RESEARCH ARTICLE

Deregulated Expression of Cry1 and Cry2 in Human Gliomas

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Abstract

Growing evidence shows that deregulation of the circadian clock plays an important role in the development of malignant tumors, including gliomas. However, the molecular mechanisms of gene chnages controlling circadian rhythm in glioma cells have not been explored. Using real time polymerase chain reaction and immunohistochemistry techniques, we examined the expression of two important clock genes, cry1 and cry2, in 69 gliomas. In this study, out of 69 gliomas, 38 were cry1-positive, and 51 were cry2-positive. The expression levels of cry1 and cry2 in glioma cells were significantly different from the surrounding non-glioma cells (P<0.01). The difference in the expression rate of cry1 and cry 2 in high-grade (grade III and IV) and low-grade (grade 1 and II) gliomas was non-significant (P>0.05) but there was a difference in the intensity of immunoactivity for cry 2 between high-grade gliomas and low-grade gliomas (r=-0.384, P=0.021). In this study, we found that the expression of cry1 and cry2 in glioma cells was much lower than in the surrounding non-glioma cells. Therefore, we suggest that disturbances in cry1 and cry2 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.

Keywords: cry1 - cry2 - glioma - circadian rhythms

Asian Pacific J Cancer Prev, **13 (11)**, 5725-5728

Introduction

Almost all physiological characteristics of animals and plants differ significantly between day and night. Central and peripheral clocks generate self-sustained circadian rhythms of about 24 hours, which coordinate physiologic processes with the rhythmically changing environment (Czeisler et al., 1999; Reppert et al., 2001; Morse et al., 2002; Reppert et al., 2002). The circadian system is not only required for proper growth control, but is also involved in the circadian regulation of cell proliferation and apoptosis (Delaunay et al., 2002; Panda et al., 2002; Storch et al., 2002). In fact, circadian rhythms regulate diverse physiological processes, including hormone secretion, metabolism, cell proliferation and apoptosis (Young et al., 2001; Lowrey et al., 2004; Lee et al., 2005). Deregulation of the circadian clock may disturb the expression of clock-controlled genes and have a profound influence on organ function.

The daily light-dark cycle synchronizes the master circadian pacemaker, located in the suprachiasmatic nuclei (SCN) of the brain, which in turn synchronizes the organism's central clock, as well as the peripheral clocks in each cell (Reddy et al., 2005). The molecular mechanisms of circadian oscillation in the SCN and peripheral cells are based on a negative transcriptional-translational feedback loop generated by the core clock genes.

CLOCK and BMAL1, associated as heterodimers, bind to the E-box enhancer element and induce the expression of Period genes (Per1, Per2, Per3) and Cryptochrome genes (Cry1, Cry2). The encoded proteins, which associate in multimeric complexes, engage in a negative feedback loop to repress transactivation by CLOCK/BMAL1 in the nucleus. 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 (Morse et al., 2002) and 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 (Metz et al., 2005; Yeh et al., 2005; Hua et al., 2007; Huttmann et al., 2012), including breast tumors, human endometrial carcinoma (Huttmann et al., 2012), Lewis lung carcinoma and lymphocytic leukemia (Yeh et al., 2005).

However, the molecular mechanism(s) in these circadian genes in glioma cells have yet to be explored in detail. In the present study, the expression of cry 1 and cry 2 was examined by real-time reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry in 69 gliomas and corresponding non-tumor brain samples.

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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)
Cry1	CTTGATGCAGATTGGAGCAT	CCATTGGGATCTGTTCTCCT	122
Cry2	AGTCAAGCAAACCTGGAAGG	AATCATCCTGCTACCCGAAG	101
hGAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGGCCATCCACAGTCTTC	266

Materials and Methods

Patients

From January 2010 to July 2012, 62 patients with diffuse subcortical gliomas were operated on in the First People's Hospital of Jingmen, and the glioma tissue samples of these patients were used for this study. Sixty nine resected glioma tissue samples with the surrounding non-tumorous tissues were collected. The paired non-tumorous 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 17 to 69 with a mean of 39.4 years, with 38 men and 31 women. The tissues were frozen or formalin-fixed immediately after surgical resection and stored in liquid nitrogen as Hui et al previously described (Hua et al., 2007). The glioma tissue and tumor free specimens were surgically obtained between 11:00 and 15:00. The glioma tissues in our study include 36 astrocytomas, 23 oligodendrogliomas and 10 glioblastoma. According to WHO pathology grading, 21 cases were stage I, 16 were stage II, 19 were stage III, and 13 were stage IV.

Real-time PCR

Let the tumor and non-tumor tissues steep in 0.4% DEPC after surgical resection. Total RNA was isolated with Trizol reagent (Invitrogen), and Real-time PCR for human cry1, cry2, hGAPDH primers were used as an internal control. Details of the primer are given in Table 1. RNA (2 μg) was reverse-transcribed into a single-stranded cDNA with oligo (dT) primer. Synthesized cDNA was used as a template in Real-time PCR , in each set of six samples run in parallel, one sample was used to run a calibration reaction. Relative expression of cry1 and cry2 mRNA levels was determined using the relative quantification method and $2^{-\Delta\Delta Ct}$ analysis.

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% H2O2 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 cry1(1:200, Santa Cruz Biotechnology, CA) and cry 2 (1:300, Abnova Co., Taiwan) for two hours at 37°C.

The slides were washed three times with PBS and incubated with secondary anti-body, 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. The numbers of positive and negative cells were determined in four random fields (×100 magnification), and the percentage of positive cells was then calculated. 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% as2, 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.

Table 2. Expression of Cry1 cDNA and Cry2 cDNA in Gliomas and Normal Tissue Cells Evaluated by RealTime-PCR

	n	cry 1
Normal tissue	69	0.9744±0.10143
Gliomal tissue	69	0.8764±0.09025*
	n	cry 2
Normal tissue	69	0.5172±0.11748
Gliomal tissue	69	1.0190±0.25407[&]

*Two-sample t-test , P>0.05; [&]Two-sample t-test , P<0.01

Statistical analysis

Real-time PCR results are reported as mean ± standard deviation and were analyzed statistically using the ANOVA test and Student's t-test. 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.

Results

Real-time PCR

The expression levels of cry2 cDNA in the glioma cells were significantly different from those in normal brain tissue cells (P/,0.01, Two-sample t-test, Table 2). There was no significant difference in the intensity of Real time-PCR for cry1 cDNA between high-grade gliomas and low-grade gliomas (r=-0.195, P=0.618>0.05), whereas the expression of cry 2 cDNA in high-grade is significantly higher than that of lowgrade (r=-0.726, P=0.011<0.05).

Immunohistochemistry

The expression of cry 1 and cry 2 can be observed in gliomas and normal tissues (non-tumor brain sample) at

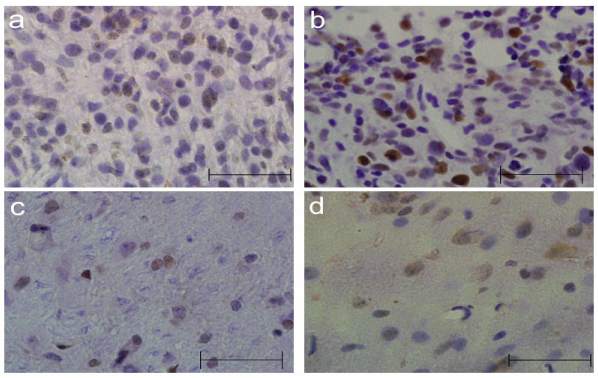

Figure 1. Immunohistochemical Analysis of Cry2 for Representative Cases and Positive Staining in the Nucleus are Found (×20). a, c were the tissues of grade I or II glioma and the non-tumor brain tissues around grade I or II glioma tissue ; b, d were the tissues grade III or IV glioma and the non-tumor brain tissues around the grade of III or IV glioma

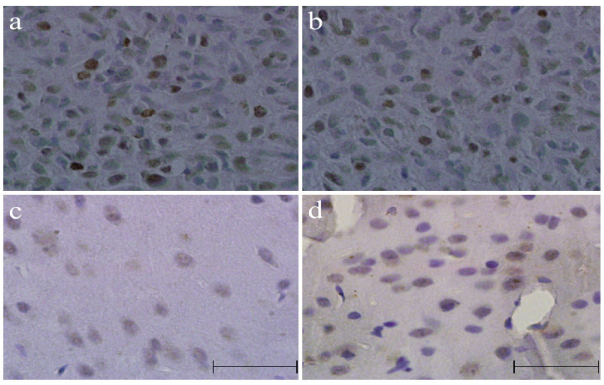

Figure 2. Immunohistochemical Analysis of Cry1 for Representative Cases and Positive Staining in the Nucleus are Found (×20). a, c were the tissues of grade I or II glioma and the non-tumor brain tissues around grade I or II glioma tissue ; b, d were the tissues grade III or IV glioma and the non-tumor brain tissues around the grade of III or IV glioma

different levels, all the staining was in the cell nucleus (Figure 1 and Figure 2). The expression of cry1 and cry 2 in normal tissues is 100% (69/69), while out of 69 gliomas, 38 were cry 1-positive (55.07%) and 51 were cry 2-positive (73.91%). The expression levels of cry 2 in the glioma cells were significantly different from those in normal brain tissue cells ($P<0.01$, Two-sample t-test, Table 3). There was no significant difference in the expression rates of cry 1 and cry 2 between high-grade (III, IV) and low grade (I, II) gliomas ($P>0.05$, Chi-Square test, Table 4). There was no significant difference in the intensity of immunoactivity of Cry1 between high-grade gliomas and low-grade gliomas ($r=-0.25$, $P=0.0714$), whereas the expression intensity of immunoactivity of cry 2 in high-grade gliomas was significantly higher than that in the low-grade gliomas ($r=-0.384$, $P=0.021$).

Discussion

The circadian clock and cell cycle are two global regulatory systems in most eukaryotic organisms. It has been known for some time that disruption of the circadian rhythm by genetic or environmental factors causes a variety of disorders in humans, such as sleep disturbances,

Table 3. Expressions of Cry1 and Cry2 in Gliomas and Normal Tissues Cells

	n	cry 1
Normal tissue	69	2.0153±1.86213
Glioma tissue	69	2.4225±1.63030*
	n	cry 2
Normal tissue	69	1.9732±0.98587
Glioma tissue	69	4.8038±1.95821[&]

*p>0.05; [&]p<0.05

Table 4. Expression of Cry1 and Cry2 in Glioma Cells

Grade	Positive	Negative	Positive Rate (%)	Total
Cry1				
I-II	21	16	56.76	37
III-IV	17	15	53.13	32
Cry2				
I-II	27	10	72.97	37
III-IV	24	8	75.0	32

seasonal affective disorder, and jet lag (Reddy et al., 2002). Disruption of cell cycle regulation causes cancer. Recent epidemiologic studies have raised the possibility that disruption of the circadian clock may also increase cancer risk in humans (Lee et al., 2010)and adversely affect prognosis in cancer patients (Destici et al., 2011). As the set of core circadian genes. CRY1 and CRY2 play a central role in the circadian clock independent of light and CRYs could inhibit the CLOCK-BMAL1 heterodimer in mammalian cells by forming direct contacts with it, pospossibly within a multiprotein complex including PER and TIM proteins (Destici et al., 2011).

The recent studies reported that, liver regeneration after partial hepatectomy is gated by the circadian clock and the proliferation of Cry1-/- /Cry2-/- hepatocytes was slower (Nagashima et al., 2005). It means that CRYs have a role in a wide variety of physiological processes, including the circadian rhythm of cells and sustaining the normal cell cycle. Therefore, the development of glioma may be related to the disruption of cry.

In present study, we analyzed and compared the expression status of transcriptional activation of cry1and cry2, and the CRYs proteins in tumor and non-tumor tissues obtained at the same time in each case so that the tissue pairs were synchronized with respect to the same circadian clock. we foud differential expression patterns in the CRY1 and CRY2 in glioma cells in most of the glioma cases (CRY 1 is 53/69 and CRY 2 is 43/69) when analyzed in comparison with their paired nearby non-tumor brain tissues. The deregulation of the circadian clock may be one of the most important factors in the proliferation of glioma. Since expression of cry1 and cry 2 play a central role in circadian rhythm, our results suggest that the circadian clock in the cancer cells of most glioma cases.

Furthermore, we also observed cry 1 and cry 2 expression patterns in different cell populations of the different grade glioma tissue to be 56.76% for cry 1 in high-grade and 53.13% for cry1 in low-grade and to be 72.97% for cry 2 in high-grade and 75.0% for cry2 in low-grade . In nearby non-tumor brain tissue around high-grade and low-grade, the expression of cry1 are 100% and

cry 2 are 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 that supported the hypothesis that the heterogeneity has been in glioma cell population in glioma tissue.

We observed a statistically significant difference of expression of cry1 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 cry 2 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 cry1 and cry 2 proteins, probably related with the development and progression stage of gliomas which need to be investigated further more. Henrik et al. (2002) report that the functional mCry2 gene product interferes with mPER1 or mCRY1 which leads to a gradual loss of circadian rhythmicity in Per2Brdm1 single mutant mice and suggest that mCry2 can act as a nonallelic suppressor of mPer2. Matsuo et al. (2003) report that the cell cycle progression from S to M phase was impaired during liver regeneration in Cry-deficient mice. Based on these results, the authors suggested that CRY2 proteins influence cell cycle progression and circadian rhythm in gliomas cells.

In this study, the use of primary human glioma tumors allowed us to examine correlations between tumor and cry 1 and cry 2 expression levels. We found a weak association of expression of cry2 among tumor grades (r=-0.712, P=0.001<0.05) and the expression of cry2 mRNA among tumor grade (r=-0.427, P=0.014<0.05), but there is no association in the expression of cry 1 between low grade and high grade glioma tissues (r=-0.217, P=0.051>0.05) and the expression of cry 1 mRNA among tumor grade (r=1.078, P=0.397>0.05),which need to be investigated further in larger samples. Taken together, we suggest the high expression of cry2 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 and noted that the intracellular circadian clockwork is able to control the celldivision cycle directly and in a unidirectional mode in proliferating cells. In addition,the mice lacking the cry 1 and 2 genes lose periodicity in wheel-running behavior (Vander et al., 1999) as well as electrophysiological activity in the SCN cells under constant darkness (DD) (Bonnefont et al., 2003). In another sepatrae study, CRY1 has been previously shown to undergo aberrant DNA methylation events in Human Chronic Myeloid Leukemia (Huttmann et al., 2012; Li et al., 2012). Based on these results, we propose that inactivation of the cry1 and cry2 in glioma cells may result in deregulation of the cell cycles thus favoring the proliferation of glioma cells, as deregulated expression of the cry1 genes is common in gliomas.

In conclusion, we found that deregulated expression of the cry1 and cry2 genes is common in glioma; and these findings provide further evidence in support of a role for circadian genes in glioma development by benefiting the gowth and proliferation of cancer cells.

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