ORIGINAL ARTICLE

Deregulated Expression of the *Per1* and *Per2* in Human Gliomas

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ABSTRACT: ***Background:*** Growing evidence shows that the deregulation of the circadian clock plays an important role in the development of malignant tumors, including gliomas. However, the molecular mechanisms of genes controlling circadian rhythm in glioma cells have not been explored. ***Methods:*** Using reverse transcription polymerase chain reaction and immunohistochemistry techniques, we examined the expression of two important clock genes, Per1 and Per2, in 33 gliomas. ***Results:*** In this study, out of 33 gliomas, 28 were Per1-positive, and 23 were Per2-positive. The expression levels of Per1 and Per2 in glioma cells were significantly different from the surrounding non-glioma cells (P<0.01). The difference in the expression rate of Per1 and Per2 in high-grade (grade III and IV) and low-grade (grade 1 and II) gliomas was insignificant (P>0.05). While there was no difference in the intensity of immunoactivity for Per2 between high-grade gliomas and low-grade gliomas (r=-0.330, P=0.061), the expression level of Per1 in high-grade gliomas was significantly lower than that in low-grade gliomas(r=-0.433, P=0.012). ***Conclusions:*** In this study, we found that the expression of Per1 and Per2 in glioma cells was much lower than in the surrounding non-glioma cells. Therefore, we suggest that disturbances in Per1 and Per2 expression may result in the disruption of the control of normal circadian rhythm, thus benefiting the survival of glioma cells. Differential expression of circadian clock genes in glioma and non-glioma cells may provide a molecular basis for the chemotherapy of gliomas.

RÉSUMÉ: Expression dérégulée de Per1 et Per2 dans les gliomes humains. ***Contexte :*** Il existe de plus en plus de données démontrant que la dérégulation de l'horloge circadienne joue un rôle important dans l'apparition de tumeurs malignes, dont les gliomes. Cependant, les mécanismes moléculaires au niveau des gènes qui contrôlent le rythme circadien dans les cellules de gliomes n'ont pas encore été étudiés. ***Méthodes :*** Nous avons examiné l'expression de deux gènes importants de l'horloge biologique, Per1 et Per2, dans 33 gliomes au moyen du RT-PCR et de techniques d'immunohistochimie. ***Résultats :*** Dans cette étude, 28 des 33 gliomes étaient positifs pour Per1 et 23 étaient positifs pour Per2. Les niveaux d'expression de Per1 et de Per2 dans les cellules gliomateuses étaient significativement différents de ceux des cellules non gliomateuses avoisinantes ($p < 0,01$). La différence du taux d'expression de Per1 et de Per2 dans les tumeurs de haut grade (grades III et IV) et de bas grade (grades I et II) n'était pas significative ($p > 0,05$). Bien qu'il n'existait pas de différence dans l'intensité de l'immunoactivité pour Per2 entre les gliomes de haut grade et ceux de bas grade ($r = -0,330$; $p = 0,061$), le niveau d'expression de Per1 dans les gliomes de haut grade était significativement plus faible que dans les gliomes de bas grade ($r = 0,433$; $p = 0,012$). ***Conclusions :*** Dans cette étude, nous avons constaté que l'expression de Per1 et de Per2 dans les cellules gliomateuses était beaucoup plus faible que dans les cellules non gliomateuses avoisinantes. Nous suggérons donc que la perturbation de l'expression de Per1 et de Per2 pourrait provoquer la perturbation du contrôle du rythme circadien normal, ce qui favoriserait la survie des cellules gliomateuses. L'expression différentielle des gènes de l'horloge circadienne dans les cellules gliomateuses et non gliomateuses pourrait offrir une base moléculaire en chimiothérapie des gliomes.

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For years, researchers puzzled over gliomas, an aggressive form of brain cancer. Glioma tumors often surmount traditional cancer treatments such as surgery, and quickly invade healthy brain tissue. Many experimental therapies have been put into practice[1-5], and recent study has shown that a new risk factor, the circadian rhythm, may play an important role in glioma development[6]. An understanding of the biological makeup and survival mechanisms of glioma tumors may help researchers develop a new method that they hope will kill the cancer.

In mammals, physiological and hormonal processes as well as behavioral reactions follow circadian rhythms that are driven by an endogenous master clock. The master clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and produces self-sustaining circadian rhythms that are synchronized by external cues. Recent studies have shown that the circadian system is a master-and-slave structure: the master pacemaker SCN synchronizes slave oscillators (peripheral tissues) of mammals[7-9]. Recently, it is circadian rhythms similar to those operating in the SCN that have been found in most mammalian cells and peripheral tissues, and these peripheral circadian rhythms may be driven or synchronized by the central

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pacemaker in the SCN[10]. The basic molecular clockworks underlying the generation of circadian rhythms consist of interacting positive and negative transcriptional/translational feedback loops. The dimerization of two basic helix–loop– helix Per-Arnt-Sim (bHLH-PAS) family members, circadian locomotor output cycles kaput (CLOCK) and brain and muscle ARNT-like protein (BMAL1), drives the rhythmic transcription of three Period genes (Per1, Per2, and Per3) and two Cryptochrome genes (Cry1 and Cry2) at the beginning of each day. By mid-day, CRY and PER proteins begin to accumulate and enter the nucleus and then interact with CLOCK and/or BMAL1 to inhibit their own transcription and enhance Bma1l transactivation. Thus, increased CLOCK:BMAL1 heterodimers activate Per and Cry gene expression, and the clock is reset at the beginning of the next day[11]. The expression of these clock genes and their rhythmic regulation are not unique to the SCN but, instead, are widely distributed in many cells and tissues[12].

It is well-known that the alterations in circadian rhythm can be a risk factor for the development of cancers in both animal and human tumors[13-16]. Deregulation of the circadian clock has been implicated in many types of cancers, including breast tumors[13], human endometrial carcinoma[15], Lewis lung carcinoma and mouse mammary carcinoma[16]. However, molecular changes in these circadian genes in glioma cells have not been explored. In the present study, the expression of Per1 and Per2 was examined by real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry in 33 gliomas and corresponding non-tumor brain samples.

Table 1: Gene nomenclature, primer sequences and predicted size of the amplified products for the different genes studied

Gene	Forward primer	Reverse primer	Size(bp)
Per1	ATCTTCCACCTCACTCCCTC	CTCATTGCCACTTGAACCAT	266
Per2	TTCTCCCATTCGGTTTCG	CCTGACTTTGTGCCTCCC	121
β-actin	GAGCTACGAGCTGCCTGACG	CCTAGAAGCATTTGCGGTGG	416

Materials and Methods

Patients

From May 2007 to April 2008, 33 patients with diffuse subcortical gliomas were operated on in the Affiliated Hospital of Ningxia Medical University, and the glioma tissue samples of these patients were used for this study. Thirty three resected glioma tissue samples with the surrounding non-cancerous tissues were collected. The paired non-cancerous tissue confirmed by histopathological analysis was collected from the normal part of glioma tissue without contamination from glioma cells. The age of the patient ranged from 8 to 74 with a mean of 42.2 years, with 20 men and 13 women. The tissues were

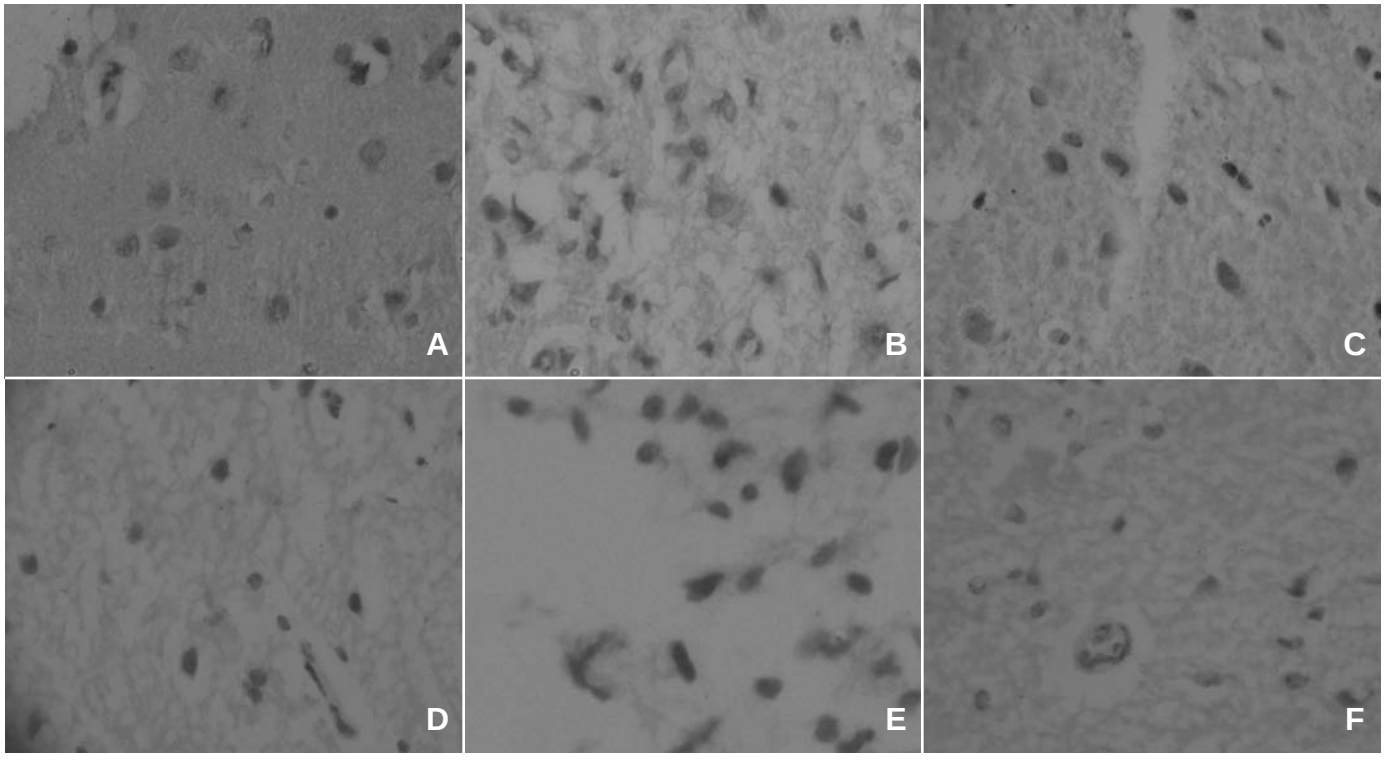

Figure 1: *Per1 and Per2 expression (X40): A) Per1 expressed in normal brain tissue; B) Per1 in glioma, stage III; C) Per1 in glioma, stage II; D) Per2 in normal brain tissue; E) Per2 in glioma, stage III; F) Per2 in glioma, stage II.*

Table 2a: Expressions of Per1 in gliomas and normal tissues cells

	n	Per1
Normal tissue	33	4.5455±1.43812
Gliomas Tissue	33	2.2727±0.94448**

P <0.01

Table 2b: Expressions of Per2 in gliomas and normal tissues cells

	n	Per2
Normal Tissue	33	4.1515±1.27772
Glioma tissue	33	2.1515±1.22783**

P <0.01

frozen or formalin-fixed immediately after surgical resection and stored in liquid nitrogen as Hui et al previously described[16]. The glioma tissue and tumor free specimens were surgically obtained between 12:00 and 14:00. The glioma tissues in our study include 15 astrocytomas, 12 oligodendrogliomas and 6 glioblastoma. According to WHO pathology grading, 2 cases were stage I, 9 were stage II, 17 were stage III, and 5 were stage IV.

Immunohistochemistry

Paraffin-embedded tissue sections (4μm) on poly-1-lysine coated slides were deparaffinized. The sections were treated with EDTA in a pressure cooker, heated at boiling temperature for 2.5 minutes, cooled and incubated with 3% H_2O_2 for ten minutes to block endogenous peroxidase, and then incubated with gradient alcohol and washed in phosphate buffered saline (PBS) three times for two minutes each time. After being bathed in PBS, sections were incubated with antibodies for Per1 (1:300, H-120, Santa Cruz Biotechnology, CA) and Per2 (1:300, M01, Abnova Co., Taiwan) for two hours at 37°C.

The slides were washed three times with PBS and incubated with secondary anti-body, anti-rabbit antibody (PV-6001, Zhongshan Goldenbridge Biotechnology Co., China) and anti-mouse antibody (PV-6002, Zhongshan Goldenbridge Biotechnology Co., China) at 37°C for 30 minutes, then thoroughly washed three times with PBS. Detection of immunostaining was performed using diaminobenzidine (DAB, Zhongshan Goldenbridge Biotechnology Co. China.) as chromogene, and then a counterstain was performed using haematoxylin.

Staining was evaluated by a pathologist and an investigator blind to diagnosis and sections were classified as positive or negative. Cell nucleus can be observed through staining.

Positive cells had yellow staining in the cell nucleus. Cells were quantified by cell numbers per one high power field , with no positive cell graded as 0, 1-25 % of the cells as 1, 26 -50% as 2, 51-75% as 3, 75-100% as 4. The staining intensity was graded, with no coloration graded as 0, light yellow as 1, yellow as 2, and brown as 3. The two scales were multiplied, the cells with a value greater than or equal to 2.0 were counted as positive[17].

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol reagent (15596-026, Invitrogen) and converted to a single-stranded cDNA by reverse transcriptase with oligo (dT) primer. Synthesized cDNA was used as a template in PCR. β-actin primers were used as an internal control. Reverse transcription polymerase chain reaction for human Per1, Per2 and β-actin mRNA was performed. Details of the primer are given in Table 1.

Table 3a: Expression of Per1 in glioma cells

Grade	Positive	Negative	Positive Rate (%)	Total
Ⅰ～Ⅱ	10	1	90.91	11
Ⅲ～Ⅳ	18	4	81.82	22

Table 3b: Expression of Per2 in glioma cells

Grade	Positive	Negative	Positive Rate (%)	Total
Ⅰ～Ⅱ	10	1	90.91	11
Ⅲ～Ⅳ	13	9	59.09	22

After gel electrophoresis, we observed the results on a UV detector and scanned the gel using a BioCaptMW gel imaging analysis system. We then analyzed the determination of amplification products of gray value bands to β-actin as internal reference for the ratio, semi-quantitative analysis of Per1 mRNA and Per2 mRNA expression level for statistical analysis.

Statistical analysis

The association between tumor grade (high-grade/low-grade gliomas) and expression of the investigated proteins (negative/positive) was assessed using the Spearman, Chi-Square Test and Two-sample t-test, included in the Statistical Package for the Social Science, version 13.0.

Result

Immunohistochemistry

The expression of Per1 and Per2 can be observed in gliomas and normal tissues (non-tumor brain sample) at different levels, all the staining was in the cell nucleus (Figure 1). The expression of Per1 and Per2 in normal tissues is 100% (33/33), while out of 33 gliomas, 28 were Per1-positive (84.85%) and 23 were Per2-positive (69.70). The expression levels of Per1 and Per2 in the glioma cells were significantly different from those in normal brain tissue cells ($P<0.01$, Two-sample t-test, Table 2a and 2b). There was no significant difference in the expression rates of Per1 and Per2 between high-grade (III, IV) and low grade (I, II) gliomas ($P>0.05$, Chi-Square test, Table 3a and 3b). There was no significant difference in the intensity of immunoactivity of Per2 between high-grade gliomas and low-grade gliomas ($r=-0.33$, $P=0.061$), whereas the expression of Per1 in high-grade gliomas is significantly lower than that of low-grade gliomas ($r=-0.433$, $P=0.012$).

RT-PCR

Lower levels of Per1 and Per2 mRNA were identified in gliomas compared with normal brain tissues (Figure 2 and Figure 3). The expression levels of Per1 mRNA and Per2 mRNA in the glioma cells were significantly different from those in normal brain tissue cells ($P<0.01$, Two-sample t-test, Table 4a and 4b).

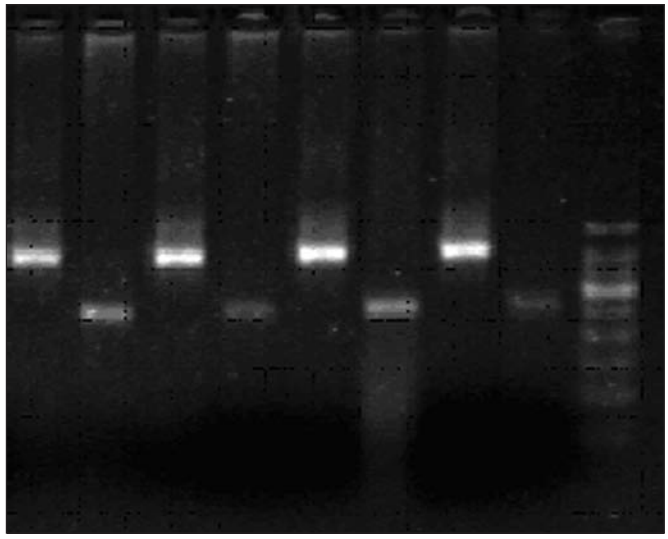

Figure 2: *Expression of Per1 mRNA was significantly different in glioma and normal brain tissue. The 1st, 3rd, 5th, and 7th lanes show the expressions of Per1 mRNA to corresponding treatments of β-actin. The 2nd lane is the expression of Per1 mRNA in grade II of corresponding non-cancer tissue. The 4th lane is the expression of Per1 mRNA in grade II of glioma tissue. The 6th lane is the expression of Per1 mRNA in grade III of corresponding non-cancer tissue. The 8th lane is the expression of Per1 mRNA in grade III of glioma tissue. The 9th lane is the expression of a 50bp Mark; from the lowest arrows, the base pair lengths are: 50bp, 100bp, 150bp, 200bp, 250bp, 300bp, 350bp, 400bp, 500bp.*

There was no significant difference in the intensity of RT-PCR for Per2 mRNA between high-grade gliomas and low-grade gliomas ($r=-0.195$, $P=0.567>0.05$), whereas the expression of Per1 mRNA in high-grade is significantly lower than that of low-grade ($r=-0.726$, $P=0.011<0.05$).

Discussion

Generally speaking, circadian clock and cell division cycles are two basic biological processes. Recently, research has shown

Table 4a: Expression of Per1 mRNA and Per2 mRNA in gliomas and normal tissue cells evaluated by RT-PCR

	n	Per1
Normal tissue	33	0.4682±0.1010
Gliomas Tissue	33	0.2905±0.0727**

P <0.01

Table 4b: Expression of Per1 mRNA and Per2 mRNA in gliomas and normal tissue cells evaluated by RT-PCR

	n	Per2
Normal tissue	33	0.4834±0.1332
Gliomas Tissue	33	0.2825±0.1402**

P <0.01

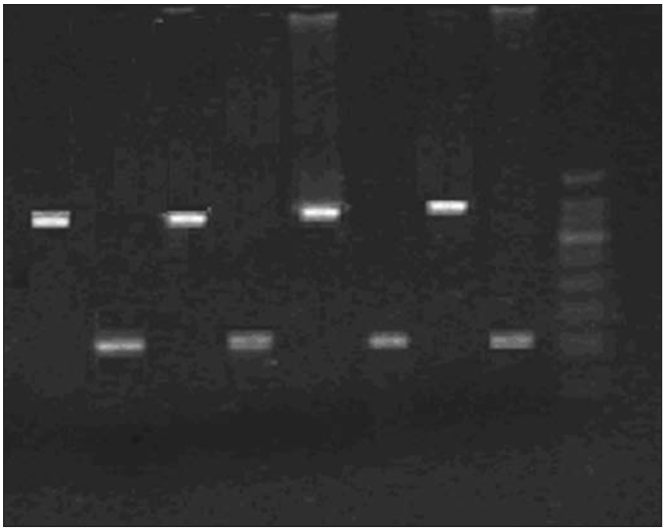

Figure 3: *Expression of Per2 mRNA was significantly different in glioma and normal brain tissue. The 1st, 3rd, 5th, and 7th lanes show the expressions of Per2 mRNA to corresponding treatments of β-actin. The 2nd lane is the expression of Per2 mRNA in grade II of corresponding non-cancer tissue. The 4th lane is the expression of Per2 mRNA in grade II of glioma tissue. The 6th lane is the expression of Per2 mRNA in grade III of corresponding non-cancer tissue. The 8th lane is the expression of Per2 mRNA in grade III of glioma tissue. The 9th lane is the expression of a 50bp Mark; from the lowest arrows, the base pair lengths are: 50bp, 100bp, 150bp, 200bp, 250bp, 300bp, 350bp, 400bp, 500bp.*

that the circadian system is not only essential for proper growth control, but it also regulates cell proliferation and apoptosis[18-20].

As a set of core circadian genes, PERs have the function of not only maintaining the circadian rhythm of cells, but also sustaining the normal cell cycle. It has been reported that 2-10% of all mammalian genes are clock-controlled[21-25], and recent studies reported that approximately 7% of all clock-controlled genes identified in rodents regulate either cell proliferation or apoptosis[24]. Therefore, the development of glioma may be related to the disruption of PERs. The molecular changes in circadian rhythm controlled genes in glioma cells are still unexplored. In this study, we analyzed and compared the expression status of the Per1 and Per2 proteins in glioma and non-cancer brain tissues obtained at the same time in each case so that tissue pairs were synchronized with respect to their circadian clocks, and we find differential expression patterns in the Per1 and Per2 genes in glioma cells in most of the glioma cases (Per1 is 28/33 and Per2 is 23/33) when analyzed in comparison with their paired nearby non-cancer brain tissues. The deregulation of the circadian clock may be one of the most important factors in the proliferation of glioma. Since expression of Per1 and Per2 play a central role in circadian rhythm, our results suggest that the circadian clock in the cancer cells of most glioma cases behaves differently than in nearby non-cancer brain tissues.

Furthermore, we also observed Per1 and Per2 expression patterns in different cell populations of the same glioma tissue to be 84.85% for Per1 and 69.70% for Per2. In nearby non-cancer brain tissue, the expression of Per1 and Per2 is 100%, suggesting that several asynchronized circadian clocks may be in operation in the same glioma tissue. This can show that the heterogeneity has been in glioma cell population in glioma tissue. Our results may provide a molecular basis for designing clinical protocols for chronotherapy in glioma treatment based on differences in the circadian clock between glioma and non-cancer brain tissue and, therefore, differences in radiosensitivity[26-29].

We observed a statistically significant difference of expression of Per1 between gliomas tissues and normal brain tissues (the results of immunohistochemistry and RT-PCR is individually $P < 0.01$). We also observed a statistically significant difference of expression of Per2 between gliomas tissues and normal brain tissues (the results of immunohistochemistry and RT-PCR are individually $P < 0.01$). Our results may show that different biological functions for Per1 and Per2 proteins, probably related with the development and progression stage of gliomas which need to be investigated further more. Fu et al[30] reported that mice deficient in the mPer2 gene were cancer prone and that these mice showed a markedly increased rate in tumor development and reduced apoptosis in thymocytes after radiation. Based on these results, the authors suggested that mPer2 was a tumor suppressor gene. In this study, the use of primary human glioma tumors allowed us to examine correlations between tumor and Per1 and Per2 expression levels. We found a weak association of expression of Per1 among tumor grades (r=-0.433, $P=0.012<0.05$) and the expression of Per1 mRNA among tumor grade (r=-0.726, $P=0.011<0.05$), but there is no association in the expression of Per2 between low grade and high grade glioma tissues (r=-0.33, $P=0.061>0.05$) and the expression of Per2 mRNA among tumor grade (r=-0.195, $P=0.567>0.05$), which need to be investigated further in larger samples. Taken together, we suggest the low expression of Per1 may play a more important role in the development of glioma. Recently, studies have shown that the circadian clock controls the expression of cell cycle related genes[19]. It was also noted that the intracellular circadian clockwork is able to control the cell-division cycle directly and in a unidirectional mode in proliferating cells. Interestingly, Genetic ablation mPer1 and mPer2 results in a complete loss of circadian rhythm control based on wheel running activity in mice. In addition, these animals also display apparent premature aging and significant increase in neoplastic and hyperplastic phenotypes[31]. In another separate study, it was observed that the loss of Per1 protein was commonly observed in human endometrial carcinoma but not in the adjacent normal cells[15]. Based on these results, we propose that inactivation of the Per1 and Per2 in glioma cells may result in deregulation of the cell cycles thus favoring the proliferation of glioma cells, as deregulated expression of the Per1 genes is common in gliomas.

Conclusions

In conclusion, deregulated expression of Per1and Per2 genes is common in gliomas, and may play a role in the development of glioma by benefiting the survival and proliferation of glioma cells.

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