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Analysis of Up-Regulation of DNA-PKcs and Its Mechanism in Human Gliomas

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OBJECTIVE To detect the differences in gene expression of nonhomologous end-joining pathway including Ku70, Ku80, ERCC4, lig4 and DNA-PKcs between human primary gliomas and normal brain tissues, and furthermore, to explore the underlying mechanism for the expression alteration.
METHODS The expression levels of Ku70, Ku80, ERCC4, lig4 and DNA-PKcs in 36 specimens of glioma and 12 specimens of normal brain tissue were measured using SYBR green-based real-time quantitative PCR. Methylation of DNA-PKcs was detected through methylation-specific PCR (MSP).
RESULTS There was no significant difference in expression of Ku70, Ku80, ERCC4 and lig4 between human primary gliomas and normal brain tissues ($P < 0.05$), while DNA-PKcs were significantly up-regulated ($P = 0.002$). The expression of DNA-PKcs was significantly higher in patients with grade III and IV diseases compared to patients with grade II disease or in normal brain tissues ($P < 0.05$). Moreover, glioma tissue showed weaker methylation than normal brain tissue.
CONCLUSION The up-regulation of the DNA-PKcs may be associated with pathogenesis of glioma. Demethylation of DNA-PKcs promoter is an important reason for its up-regulation.

KEY WORDS: glioma, non-homologous end joining, NHEJ, DNA-PKcs, methylation, real-time PCR.

Introduction

Glioma is one of the most common types of cancer in the CNS, composing greater than 80% of all kinds of CNS cancers[1]. Most of these gliomas, which have a complex development, are primary cancers[2], and are affected by genes, environment, and other factors. Damage to DNA causes gene mutations and chromosome abnormalities which result in carcinogenesis. However, little is understood concerning the underlying mechanism of glioma formation[3]. DNA double-strand breaks caused by x-ray and γ-ray are the most serious effects, therefore, repairing the breaks is very important to maintain the stability of the genome and to reduce chromosome abnormalities[4,5].

There are two ways to repair double-strand breaks: homologous recombination, HR, and non-homologous end joining, NHEJ[6,7]. HR involves repairing double-strand breaks based on homologous chromosome as template in the S/G2 phase, whereas NHEJ involves

connecting fractured chromosome directly by some proteins. HR is the main way for repairing double-strand breaks in yeast. However, in mammals, NHEJ is more commonly employed[8,9].

When DNA double strand is broken, caused by some intrinsic or extrinsic factors, the protein complex composed of Ku70 and Ku80 first binds to the fractured DNA chains, and then makes the structure into a cyclic structure[10,11]. This cyclic protein combines DNA-PKcs to form DNA-dependent protein kinase, which recruits proteins, such as ERCC4 and Lig4 to conduct conjunction of DNA chain and to repair gaps[12-15]. NHEJ assures the integrity of chromosomes when DNA is broken. Therefore, the repairing ability of NHEJ affects gene exoression and tumor progress[16,17]. However, NHEJ gene expression in gliomas has not been reported. We analyzed the expression of DNA-PKcs genes, Ku70, Ku80, ERCC4, and Lig4, which are main proteins involved in NHEJ. In addition, we investigated the nature of the difference in these DNAs by the means of DNA methylation. The pathogenesis of glioma, the theoretical guide for biological diagnosis and individualized therapy were also discussed in this study.

Patients and Methods

Patients

After obtaining informed consent, biopsies were taken from 36 patients with gliomas during their surgery at the Second Affiliated Hospital of Soochow University. According to the WHO 2007 classification[18], all the cases were given pathologic grades. Twelve of the cases were in grade II, (6 males and 6 females), with ages ranging from 17 to 56 years, and a mean of 39.4. Twelve were in grade III (5 males and 7 females), with ages ranging from 19 to 55 years, and a mean of 43.2. The remaining 12 were in grade IV (6 males and 6 females) with ages ranging from 16-66 years, and a mean of 44.6. For controls, normal brain tissues obtained from the 12 patients with cranio-cerebral trauma, who were undergoing a decompression surgery, was synchronously collected. These patients included 8 males and 4 females, aging 22-53 years, with a mean of 40.1.

Reagents

TRIzol reagent was obtained from Invitrogen in the United States. DNase I and the Wizard DNA purification kit were obtained from Promega in the United States. Reverse transcriptase, random primers, dNTP, RNase inhibitor, SYBRTM Green, Realtime Master Mix, and other products were obtained from Japan TOYOBO.

Experimental methods

Collection of tissue samples

Blood vessels and connective tissue were eliminated from the fresh specimens taken during surgery when the glioma was removed. The specimens were cut into several wads with 0.3-0.5 cm, and rinsed in 0.9% RNAase-free saline. Immediately after, the tissues were put into liquid nitrogen and then deposited in freezer at -80°C.

Designing and ordering primer

Gene primers of Ku70, Ku80, ERCC4, Lig4, and DNA-PKcs were designed based on a GeneBank sequence. In order to avoid contamination of genomic DNA, we designed an upstream or downstream primer to cross 2 exons. The primers of reference gene GAPDH were designed by ABI. All primers were synthesized by the Shanghai Biological Engineering Technology Services. Primers are presented in Table 1.

Table 1. Primers sense of the target genes and internal control GAPDH.

Gene	Primer	Primer Sequence (5'-3')
Ku70	Sense	CCTTTTGACATGAGCATCCA
	Antisense	ATACCGTGGCTCTTTCTGTT
Ku80	Sense	CAAAGAGGAAGCCTCTGGAA
	Antisense	TCAAAGACCGGGGGTTTC
ERCC4	Sense	GAGTGATGAGGAACCTTTTTGTGA
	Antisense	TGTTGTTCCAAGAACGAGTTGACT
Lig4	Sense	CTGCCCCAAAGATGAAGAAA
	Antisense	GTCTGGGCCTGGAT TTGTA
DNA-PKcs	Sense	CCACCTTGCTGAGTGGAAAT AAATGTCAGCAGGGACTGGT
	Antisense	CCACCTTGCTGAGTGGAAAT AAATGTCAGCAGGGACTGGT
GAPDH	Sense	GAAGGTGAAGGTCGGAGTC
	Antisense	GAAGATGGTGATGGGATTTC

Extraction of total RNA by the TRIzol method

To digest residual genomic DNA in RNA by DNase I.

Preparation of cDNA

First, a 16.5 μl reaction system was prepared, including total RNA 10 μl, oligo dT 2 μl, random primer 0.5 μl, and DEPC water 4 μl; Second, they were put into a 72°C water bath for 7 min; Third, the reverse transcriptase 1 μl, 5×buffer 5 μl, dNTP 2 μl, RNase inhibitor 0.5 μl was added in the reaction system mentioned above; and then, the reverse transcription of the total system was conducted in the 2700 PCR instrument. The reaction conditions were 42°C for 1 h or 95°C for 5 min; finally, cDNA prepared by reverse transcription was placed in a freezer at - 20°C for storage.

Detecting the expression of target genes by SYBR green-based real-time quantitative PCR

The reaction system of 8 μl was prepared, which included 2 × Master Mix 4 μl, the target gene or reference primers 1 μl, sample cDNA 1 μl and MilliQ water 1 μl. The reaction involved fluorescence quantitative and PCR

amplification according to the following conditions: pre-denaturation, at 95°C for 1 min; PCR amplification, 40 cycles, at 95°C, for 15 s, at 60°C, for 1 min; dissolution curves, at 95°C, for 15 s, at 60°C, for 15 s, at 95°C, for 15 s. The sample value of the target gene expression was calculated using the method of reference genes ΔCt, where the ΔCt value represented the amount of reaction cycles when the fluorescence of the gene reaction products reached the threshold. The sample value had been set (the value of target gene expression = 2Ct (GAPDH) – Ct (target gene).

Extraction and modification of DNA

Genomic DNA was extracted using the method of SDS and proteinase K. DNA methylation was conducted by the method described by Grunau et al.[19] First, 1 μg of DNA was denatured with 3M NaOH at 40°C for 15 min; Second, 520 μl 3M sodium sulfite was added and processed at 55°C for 14 h; Third, the treated DNA was purified using a Wizard DNA Clean-Up (Promega) kit, and then methylation of the products was amplified by PCR.

Detection of methylation-specific PCR (MSP)

The methylation primers of DNA-PKcs were: 5'-ATT ATG TTG TTT AGA TTG GTT TCG A-3'(upstream primer), 5'-AAA ATT CCA AAT TTA TTC CTC GAT-3' (downstream primer), a product of 135 bp, and annealed at 54°C; unmethylation primers were: 5'-ATT ATG TTG TTT AGA TTG GTT TTG-3' (upstream primer), 5'-AAA AAT TCC AAA TTT ATT CCT CAA T-3' (downstream primer), a product of 135 bp, and annealed at 54 °C. The PCR reaction system for DNA template was 30 ng, and the PCR Master Mix was 5 μl. The upstream and downstream of each primer was 0.5 μl (10 μM), template 1 μl, water 3 μl. Reaction conditions were as follows: the pre-denaturation at 95°C for 5 min, at 95°C for 30 s, annealing at 72°C for 45 s, 35 cycles in total, and extention at 72°C for 5 min.

Statistical analysis

Statistical analysis was conducted using SPSS 15.0 software. The relative value of different gene expression used independent samples *t*-test analysis, $P < 0.05$ was considered statistically significant.

Results

The optimization of the concentration of the sample template

The cycle number of the reference GAPDH gene achieving the threshold was below 15 when the concentration of the sample cDNA was diluted 2 times, above 30 when diluted 20 times, and in a range between 15 and 30 when diluted 8 times. Therefore, when the sample cDNA was diluted 8 times, the template concentration was optimal for the SYBR Green real-time quantitative PCR.

The results of real-time quantitative PCR

The melting curves and results of amplification of Ku70, Ku80, ERCC4, Lig4, and DNA-PKcs gene are clearly displayed in Fig.1. The melting curve analysis showed that the melting temperature was uniform, and peak of the readout was relatively sharp. It showed that the product of the Real-Time PCR of DNA had a high specificity without a hybrid band.

The quantitative analysis of Ku70, Ku80, ERCC4, Lig4, and DNA-PKcs gene in glioma and in normal brain tissue

The results of the target gene expression in 36 patients with primary gliomas and in 12 cases with normal brain tissue are shown in Fig.2. The differences in expression

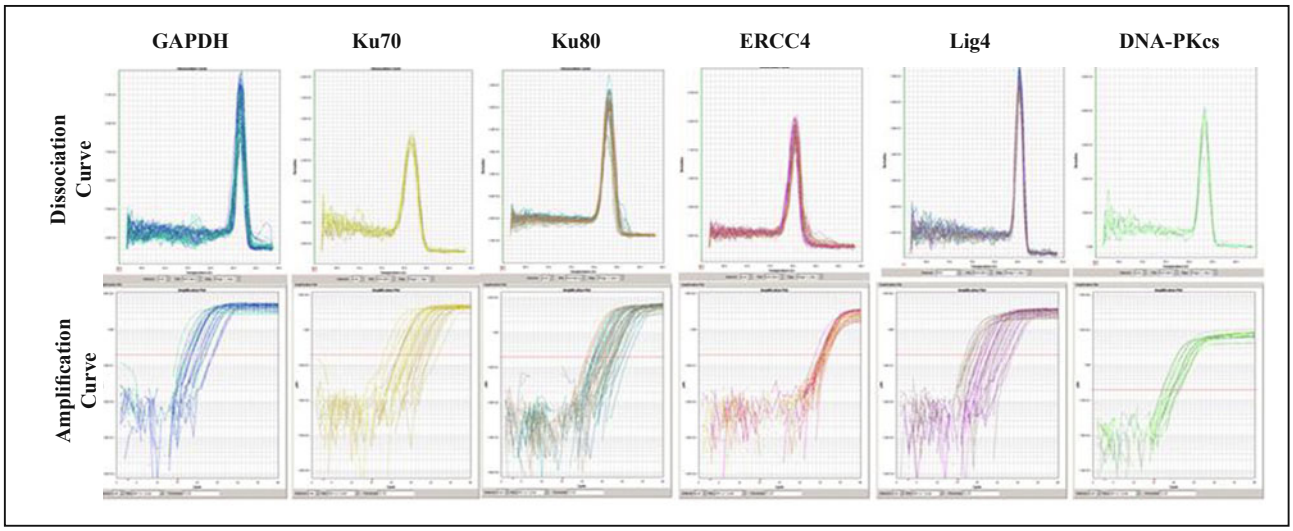

Fig. 1. Dissociation curve and amplification curve of Ku70, Ku80, ERCC4, Lig4 and DNA-PKcs.

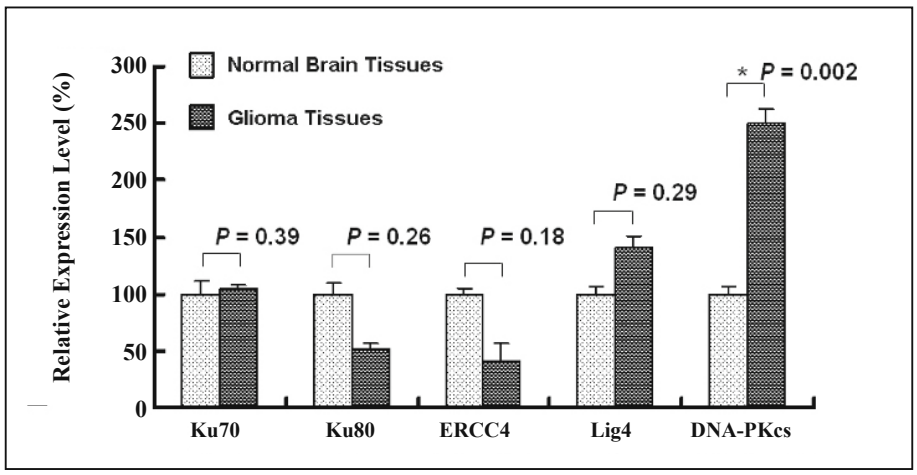

Fig.2. Relative expression levels of Ku70, Ku80, ERCC4, Lig4 and DNA-PKcs between normal brain tissues and glomas. Data were considered significant and indicated by "*" if $P < 0.05$.

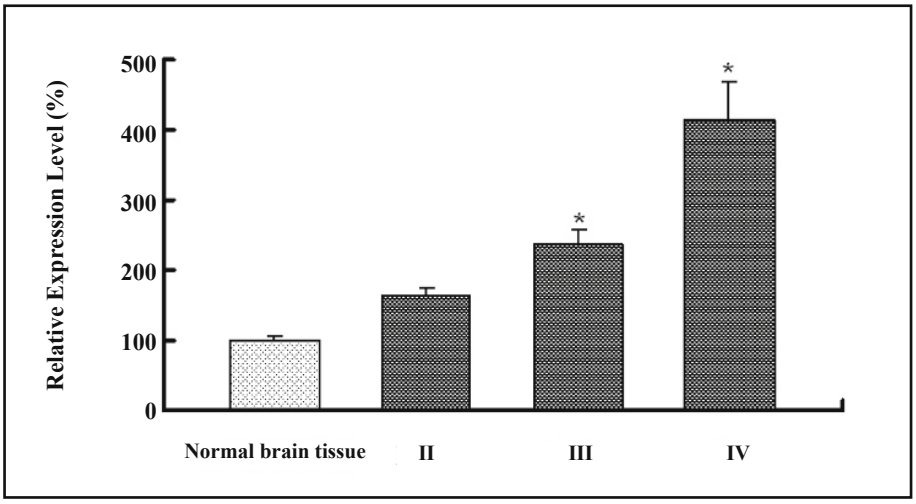

Fig.3. Relative expression levels of DNA-PKcs in gliomas of different grades.

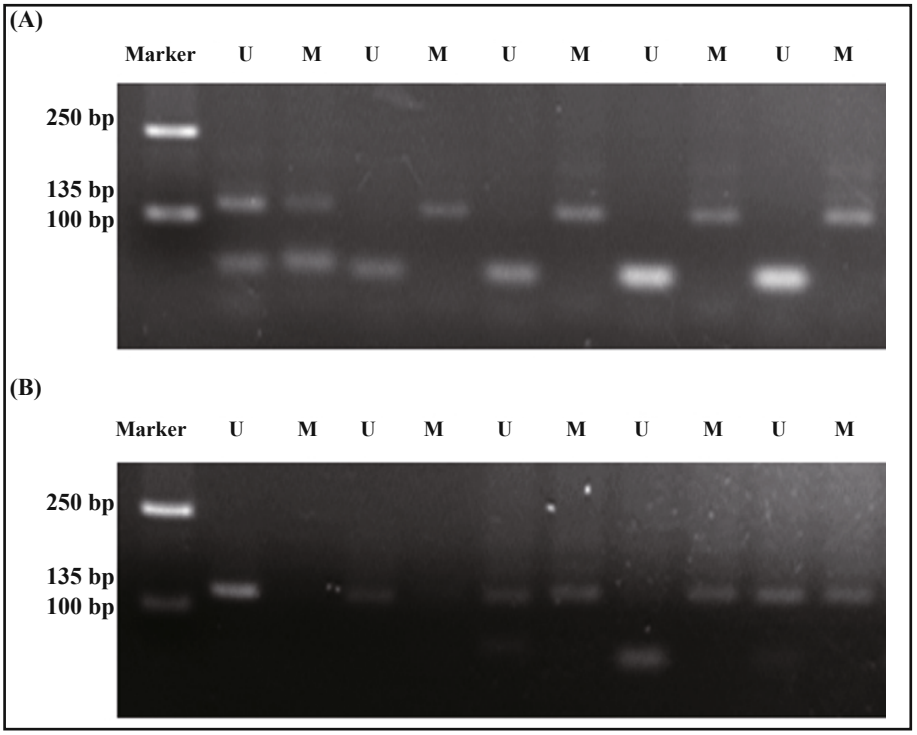

Fig.4. Methylation status of DNA-PKcs in (A) normal brain tissues and (B) in gliomas.

of Ku70, Ku80, ERCC4 and Lig4 gene between patients with glioma and the cases with normal brain tissues were not significant ($P > 0.05$). However the expression of the DNA-PKcs gene in human gliomas was increased 2.51 times compared with that in normal brain tissues ($P = 0.002$). The expression of the DNA-PKcs gene was gradually increased with the increase in the pathological grade of the tumor tissue. And it was also found that the mRNA content of DNA-PKcs gene in gliomas of Class III and IV level were as 2.37 and 4.14 times as those in the normal brain tissues.

Methylation of the DNA-PKcs gene promoter

The promoter methylation status of DNA-PKcs gene in 5 cases with normal brain tissues, and in 5 cases with brain gliomas, for exploring why the DNA-PKcs gene expression became upward in the tumor tissues, were detected. As shown in Fig.4, the methylation of the DNA-PKcs gene could be detected in all the glioma tissues, and 80% of these were fully methylated. The methylation of the gene in normal tissues was found in only 1 case. Non-methylation was found in 2 cases. Thus, the methylation level in glioma tissue was significantly lower than that in brain tissue. It may probably explain why the expression of the DNA-PKcs gene was increased.

Discussion

DNA constantly is damaged by internal (oxygen free radicals, etc.) and external (ultraviolet light, ionizing radiation, etc.) factors. During long-term evolution, cells have formed a number of ways to repair the DNA damage. There are mechanisms to detect DNA damage and to maintain stability and integrity of the genes[20]. If the DNA repair is not carried out correctly, it may result in DNA mutations and chromosome abnormalities, which lead to abnormal expression of genes within cells. As a result, uncontrolled cell growth and lack of differentiation occur, which eventually result in cancer[21].

The break of DNA double-strand is the most serious damage to DNA. Gene deletion lacking timely repair, the aberrations of chromosome, genomic instability, and so on, may accelerate the development of tumorigenesis[22]. We have known that non-homologous end joining is the main route for repairing DNA double-strand break damage. The heterodimers that are composed of Ku70 and Ku80 will be used to form a ring structure when the DNA fractured ends occur. Then DNA-PKcs protein is recruited for stabilizing the binding with DNA[23].

DNA-PKcs is a DNA-dependent serine/threonine protein kinase which is activated when combined with the Ku protein complex. It can also cause the P53 protein to be phosphorylated so as to start the apoptotic process[24]. It may further recruit the DNA repair and connection proteins to repair DNA when DNA-PKcs and Ku become complexed[25-27]. Thus, the proteins with nonhomologous end joining pathway play an important role in maintaining the stability of the genome.

Our study examined the mRNA levels of Ku70, Ku80, ERCC4, Lig4, and DNA-PKcs gene expression in normal brain tissues and in gliomas by means of nonhomologous end joining. We found that the difference in mRNA levels of Ku70, Ku80, ERCC4 and Lig4 gene between normal brain tissues and gliomas was not significant ($P > 0.05$). However the degree of DNA-PKcs gene expression was significantly higher in glioma tissues than in normal tissues ($P = 0.002$). Furthermore, the levels of DNA-PKcs gene mRNA were increased consistently with the increased pathological grades of the gliomas. These findings show that the expression of DNA-PKcs may play a key role in the incidence of glioma, and its expression shows that it has positive correlation with pathological grades of glioma.

Bartkova and other researchers[28] found that the expression of ERCC4 in star-shaped glioma cells at grade II- III and grade IV was lower than that in normal brain tissues. Beskow et al.[29] demonstrated that DNA-PKcs was highly expressed in cervical cancer and had a positive correlation with the P53 gene expression. Bertolini et al.[30] also reported that the expression of DNA-PKcs, Ku86 and Ku70 was significantly higher in cervical cancer tissue than in the adjacent non-tumor tissues. In summary, as the expression of the DNA-PKcs gene in gliomas is increased, we suggest that it may be a form of feedback regulation for DNA double-strand breaks.

In order to study the mechanism of increased expression of the DNA-PKcs gene in gliomas, we studied the methylation of the DNA-PKcs gene promoter in gliomas and in normal brain tissue. We found that the CpG islands existed around the site of upstream-1000 bp where DNA-PKcs gene transcription started, utilizing the tools of bioinformatics. Our study demonstrated that the DNA-PKcs promoter in normal brain tissue was hypermethylated. Contrarily, the level of methylation was decreased significantly in gliomas, therefore, we suggest that the demethylation of the DNA-PKcs promoter may cause the up-regulated expression of the gene.

In conclusion, our study shows that the expression of the DNA-PKcs gene was increased in gliomas, and may have a relation with the pathological grade of glioma. We also suggest that the demethylation of the DNA-PKcs promoter may be the cause of the up-regulated expression of the gene.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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