Int J Clin Exp Pathol 2017;10(1):530-536
www.ijcep.com /ISSN:1936-2625/IJCEP0035383

Original Article

Effects of toll-like receptor domain-containing adaptor protein on human gliomas cells

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Received July 9, 2016; Accepted September 6, 2016; Epub January 1, 2017; Published January 15, 2017

Abstract: By using small interfering RNA (siRNA), this study investigated the expression of toll-like receptor domain-containing adaptor protein (TIRAP) of human gliomas cell (U251) and the impact on cell proliferation, apoptosis and invasion. Using lipofectamine, we transferred TIRAP siRNA into the cells, and classified the cells into three groups, including the transfection positive group (I) negative transfection group (II) and control group (III). Then the transfection effect was evaluated by using real-time PCR (RT-PCR). MTT and EdU were performed to test the cell proliferation; cell apoptosis was evaluated by observing the expression of Bcl-2 with western blot. To explore the associated biological mechanism, mRNA and protein of TIRAP, NF-κB were tested by using RT-PCR or western blot, additionally, the expression of AKT was also been observed. Compared with groups II and III, results showed that transfection of TIRAP siRNA could inhibit the expression of TIRAP mRNA and protein effectively; The down-regulation of the expression of TIRAP could inhibit the expression of Bcl-2; simultaneously, the expression of NF-κB and AKT was also downregulated. Based on the results, we implied that the mechanism of siRNA down-regulated the expression of TIRAP was mediated by the down-regulation of the related pathway of AKT/NF-κB/Bcl-2, by which the proliferation and invasion activity of U251 was inhibited, the apoptosis was induced, implying that TIRAP could be the potential therapeutic target of gliomas.

Keywords: Gliomas, TIRAP, NF-κB, Bcl-2

Introduction

Gliomas is the most commonest primary neoplasms of the central nervous system (CNS), 35-60% of which has been reported in China, which takes up 1%-3% of human malignancies [1, 2]. The difficulty of cure for gliomas is due to the strong invasion activity, showing the higher malignancy.

The conventional methods including operation, chemotherapy and radiotherapy are unsatisfactory, while the invasion activity of gliomas cells to the periphery normal tissues is the reason why some patients quickly deteriorated, therefore, the invasion characteristic of gliomas is the main obstacle for cure gliomas [3]. It's well known that the occurrence and development of tumors are especially complicated process with multi-factors and multi-stages, and the abnormal expression or silence of several genes was the common phenomena [3-5]. Nowadays, with the development of oncology molecular biology, many associated genes of genesis for gliomas were found, but the pathology of gliomas still can't be fully explained. On the basis of the previous studies, exploring the new therapeutic stratagem, new target is the major research on neurosurgery.

Small interfering RNA (siRNA) is the process of classic double strand inducing specific inhibition of corresponding complementary base of gene expression, with the characteristic of highly specificity, small molecules and transitivity. At present, this technology has been the major instrument to study on genes, and already been applied on the gene therapy of oncology gradually [6-8].

Toll-like receptor (TIR) is considered to be the important receptor of natural immune response, which could recognize the invading organism of various ingredient including lipopolysaccharide,

lipopeptides, flagella, DNA, RNA and so on. TIR domain-containing adaptor protein (TIRAP) has also been considered as MyD88-like adaptor (Mal), which is another adaptor receptor with structural domain of TIR, working as aptamer in the Toll-like receptor 2 (TIR-2)/TIR-4 signaling pathway.

TIRAP has the characteristic of importance and complexity in the member of TLR family signal transduction. In the process of early stage of natural immune system defending on the pathogen invading, the structural domain of TLR has played a key role [9, 10]. Studies have shown that TLR2 exists in the gliomas cells, while TIR-2 is the critical molecule of TIRAP, consequently, we hypothesis that TIRAP exists in the gliomas cells. With transfection siRNA TIRAP into U251, we screened the expression of TIRAP mRNA, the cell proliferation, apoptosis and invasion, and the associated mechanism was explored in order to make some gene experimental foundation for gliomas.

Materials and methods

Dulbecco's modified eagle medium (DMEM) was purchased from Gibco in the United States; Trypsin and A0/EB were purchased from Sigma in the United States; Lipofectamine 2000, Rat polyclonal antibody against people, HRP labeled rabbit anti-rat polyclonal antibody and Negative siRNA were purchased from Invitrogene in the United States; Protein extraction reagent was purchased from Novogen in the United States; Protein assay was purchased from Pierce in the United States; MTT and BCA staining kits were purchased from Ribobio in China.

Cell culture

The human gliomas cell line of U251 was obtained from Pathology Experimental Lab of our college. Then the frozen U251 cells were thawed quickly in water at 37°C, and cultured it in the DMEM with 10% fetal bovine serum (FBS) in incubator at 37°C, changed the medium regularly; when the cells grew more than 80% of the culture bottle, digested it with 0.25% trypsin, cultured in the fresh medium.

Synthesis and design of the siRNA

Referred to Gene bank, the corresponding sequences of TIRAP siRNA was designed. The positive-sense strand was 5'-GGAACUUGCCAUCAAGAUCTT-3'; the antisense strand was 5'-AAUGUCGAUAGGAACUUGCTT-3'. And the negative siRNA sequences were 5'-CGAACGAGUACCGUACACUdTdT-3'; 5'-dTdTGCUUGCUCAUGGCAUGUGA-3'.

Subgroup and transfection

The cells were divided into three groups including group I (TIRAP siRNA), group II (negative siRNA) and group III (control group). Proper cells were seeded into the six well plates (1×10^6/plate), according to the instruction of Invitrogene, TIRAP siRNA and corresponding negative siRNA were transfected. After 48 hours, related detection indexes were screened.

mRNA expression of TIRAP and NF-κB tested by RT-PCR

48 hours after transfection, referred to the instruction of Invitrogene, the total RNA was extracted. The forward primer of TIRAP mRNA was 5'-CGT CAC GAC CTA CGA TA-3', and the reverse primer was 5'-GCC CCG CGC CGA GGC AG-3'; while the forward primer of NF-κB mRNA was 5'-GGT GTG ATGGTG GGT ATG GGT-3'; and the reverse primer was 5'-CTG GGT CATCTT TTC ACG GTC-3'. Referred to the protocol of Takara, RT-PCR was performed. According to the $2^{-\Delta\Delta Ct}$, the expression of TIRAP and NF-κB was calculated.

Proliferation of U251 using MTT

The cells (1×10^5/ml) were seeded in the 96-well plate, 100 μL cell suspension was added in every well. When the cells grew into more than 70% of the total well, TIRAP siRNA was transfected. 48 hours later, according to the MTT kit instruction of Ribobio, the absorbance density (OD) was tested at 490 nm by ELIASA.

Proliferation of U251 using EdU

5-Ethynyl-2'-deoxyuridine (EdU) is a kind of thymus pyrimidine nucleoside analogue, which could examine the replication activity of DNA directly and accurately. In order to evaluate the down-regulation of TIRAP impact on cell proliferation, the results were analyzed under fluorescence microscope referred to the EdU kit instruction of Ribobio.

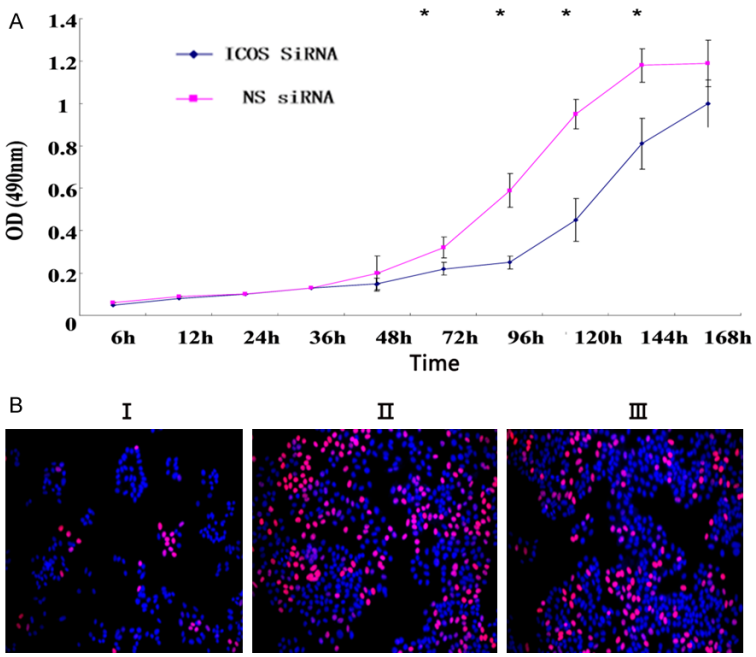

Figure 1. Proliferation of U251 cells transfected with TIRAP siRNA A: Growth curve of U251 after transfection, *P<0.05; B: Cell morphology of U251 stained with EdU, red stands for the cells of S phase.

Cell invasion using transwell

At first, the cells were transfected with TIRAP siRNA in the 24-well Borden chamber (8 μm/well). The Matrigel was diluted with serum free DMEM with the ratio of 1:3 in 12 hours before, then the medium was put upon the polycarbonate of the upper Transwell chamber evenly, placed at the room temperature for 1 hour, the Matrigel would freeze into substrate membrane. After that, 700 ul DMEM with 10% FBS was added into the underneath chamber, with 200 ul cell suspension was seeded into the chamber, 18 hours later, the Transwell membrane could be took out. Washed twice with precooling PBS, fixed with paraformaldehyde for 15 min, then stained with 0.1% crystal violet for 5-10 min, photographed at microscope, the number of cells across the Transwell membrane was recorded. Additionally, the experiment was performed for more than 3 times.

Expression of TIRAP, NF-κB, AKT and Bcl-2 with western blot

After medium of U251 was removed, RIPA buffer (Zhongzhi Biotechnologies, China) was added. Then the extract protein was conducted electrophoresis with SDS-PAGE, transferred the protein onto the nitrocellulose membrane (NC), and blocked the NC with 5% skim milk for 2 hours. Primary antibody (Santa Cruz) was added overnight at 4°C, with PBS washed once, and TBST washed twice, the second antibody (Santa Cruz) was added, cultured for 2 hours at room temperature. Electrogenerated chemilummescence (ECL) was performed; finally, x-ray film was developed, fixed and scanned. And the internal reference was β-actin.

Statistical analysis

The quantitative data was represented with mean ± standard deviation, by using SPSS 17.0 software package; the Student's t-test was performed. For multiple comparison between groups, analysis of variance (ANOVA) was performed. And P<0.05 was considered statistical significant.

Results

Expression of TIRAP mRNA was down-regulated by using TIRAP siRNA transfection

As **Figure 2A** shown, compared with group II and III, the expression of TIRAP siRNA in group I was significantly down-regulated, which showed that vector of constructed TIRAP siRNA could transfect U251 efficiently, laying solid foundation for the next experiment.

Cell proliferation was inhibited with TIRAP-siRNA transfection

As shown in **Figure 1A** of growth curve, compared to the cells of control, the growth rate of U251 was decreased obviously after TIRAP siRNA transfection. In order to evaluate the inhibition rate of TIRAP siRNA, continuous observation for 7 days, results show that the growth rate of U251 was significant deceased (P<0.05). Additionally, EdU was performed to show the S phase of the cells directly, namely the condition of proliferation. In **Figure 1B**, under the fluorescence microscope, red stands

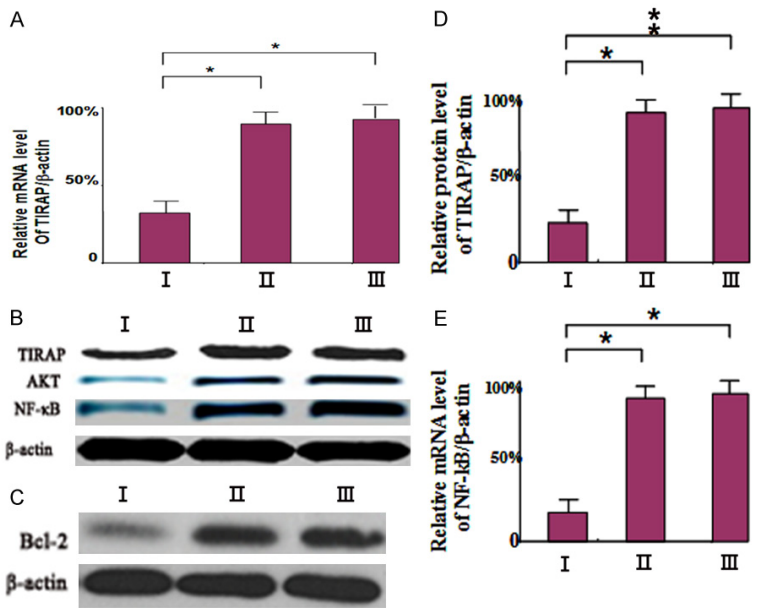

Figure 2. Expression of associated protein and mRNA after transfection. A: The expression of TIRAP mRNA in different groups; B: β-actin as internal reference, the protein of TIRAP, AKT, NF-κB in different groups; C: The expression of Bcl-2 in different groups; D: The protein expression of TIRAP in different groups more than three experiments; E: The mRNA expression of NF-κB in different groups more than three experiments.

for the cells of S phase, compared to the cells of group II and III, the percentage of cells in S phase were decreased obviously, showing that down-regulation of TIRAP inhibited the proliferation of U251 significantly.

Expression of associated protein and mRNA transfected with TIRAP siRNA

As shown in **Figure 2B** and **2D**, transfection with TIRAP siRNA, the expression of TIRAP was decreased obviously in group I ($P<0.05$). In order to explore the relative mechanism, the expression of NF-κB and AKT was detected, and results show that both the expression of NF-κB and AKT were down-regulated as the down-regulation of TIRAP ($P<0.05$). Furthermore, the mRNA of NF-κB was also screened, showing that down-regulation of TIRAP could down-regulate both protein and mRNA of NF-κB.

In order to screen the apoptosis of U251 after transfection with TIRAP siRNA, the expression of Bcl-2 was observed. As shown in **Figure 2C**, compared with group II and III, the expression of Bcl-2 was down-regulated obviously ($P< 0.05$), showing that the regulation of TIRAP could have an impact on cell apoptosis.

Results of cell invasion using transwell

Under microscope, the number of cells across the Transwell membrane were shown as **Figure 3A**, compared with group II and III, the number of cells getting through decreased obviously. After more than three independent experiments, the number of cells crossing the membrane decreased significantly, showing the ability of inhibition invasion for TIRAP siRNA.

Discussion

Gliomas is the most common intracranial malignancy of human CNS. Although the incidence was different from the time and region, the total incidence occupies the half of the intracranial tumors, in which astrocytoma is the common type. Gliomas is with the characteristics of diffuse infiltrative growth, none obvious boundary with periphery brain tissue, invasion into the critical nerve tissue and difficulty completely removal on operation. However, chemistry and radiation are impossible to kill gliomas cells distinctively, with bad therapeutic effect, even toxic and side effect the whole body and CNS can't endure could occur. Therefore, with poor prognosis and short survival time, Gliomas is with high recurrence and mortality. Overall mortality of 5 years is next to lung cancer and pancreatic cancer in all body system of malignancy [1-3]. Many studies have recorded that including gliomas, the regulation of relative genes in circulation or tissues could change the biological characteristic of cancer cells [4-8]. Therefore, exploration the associated mechanism is critical to clinic for gliomas.

Toll-like receptor (TLR) plays an important part in the innate immunity but not adaptive immunity, while innate immunity is the element immune system of human body [11-14]. And TIRAP is the critical media of TLR, which mediates the innate immune in somatic cells [15-17]. As is known to us, there hasn't been any

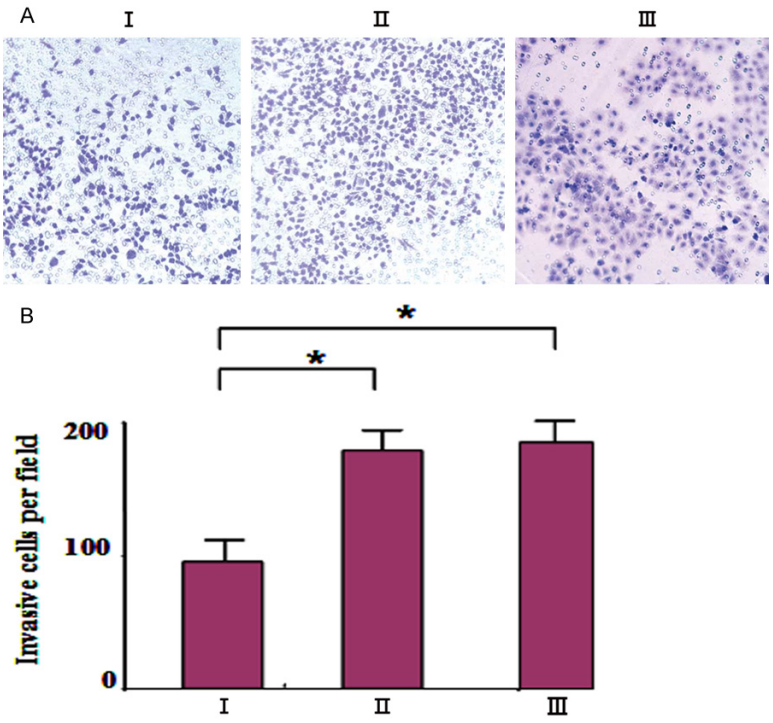

Figure 3. *Results of cell invasion using Transwell.* A: The cells across the Transwell membrane in different groups under microscope; B: The cell number of cells across the Transwell membrane in three independent experiments of different groups, *P<0.05.

full and detail mechanism reported of TIRAP. Initially, some reports have implied that TIRAP has played important part for gliomas to avoidance for the innate immunity. Furthermore, TIRAP upon the tumor cells surface could induce apoptosis of tumor specific CTL and inhibition the immune activity for tumor of human body. In lymphoid organ, TIRAP on the surface of antigen-presenting cell interplays with native T lymphocyte, inducing the inadequacy of T lymphocyte [18, 19]. Consequently, TIRAP has been reported to involve in tumor immune escape, which has the ability of preventing itself bonding from its receptor. Overall, it's possible for TIRAP to inhibit the tumor growth directly, prevent the tumor immune escape, which improves the activity of T cell activation and kill ability of CTL, showing its ability to improve the immune activity of other antigen.

Other researchers have shown that neutralize the innate immune could improve the production and activity of IFN-γ, simultaneously cytotoxic T cells of antigen-presenting cell are strengthened. However, in the next animal experiments, TIRAP was injected into the rats model with melanoma, then results show that cytotoxic $CD8^+$ T cells of anti-melanoma increased [20, 21]. Therefore, TIRAP is not only the new pathological element but also the new research topic and thought for the gene diagnosis and gene therapy for tumor in the future.

Recently, as the research of tumor molecule mechanism has been deep explored, many researchers propose that the occurrence and development of cancer have been connected with the series of immune avoidance. The point is that gliomas is a kind of tumor with immune inhibition, with the ability to anti-tumor immune reaction such as release IL-10 and transforming growth factor (TGF), to avoid the monitoring and killing for body immune system. Whereas there hasn't any immune therapy strategy for gliomas at present, abolishment the immune mechanism or improvement the anti-cancer immune response would be the possible approach for cure gliomas [14-17].

Studies have shown that the expression of TIRAP is up-regulated in gliomas, but not in the normal brain tissue and the adjacent cancer tissue, implying the possible closed relationship of such difference between the clinic pathological characteristics and prognosis. Another research found that the change of some genes and protein correlated with the gliomas pathological degree, proliferation activity and prognosis of patients such as Ki-67, TGF-β2, EGFR, p16 and survivin. Additionally, studies have shown that the deletion of tumor suppressor gene PTEN could induce immune resistance, while the over-expression of TIRAP mediates the pathway [13-17].

AKT and NF-κB are critical factors in the cell-mediated immunity, also play an important part in the innate immune activity. AKT is also called

protein kinase B, which is a critical kinase for the downstream of PI3K, also as the element molecule to promote cell survival and function, and it has been prove to play an important role in the occurrence and development of liver cancer [22]. At the same time, targeting on AKT could improve the chemotherapy sensitivity for melanoma [23], another study has shown that mutation of PI3K and AKT was found in the brain cancer tissue of patients, showing that targeting on PI3K could be the possible approach to cure for gliomas [24]. Therefore, in this study, we evaluated the protein expression of NF-κB and AKT quantificationally. Results have shown that as down-regulation of TIRAP, the expression of NF-κB and AKT was also down-regulated. Consequently, it's possible that TIRAP regulates the proliferation, invasion and apoptosis of U251 by regulating NF-κB and AKT.

In summary, TIRAP is a negative regulation signal to mediate immune activity, which plays specific role in the process of tumorgenesis, virus infection and autoimmune diseases. Our results show that downregultion of TIRAP could inhibit the cell proliferation, invasion and induce cell apoptosis, showing its potential target molecule for cure gliomas. Base on the specific expression of TIRAP for gliomas cells, TIRAP siRNA could be tried to intervene the animal model with gliomas in the next experiment. Although this research suggests that TIRAP has played an important role in gliomas, the mechanism should be elusive in the following research.

Disclosure of conflict of interest

None.

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References

[1] Kleber S, Sancho-Martinez I, Wiestler B, Beisel A, Gieffers C, Hill O, Thiemann M, Mueller W, Sykora J, Kuhn A, Schreglmann N, Letellier E, Zuliani C, Klussmann S, Teodorczyk M, Gröne HJ, Ganten TM, Sültmann H, Tüttenberg J, von Deimling A, Regnier-Vigouroux A, Herold-Mende C, Martin-Villalba A. Yes and PI3K bind CD95 to signal invasion of glioblastoma. Cancer Cell 2008; 13: 235-48.

[2] Ostrom QT, Gittleman H, Stetson L, Virk SM, Barnholtz-Sloan JS. Epidemiology of gliomas. Cancer Treat Res 2015; 163: 1-14.

[3] Reardon DA, Desjardins A, Peters K, Gururangan S, Sampson J, Rich JN, McLendon R, Herndon JE, Marcello J, Threatt S, Friedman AH, Vredenburgh JJ, Friedman HS. Phase II study of metronomic chemotherapy with bevacizumab for recurrent glioblastoma after progression on bevacizumab therapy. J Neurooncol 2011; 103: 371-9.

[4] Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. Identification of human brain tumour initiating cells. Nature 2004; 432: 396-401.

[5] Maher EA, Brennan C, Wen PY, Durso L, Ligon KL, Richardson A, Khatry D, Feng B, Sinha R, Louis DN, Quackenbush J, Black PM, Chin L, DePinho RA. Marked genomic differences characterize primary and secondary glioblastoma subtypes and identify two distinct molecular and clinical secondary glioblastoma entities. Cancer Res 2006; 66: 11502-13.

[6] Parsons DW, Jones S, Zhang X, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Siu IM, Gallia GL, Olivi A, McLendon R, Rasheed BA, Keir S, Nikolskaya T, Nikolsky Y, Busam DA, Tekleab H, Diaz LA, Hartigan J, Smith DR, Strausberg RL, Marie SK, Shinjo SM, Yan H, Riggins GJ, Bigner DD, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. An integrated genomic analysis of human glioblastoma multiforme. Science 2008; 321: 1807-12.

[7] Lee RC, Feinbaum RL and Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843-54.

[8] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783-801.

[9] Hardy MP, Mc GAF, O'Neill LA. Transcriptional regulation of the human TRIF (TIR domain-containing adaptor protein inducing interferon beta) gene. Biochem J 2004; 380: 83-93.

[10] Oshiumi H, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. J Biol Chem 2003; 278: 49751-62.

[11] Takeuchi, Akira OS. Pattern recognition receptors and inflammation. Cell 2010; 140: 805-20.

[12] Paludan SR, Bowie AG. Immune sensing of DNA. Immunity 2013; 38: 870-80.

[13] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004; 5: 987-95.

[14] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005; 17: 1-14.
[15] Basith S, Manavalan B, Yoo TH, Kim SG, Choi S. Roles of toll-like receptors in cancer: a double-edged sword for defense and offense. Arch Pharm Res 2012; 35: 1297-316.
[16] Hoving JC, Wilson GC, Brown GD. Signalling C-type lectin receptors, microbial recognition and immunity. Cell Microbiol 2014; 16: 185-94.
[17] Drummond RA, Brown GD. Signalling C-type lectins in antimicrobial immunity. PLoS Pathog 2013; 9: e1003417.
[18] Geijtenbeek TB, Gringhuis SI. Signalling through C-type lectin receptors: shaping immune responses. Nat Rev Immunol 2009; 9: 465-79.
[19] Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature 2006; 442: 39-44.
[20] Elinav E, Strowig T, Henao-Mejia J, Flavell RA. Regulation of the antimicrobial response by NLR proteins. Immunity 2011; 34: 665-79.
[21] Goubau D, Deddouche S, Reis e Sousa C. Cytosolic sensing of viruses. Immunity 2013; 38: 855-69.
[22] Zhang CZ, Wang XD, Wang HW, Cai Y, Chao LQ. Sorafenib inhibits liver cancer growth by decreasing mTOR, AKT, and PI3K expression. J BUON 2015; 20: 218-22.
[23] Niessner H, Forschner A, Klumpp B, Honegger JB, Witte M, Bornemann A, Dummer R, Adam A, Bauer J, Tabatabai G, Flaherty K, Sinnberg T, Beck D, Leiter U, Mauch C, Roesch A, Weide B, Eigentler T, Schadendorf D, Garbe C, Kulms D, Quintanilla-Martinez L, Meier F. Targeting hyperactivation of the AKT survival pathway to overcome therapy resistance of melanoma brain metastases. Cancer Med 2013; 2: 76-85.
[24] Ströbele S, Schneider M, Schneele L, Siegelin MD, Nonnenmacher L, Zhou S, Karpel-Massle G, Westhoff MA, Halatsch ME, Debatin KM. A Potential Role for the Inhibition of PI3K Signaling in Glioblastoma Therapy. PLoS One 2015; 10: e0131670.