRS-2. Our findings support this hypothesis that miRNA-7 could enhance the antitumour effects of EGFR-TKIs in glioblastoma cells via the blockage of IRS/Akt pathway.

Table 2 CI analysis of miRNA-7 and erlotinib combination in U373-MG cells

| Erlotinib concentration (µM) | 24 h | Combined effect | 48 h | Combined effect |
|-----------------------------|------|----------------|------|----------------|
|                             | Fa   | CI  | Fa  | CI  | Fa  | CI  |
| 2.5                         | 0.17 | 0.94 | S   | 0.20 | 0.91 | S   |
| 5                           | 0.19 | 0.92 | S   | 0.25 | 0.89 | S   |
| 10                          | 0.26 | 0.89 | S   | 0.33 | 0.87 | S   |
| 20                          | 0.46 | 0.87 | S   | 0.52 | 0.83 | S   |
| 40                          | 0.70 | 0.81 | S   | 0.80 | 0.80 | S   |
| 80                          | 0.93 | 0.79 | S   | 0.97 | 0.79 | S   |
| 160                         | 0.99 | 0.78 | S   | 1    | 0.72 | S   |

The combination index (CI) values were calculated with Chou-Talalay combination index model and CompuSyn program. Synergistic (S), additive and antagonistic effects are defined by CI value <1, =1 or close to 1, or >1, respectively.
The above-mentioned results are consistent with the findings of previous studies. For example, Kefas et al.\(^\text{[31]}\) found that miRNA-7 expression levels were decreased in cells compared with surrounding brain tissue. The results of their study show that miRNA-7 potently inhibits EGFR, IRS-1 and IRS-2 expression and reduces viability and invasion of glioblastoma cells. Furthermore, evidence has emerged that miRNA-7 has the capacity to increase the therapeutic sensitivity of a variety of cancer types. Kalikowski et al.\(^\text{[3]}\) demonstrated that over-expression of miRNA-7 sensitizes head and neck cancer cells to erlotinib by inhibition of Akt activity. A previous work demonstrated that introduction of miRNA-7 reduced the expression of multidrug resistance-associated protein 1 (MRP1) and restored sensitivity to cisplatin in cisplatin-resistant breast cancer cells.\(^\text{[3]}\) Lee et al.\(^\text{[37]}\) also showed that down-regulation of miRNA-7 caused over-expression of EGFR and resistance to radiotherapy in glioma cells. Together, these results suggest that down-regulated miRNA-7 expression might be related to up-regulation of IRS-1 and IRS-2 and resistance to erlotinib in glioblastoma cells.

**Conclusion**

In conclusion, we have showed that miRNA-7 plays a pivotal role in the cell survival and sensitivity of glioblastoma cells to erlotinib. Knockdown of IRS-1 and IRS-2 with miRNA-7 was associated with the proliferation inhibition and enhancement of apoptosis in U373-MG glioblastoma cells. Moreover, the combination of miRNA-7 with erlotinib showed synergistic antitumour effects. Our findings support this hypothesis that down-regulation of miRNA-7 is associated with increased expression of IRS-1 and IRS-2, activation of Akt activity and, subsequently, EGFR-TKIs resistance. Therefore, miRNA-7 can be considered as an attractive therapeutic target in patients with glioblastoma to overcome resistance to TKIs, such as erlotinib, in part by inhibition of IRS-1 and IRS-2 expression, as well as Akt activity.

**Declarations**

**Conflict of interest**

The Authors declare that they have no conflicts of interest to disclose.

**Funding**

This work was supported by a grant from the Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, Iran (Grant number 2349).

**Acknowledgements**

We would like to thank Dr. Maryam Baazm and the staff of the Anatomy Department for their excellent technical and secretarial assistance.

**References**

1. Karsy M et al. Current progress on understanding microRNAs in glioblastoma multiforme. *Genes Cancer* 2012; 3: 3–15.
2. Garcia-Claver A et al. Gene expression changes associated with erlotinib response in glioma cell lines. *Eur J Cancer* 2013; 49: 1641–1653.
3. Kalikowski FC et al. microRNA-7: a tumor suppressor miRNA with therapeutic potential. *Int J Biochem Cell Biol* 2014; 54: 312–317.
4. Li M et al. MicroRNA in human glioma. *Cancers (Basel)* 2013; 5: 1306–1331.
5. Novakova J et al. MicroRNA involvement in glioblastoma pathogenesis. *Biochem Biophys Res Commun* 2009; 386: 1–5.
6. Yamaoka T et al. Receptor tyrosine kinase-targeted cancer therapy. *Int J Mol Sci* 2018; 19: 3491.
7. Singh M, JadHAV HR. Targeting non-small cell lung cancer with small-molecule EGFR tyrosine kinase inhibitors. *Drug Discov Today* 2018; 23: 745–753.
8. Koshkin PA et al. Role of microRNAs in mechanisms of glioblastoma resistance to radio- and chemotherapy. *Biochemistry (Mosc)* 2013; 78: 325–334.
9. Liu Q et al. EGFR-TKIs resistance via EGFR-independent signaling pathways. *Mol Cancer* 2018; 17: 53.
10. Sigismund S et al. Emerging functions of the EGFR in cancer. *Mol Oncol* 2018; 12: 3–20.
11. Kalman B et al. Epidermal growth factor receptor as a therapeutic target in glioblastoma. *Neuromolecular Med* 2013; 15: 420–434.
12. Brandes AA et al. Epidermal growth factor receptor inhibitors in neuro-oncology: hopes and disappointments. *Clin Cancer Res* 2008; 14: 957–960.
13. Raizer JJ. HER1/EGFR tyrosine kinase inhibitors for the treatment of glioblastoma multiforme. *J Neurooncol* 2005; 74: 77–86.
14. Taylor TE et al. Targeting EGFR for treatment of glioblastoma: molecular basis to overcome resistance. *Curr Cancer Drug Targets* 2012; 12: 197–209.
15. Zahnoro C, Sanchez-Gomez P. EGFR-dependent mechanisms in glioblastoma: towards a better therapeutic strategy. *Cell Mol Life Sci* 2014; 71: 3465–3488.
16. Han CH et al. Clinical significance of insulin receptor substrate-1 down-regulation in non-small cell lung cancer. *Oncol Rep* 2006; 16: 1205–1210.
17. Limesand KH et al. Impact of targeting insulin-like growth factor receptor tyrosine kinase on the clinical outcomes of patients with glioblastoma. *Drug Discov Today* 2012; 17: 434–439.
signaling in head and neck cancers. *Growth Horm IGF Res* 2013; 23: 135–140.

18. Mardilovich K et al. Expression and function of the insulin receptor substrate proteins in cancer. *Cell Commun Signal* 2009; 7: 14.

19. Boissan M et al. Overexpression of insulin receptor substrate-2 in human and murine hepatocellular carcinoma. *Am J Pathol* 2005; 167: 869–877.

20. Guix M et al. Acquired resistance to EGFR tyrosine kinase inhibitors in cancer cells is mediated by loss of IGF-binding proteins. *J Clin Invest* 2008; 118: 2609–2619.

21. Buck E et al. Feedback mechanisms promote cooperativity for small molecule inhibitors of epidermal and insulin-like growth factor receptors. *Cancer Res* 2008; 68: 8322–8332.

22. Jiang LH et al. MiR-30a: a novel biomarker and potential therapeutic target for cancer. *J Oncol* 2018; 2018: 1–9.

23. Braicu C et al. miR-181a/b therapy in lung cancer: reality or myth? *Mol Oncol* 2019; 13: 9–25.

24. Liu DK et al. MiRNA-93 functions as an oncogene in glioma by directly targeting RBL2. *Eur Rev Med Pharmacol Sci* 2018; 22: 2343–2350.

25. Ding L et al. Promising therapeutic role of miR-27b in tumor. *Tumour Biol* 2017; 39: 1010428317691657.

26. Gulluoglu S et al. Simultaneous miRNA and mRNA transcriptome profiling of glioblastoma samples reveals a novel set of OncomiR candidates and their target genes. *Brain Res* 2018; 1700: 199–210.

27. Lin H et al. MiR-324-5p reduces viability and induces apoptosis in gastric cancer cells through modulating TSPAN8. *J Pharm Pharmacol* 2018; 70: 937–951.

28. Chen Y et al. MiR-505 mediates methotrexate resistance in colorectal cancer by targeting RASSF8. *J Pharm Pharmacol* 2018; 70: 937–951.

29. Hübner M et al. Intronic miR-744 inhibits glioblastoma migration by functionally antagonizing its host gene MAP2K4. *Cancers (Basel)* 2018; 10: 400.

30. Wang G et al. Targeting strategies on miRNA-21 and PDCD4 for glioblastoma. *Arch Biochem Biophys* 2015; 580: 64–74.

31. Kefas B et al. microRNA-7 enhances erlotinib sensitivity in glioblastoma. *Oncotarget* 2014; 5: 6687–6700.

32. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402–408.

33. Chou TC, Talalay P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; 22: 27–55.

34. Webster RJ et al. Regulation of epidermal growth factor receptor signaling in human cancer cells by microRNA-7. *J Biol Chem* 2009; 284: 5731–5741.

35. Lee KM et al. microRNA-7 increases radiosensitivity of human cancer cells with activated EGFR-associated signaling. *Radiother Oncol* 2011; 101: 171–176.