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Expression of RhoA and COX-2 and their roles in the occurrence and progression of brain glioma

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Abstract: This study was conducted to detect the expression of RhoA and COX-2 in the brain glioma and to discuss their roles in the occurrence and progression of brain glioma. Brain glioma tissues were collected from 22 cases with brain glioma by surgical resection (tumor group); normal brain tissues were collected from 15 cases with brain trauma by surgical resection (healthy group). Western Blot and immunohistochemistry were applied to detect the expression of RhoA and COX-2 in the tissues. The brain glioma cell lines with silenced RhoA expression or silenced COX-2 expression were used to analyze the roles of RhoA and COX-2 in the occurrence and progression of brain glioma through the cell proliferation and invasion/migration assays. The relative expression of RhoA and COX-2 in the brain glioma was 0.82±0.13 and 0.75±0.14, respectively, which was significantly higher than that in the normal brain tissues (0.12±0.08 and 0.043±0.14) (P<0.05). The percentage of RhoA-positive brain glioma cells and COX-2-positive cells was 75.32±15.02% and 82.39±17.82%, respectively; it was significantly higher than that of the normal brain tissues (17.03±7.72 and 5.83±4.01) (P<0.05). As compared with glioma cell line SHG-44, the relative proliferation rate of C8-D9 and E5-B9 was 20.72% and 25.45%, respectively; the relative invasion/migration rate was 20.91% and 20.97%, respectively. The G0/G1 phase decreased significantly (P<0.05) and significantly increased in stage S and G2/M (P<0.05). Both RhoA and COX-2 were upregulated in the brain glioma tissues; their over-expression contributed to the proliferation and invasion/migration of the brain glioma cells.

Keywords: RhoA, COX-2, brain glioma, proliferation, invasion/migration.

INTRODUCTION

Brain glioma is one of the most common intracranial tumors, accounting for about 45% of all intracranial tumors (Witiak *et al*., 2007). Much has been revealed about the mechanism of occurrence and progression of brain glioma and considerable progress has been achieved in the diagnosis and treatment of brain glioma (Cheng *et al*., 2017). However, the mortality and postoperative survival of brain glioma (Johnson *et al*., 2019) patients are not significantly improved (Reardon and Wen, 2015). Rho proteins, with a relative molecular mass of 20-25kD, are GTP-binding proteins. Because of their GTP protease activity, Rho proteins are also known as Rho GTPases, and RhoA is an important Rho GTPase. RhoA is highly expressed in various tumors, including breast cancer (Kalpana *et al*., 2019) and pancreatic cancer (Wong *et al*., 2019), which further contributes to the proliferation and migration of the tumor cells and the postoperative clinicopathological characteristics of the patients (Liu *et al*., 2014). COX-2 (cyclooxygenase-2) expression can be induced by tissue damage, leading to intense proinflammatory effects and pain (Grzanna *et al*., 2020). It is shown that COX-2 cannot only promote the proliferation and growth of tumor cells, but also enhance the immunological tolerance of tumors by upregulating IL-10 and IL-12 (Ye *et al*., 2018). COX-2 usually functions as a cancer-promoting gene (Jian *et al*., 2015). It has been found by a diversity of researches (Song *et al*., 2017, Xu *et al*., 2017) that both RhoA and COX-2 contribute to the occurrence and progression of tumors, but their expression and roles in brain glioma are rarely understood. The expression of RhoA and COX-2 in the normal brain tissues and brain glioma was compared in this study. We further analyzed the roles of RhoA and COX-2 in the proliferation, invasion and migration of brain glioma cells by silencing either RhoA or COX-2 in the glioma cell line SHG-44.

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MATERIALS AND METHODS

Specimens

Brain glioma tissues were collected by surgical resection from 22 patients with brain glioma at Yantaishan Hospital As controls, normal brain tissues were collected by surgical resection from 15 cases with brain trauma. Of 22 patients with brain glioma, there were 12 males and 10 females, who were aged 31 to 62 years old with an average of 41.3±6.2 years. None of the brain glioma patients were combined with involvement of the ventricular system. As to the clinical staging, 9 cases were of stage T1 and 13 cases of stage T2, including 17 supratentorial tumors and 5 infratentorial tumors. Of 15 cases with brain trauma, there were 10 males and 5 females, who were aged 26 to 57 years old with an average of 32.3±5.9 years. None of the brain trauma

patients were combined with tumors. All of the experiments were approved by the Ethics Committee of Yantaishan Hospital (20180523).

Reagents

Human glioma cell line SHG-44 was purchased from ATCC (USA). C8-D9 cell line with silenced RhoA expression and E5-B9 cell line with silenced COX-2 expression were prepared by RiboBio (Guangzhou, China). Total histone extraction kit (OP-0006) was purchased from AmyJet Scientific Inc. Rabbit RhoA (sc-28565) and COX-2 (sc-7951) polyclonal antibodies as well as the goat anti-rabbit (sc-2004) polyclonal antibodies were purchased from Santa Cruz. Immunohistochemistry kit was purchased from Shanghai Rui-qi Biological Technology Co., Ltd.

Western Blot

Total histone extraction was performed, and the total protein was quantified by using the bicinchoninic acid (BCA) protein assay. Then 70ug of the protein was taken for SDS-PAGE. The proteins were wet transferred to the membrane, which were sealed, incubated with primary and secondary antibodies and stained. Band intensity was analyzed by using the Imag-J software.

Immunohistochemical detection of RhoA and COX-2 in the tissues

Immunohistochemical staining was performed after adding the primary antibodies according to the manufacturer's instructions. For negative control, phosphate buffer saline (PBS) was added instead of the primary antibodies. The cells were positive for RhoA if the cytoplasm or cytomembrane and cytoplasm (brown-yellow particles) was stained; the cells were positive for COX-2 if the cytoplasm was stained (brown-yellow or brown granules). Ten fields of view were randomly selected under the high-power microscope, and the cell positive rates were calculated.

Cell cloning assay

Into the 6-well plate 2×10^3 cells/ml were inoculated at 2ml per well. The culture medium was replaced once every 3 days, and the cells were cultured conventionally for about 3 weeks. The cell culture was discontinued when clones appeared in the wells. The supernatant was collected, washed with PBS twice and fixed in 4% formaldehyde for 15min. The supernatant was collected and stained with 0.25% crystal violet for 25min. After washed slowly with sterilized water, they were placed on the ultra-clean bench, dried, photographed and counted.

Transwell invasion/migration assay

The cells at the log phase were collected and cultured in the serum-free medium for 1d to prepare the serum-free cell suspension (cell concentration 1×105 cells/ml). Into the upper chamber, 200ul of cell suspension was added; 500ul of culture medium containing 10F FBS was inoculated into the 24-well plate in the lower chamber. After conventional cell culture for about 1d, the cells were fixed in 4% formaldehyde for 15min. The supernatant was collected and stained with 0.25% crystal violet for 25min. The cells were washed slowly with sterilized water, placed on the ultra-clean bench and dried. Pictures were taken and the cells were counted.

The expression of intracellular protein was detected by immunofluorescence

The cells were seeded into culture dishes with pretreated coverslips. When the cell grows to 95%-100%, it was washed two times with PBS. And then fixed by formaldehyde, washed by PBS, permeabilized by Triton X-100, washed by PBS, sealed, incubated overnight at 4 °C with primary antibody (anti-RhoA, anti-COX-2 rabbit polyclonal antibody), washed by PBS, incubated at room temperature for 2 h (Sheep anti rabbit IgG - H&L (Alexa Fluor, 488) polyclonal antibody) with second antibody. Then they were washed by PBS 3 times and preserved in the darkroom.

The cell cycle was detected by flow cytometry

The cells were passaged before the experiment 24 hours. Next day, digest the cells and add 70% pre-cooled ethanol, fixed overnight at 4 degrees. Then cleaned by PBS and PI treatment, apoptosis or cell cycle was detected by flow cytometry.

STATISTICAL ANALYSIS

Statistical analyses were carried out using the SPSS 19.0 software. The results were expressed as mean ± standard deviation. Intergroup comparisons were performed using independent sample t-test. The counts were expressed as percentages and the difference between the groups was analyzed by the chi-square test. $P<0.05$ indicated significant difference.

RESULTS

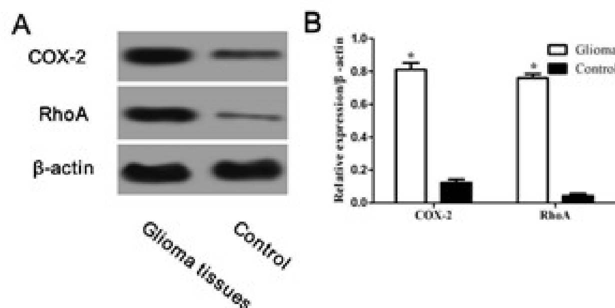
Expression of RhoA and COX-2 in different tissues

The relative expression of RhoA and COX-2 in the brain glioma tissues was 0.82 ± 0.13 and 0.75 ± 0.14, respectively, which was significantly higher compared to the normal brain tissues (0.12 ± 0.08 and 0.043 ± 0.14) ($P<0.05$), as shown in fig. 1.

Immunohistochemical staining of RhoA and COX-2 in different tissues

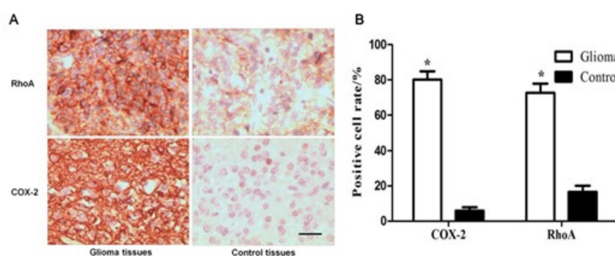
Immunohistochemical staining was performed to analyze the expression of RhoA and COX-2. The percentage of cells positive for RhoA and COX-2 was calculated for each specimen. The percentage of positive cells was compared between the brain glioma tissues and the control. The result showed that the percentage of cells positive for RhoA and COX-2 was 75.32 ± 15.02% and

82.39±17.82%, respectively, which was considerably higher compared to the normal brain tissues (17.03±7.72% and 5.83±4.01%) (P<0.05) (fig. 2).



Note: *P<0.05, indicating significant difference between the glioma tissues and the control.

Fig. 1: Expression of RhoA and COX-2 in different tissues



Note: *P<0.05, indicating significant difference between the glioma tissues and the control. Scale bar=100μm.

Fig. 2: Immunohistochemical staining of RhoA and COX-2 in different tissues.

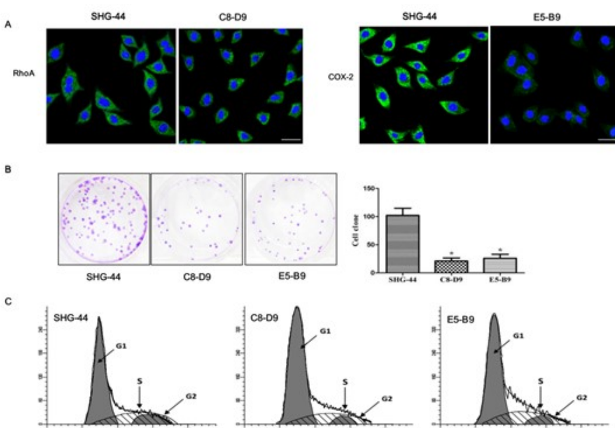


Fig. 3: Silencing expression of RhoA and COX-2 inhibits glioma cell proliferation. A: the expression of RhoA and COX-2 proteins in different cell immunofluorescence assay showed that the expression of C8-D9 cells and COX-2 silencing E5-B9 cells RhoA silencing were successfully constructed; scale bar=50 µm. B: Clone cells test indicated that silencing RhoA and COX-2 suppressed brain glioma SHG-44 cell proliferation; C: RhoA or COX-2 inhibited the expression of SHG-44 after brain cells glioma was blocked in the G0/G1 period; * stands for significant difference when compared with the SHG-44 group, P<0.05.

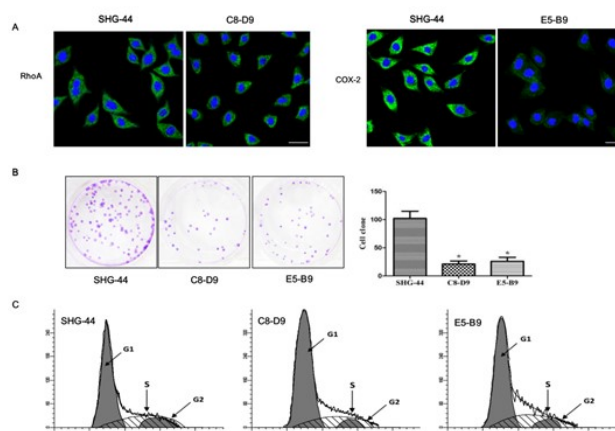


Fig. 4: Silencing expression of RhoA and COX-2 inhibits proliferation and migration of glioma cells. A: the expression of RhoA and COX-2 protein in different cells by immunofluorescence assay showed that RhoA silencing C8-D9 cells and COX-2 silencing E5-B9 cells were successfully constructed; scale bar=50 µm. B: the cell migration experiments showed that RhoA and COX-2 silencing inhibited migration of glioma cell SHG-44; C: RhoA protein through Notch pathway, COX-2 protein by wnt/ beta catenin pathway on the physical signs (E-cadherin) regulate the migration ability of the brain glioma cells; * indicated that compared with the SHG-44 group, it was significant difference, P<0.05.

Effects of RhoA and COX-2 expression on the proliferation and migration of the brain glioma cells

The cell cloning assay and cell invasion/migration assays were performed using the brain glioma cell line SHG-44, C8-D9 cells with silenced RhoA expression and E5-B9 cells with silenced COX-2 expression. The cell cycle was detected by flow cytometry. The results showed that 21.61±5.97 and 26.02±7.28 clones were formed in the C8-D9 cells and E5-B9 cells, respectively; this was much lower than the number of clones formed in the SHG-44 cells (102.26±23.94) (P<0.05). As compared with the SHG-44 cells, the relative proliferation rate of C8-D9 cells and E5-B9 cells was 20.72% and 25.45%, respectively. The percentage of G0/G1, S and G2/M phase cells in SHG-44 cells were (68.83+8.36) %, (25.00+10.25) % and (9.18+2.16) %, respectively. While, the relative proliferation rates of C8-D9 are (80.42+14.61) %, (15.24+5.97) % and (4.34+1.06) %. The relative proliferation rates of E5-B9 are (74.67+15.39) %, (20.83+9.24) % and (4.57+2.03) %, respectively (fig. 3).

Effects of RhoA and COX-2 expression on migration of brain glioma cells

The cell invasion/migration assays were performed using the brain glioma cell line SHG-44, C8-D9 cells with silenced RhoA expression and E5-B9 cells with silenced COX-2 expression. The expression of related proteins in the cells was detected by Western blot. The number of C8-D9 and E5-B9 cells migrating through the membrane

was 55.83±13.84 and 56.00±15.62, which was significantly lower than that of the SHG-44 cells (267.58±59.67) (P<0.05). As compared with the SHG-44 cells, the relative invasion/migration rate of the C8-D9 and E5-B9 cells was 20.91% and 20.97%, respectively. Compared with normal SHG-44 cells, the expression of Notch 1 and Dell protein decreased significantly in RhoA silenced SHG-44 cells (P<0.05) and E-cadherin protein increased significantly (P<0.05). SHG-44 cells silenced COX-2 showed significant decreases in beta-catenin and Wnt1 protein (P<0.05), and E-cadherin protein increased significantly (P<0.05) (fig. 4).

DISCUSSION

Brain glioma is the most common primary intracranial tumor caused by the canceration of gliocytes in the brain and spinal cord. Its annual incidence is about 3-8 every 100 thousand people. Like many other tumors (diseases), glioma results from the combined action of high-risk genetic factors and environmental carcinogenic factors (JA *et al*., 2005). Rho proteins are a family of small-mol-wt G proteins with GTPase activity. RhoA protein is an important member of the Rho family. An early research (Annabi *et al*., 2005) indicates that overexpression of RhoA can upregulate the proteins related to the MT1-MMP signaling pathway, resulting in the shedding of the CD44 molecules from the surface of the brain glioma cells. This finally causes the migration and infiltration of the brain glioma cells. As pointed out by Gu YT *et al*. (Gu *et al*., 2013), minoxidil sulfate enhanced the permeability of the blood-tumor barrier in the brain glioma model in rats by downregulating the tight junction via the ROS/RhoA/PI3K/PKB signaling pathway. The above studies indicate a close connection between RhoA gene expression and the occurrence and progression of brain glioma. The RhoA expression in the brain glioma tissues and normal brain tissues was detected, and it was found that the RhoA expression and the percentage of RhoA-positive cells in the brain glioma tissues were much higher than those in the normal brain tissues (P<0.05). According to Miao *et al*. (Miao *et al*., 2011), RhoA was over-expressed in the human brain glioma and the percentage of RhoA-positive cells positively correlated to mRNA expression and pathological staging of the brain glioma.

COX as a bifunctional enzyme exhibits the activities of both cyclooxygenase and catalase. It is involved in the regulation of cell growth and apoptosis, metabolic maintenance, malignant transformation and inflammatory response (Chen *et al*., 2015b). COX-2 is one of the two isoenzymes of the cyclooxygenase and overexpressed in many solid tumors such as gastric cancer (Qi *et al*., 2019), esophageal cancer (Chen *et al*., 2015a), pancreatic cancer (Bergmann *et al*., 2010) and bladder cancer (Gangwar *et al*., 2011). We also found that the COX-2 expression and the percentage of COX-2-positive brain glioma cells were considerably higher than those in the normal brain tissues (P<0.05). Eberstal S *et al*. found in the brain glioma model in rats that inhibiting the COX-2 expression was conducive to the treatment of brain glioma (Eberstål *et al*., 2014).

Invasion and migration of tumor cells are among the most important features of malignant tumors and also the major causes of tumor-related deaths. Since the last century, scientists began to focus on the mechanism underlying the invasion and migration of tumor cells. At present, we have established theories about the multi-factor, multi-mechanism and multi-gene regulation of the tumor cell invasion and migration. Regulatory genes of the tumor invasion/migration, genes in the relevant signaling pathways and target genes are the main focuses of study, as they are considered as important markers of clinicopathological staging and postoperative prognosis. RhoA and COX-2 are two important genes related to the invasion and migration of many tumor cells, including breast cancer, colorectal cancer and gastric cancer. It has been suggested that RhoA regulates the proliferation, invasion and migration of the tumor cells by regulating the cellular morphology, G1/S phase transition, and adhesion of cells to the matrix (Fife *et al*., 2014). COX-2 regulates the invasion and migration of tumor cells by regulating E-cadherin, MMP-2, Wnt and Notch signaling pathways and EMT-related genes (Huang *et al*., 2016). Through the present study, it was found that the expression of RhoA and COX-2 in the brain glioma tissues was significantly upregulated as compared with the normal brain tissues (P<0.05). This implies a close connection between RhoA and COX-2 and the occurrence and progression of brain glioma. We further detected the expression of RhoA and COX-2 in the SHG-44 cells with silenced RhoA expression and E5-B9 cell line with silenced COX-2 expression, so as to analyze the effect of RhoA and COX-2 on the invasion and migration of glioma cells. The result showed that the SHG-44 cells with silenced RhoA or COX-2 expression displayed a dramatic decline in the invasion/migration capacities (P<0.05).

CONCLUSION

Both RhoA and COX-2 were upregulated in the brain glioma tissues, which contributed to the proliferation and invasion/migration of the brain glioma cells.

REFERENCES

Annabi B, Bouzeghrane M, Moumdjian R, Moghrabi A & Beliveau R (2005). Probing the infiltrating character of brain tumors: Inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. *J. Neurochem.,* **94**(4): 906-916.

Bergmann F, Moldenhauer G, Herpel E, Gaida MM,

Strobel O, Werner J, Esposito I, Muerkoster SS, Schirmacher P and Kern MA (2010). Expression of L1CAM, COX-2, EGFR, c-KIT and Her2/neu in anaplastic pancreatic cancer: Putative therapeutic targets? *Histopathology,* **56**(4): 440-448.

Chen J, Fang WU, Pei HL, Wen-Dong GU, Ning ZH, Shao YJ and Jin H (2015a). Analysis of the correlation between P53 and Cox-2 expression and prognosis in esophageal cancer. *Oncol. Lett,* **10**(4): 2197-2203.

Chen Z, Wang ZC, Yan XQ, Wang PF, Lu XY, Chen LW, Zhu HL and Zhang HW (2015b). Design, synthesis, biological evaluation and molecular modeling of dihydropyrazole sulfonamide derivatives as potential COX-1/COX-2 inhibitors. *Bioorg. Med. Chem. Lett.,* **25**(9): 1947-1951.

Cheng H, Chi XS, Xin H, Ni C, Qiao Z, Dong Z and Li JM (2017). Predictors and mechanisms of epilepsy occurrence in cerebral gliomas: What to look for in clinicopathology. *Exp. Mol. Pathol.,* **102**(1): 115-122.

Eberstal S, Fritzell S, Sanden E, Visse E, Darabi A and Siesj P (2014). Immunizations with unmodified tumor cells and simultaneous COX-2 inhibition eradicate malignant rat brain tumors and induce a long-lasting CD8(+) T cell memory. *J. Neuroimmunol,* **274**(1-2): 161-167.

Fife CM, Byrne FL, Sagnella SM, Davis TP, Mccarroll J A and Kavallaris M (2014). Abstract 4987: Stathmin regulates cell migration, invasion and transendothelial migration via RhoA activation in neuroblastoma. *Cancer Res,* **74**(19): 4987-4987.

Gangwar R, Mandhani A and Mittal RD (2011). Functional polymorphisms of cyclooxygenase-2 (COX-2) gene and risk for urinary bladder cancer in North India. *Surgery,* **149**(1): 126-134.

Grzanna MW, Au RY, Au AY, Rashmir AM and Frondoza CG (2020). Avocado/soybean unsaponifiables, glucosamine and chondroitin sulfate combination inhibits proinflammatory COX-2 expression and prostaglandin e2 production in tendon-derived cells. *J. Med. Food,* **23**(2): 139-146.

Gu YT, Xue YX, Wang YF, Wang JH, Chen X, Shangguan QR, Lian Y, Zhong L and Meng YN (2013). Minoxidil sulfate induced the increase in blood-brain tumor barrier permeability through ROS/RhoA/PI3K/PKB signaling pathway. *Neuropharmacology,* **75**(12): 407-415.

Huang M, Long W, Chen J, Bai M, Zhou C, Liu S and Lin QU (2016). Regulation of COX-2 expression and epithelial-to-mesenchymal transition by hypoxia-inducible factor-1α is associated with poor prognosis in hepatocellular carcinoma patients post TACE surgery. *Int. J. Oncol.,* **48**(5): 2144-2154.

Jennifer A Doherty, Noel S Weiss, Robert J Freeman, Douglas A Dightman, Perry J Thornton, John R Houck, Lynda F Voigt, Mary Anne Rossing, Stephen M Schwartz and Chu Chen (2005). Genetic factors in catechol estrogen metabolism in relation to the risk of endometrial cancer. *Cancer Epidemiol. Biomarkers Prev,* **14**(2): 357-366.

Jian H, Di Z, Xie FQ and Lin DG (2015). The potential role of COX-2 in cancer stem cell-mediated canine mammary tumor initiation: An immunohistochemical study. *J. Vet. Sci.,* **16**(2): 225-231.

Johnson DR, Guerin JB, Ruff MW, Fang S, Hunt CH, Morris JM, Pearse Morris P and Kaufmann TJ (2019). Glioma response assessment: Classic pitfalls, novel confounders and emerging imaging tools. *Br. J. Radiol.,* **92**(1094): 20180730.

Kalpana G, Figy C, Yeung M and Yeung KC (2019). Reduced RhoA expression enhances breast cancer metastasis with a concomitant increase in CCR5 and CXCR4 chemokines signaling. *Sci. Rep.,* **9**(1): 16351.

Liu X, Chen D and Liu G (2014). Overexpression of RhoA promotes the proliferation and migration of cervical cancer cells. *Biosci. Biotechnol. Biochem.,* **78**(11): 1895-901.

Miao W, Liu XD, Fan YM, Zhao HQ and Wang HQ (2011). Expression of RhoA in human glioma and its clinical significance. *Chin.J. Clinicians,* **5** (9): 2549-53.

Qi YF, Liu M, Zhang Y, Liu W, Xiao H and Luo B (2019). EBV down-regulates COX-2 expression via TRAF2 and ERK signal pathway in EBV-associated gastric cancer. *Virus Res.,* **272**(15): 197735.

Reardon DA and Wen PY (2015). Glioma in 2014: Unravelling tumour heterogeneity-implications for therapy. *Nat. Rev. Clin. Oncol.,* **12**(2): 69-70.

Song L, Guo Y and Xu B (2017). Expressions of ras homolog gene family, member a (RhoA) and cyclooxygenase-2 (cox-2) proteins in early gastric cancer and their role in the development of gastric cancer. *Med Sci Monit,* **23**(18): 2979-2984.

Witiak DT, Lee HJ, Goldman HD and Zwilling BS (2007). Pathogenesis of brain glioma and research development of its diagnosis and treatment. *Chin J Neuromed,* **6**(9): 869-871.

Wong BS, Shea DJ, Mistriotis P, Tuntithavornwat S, Law RA, Bieber JM, Zheng L and Konstantopoulos K (2019). A direct podocalyxin-dynamin-2 interaction regulates cytoskeletal dynamics to promote migration and metastasis in pancreatic cancer cells. *Cancer Res,* **79**(11): 2878-2891.

Xu JX, Xiong W, Zeng Z, Tang Y, Wang YL, Xiao M, Li M, Li QS, Song GL and Kuang J (2017). Effect of ART1 on the proliferation and migration of mouse colon carcinoma CT26 cells *in vivo*. *Mol. Med. Rep.,* **15**(3): 1222-1228.

Ye Y, Xu Y, Lai Y, He W, Li Y, Wang R, Luo X, Chen R and Chen T (2018). Long non-coding RNA cox-2 prevents immune evasion and metastasis of hepatocellular carcinoma by altering M1/M2 macrophage polarization. *J. Cell Biochem.,* **119**(3): 2951-2963.