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Original Article

Blood-based DNA methylation of DNA repair genes in the non-homologous end-joining (NEHJ) pathway in patient with glioma

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Abstract: To investigate the blood-based DNA methylation of repair genes including LIG4, XRCC4, XRCC5, XRCC6 and XRCC7 that involved in non-homologous end-joining (NEHJ) DNA repair pathway in patients with glioma. Blood samples were obtained from 114 glioma patients, 96 normal controls, and 81 glioma patients after radiotherapy and chemotherapy. Blood-based DNA methylation of the five NHEJ repair genes was assayed by methylation-specific polymerase chain reaction (MSP). The DNA methylation level of XRCC5 and XRCC7 in glioma group are significantly higher than those of normal group ($P<0.001$). Moreover, radiotherapy treatment significantly increased methylation level of XRCC5 and XRCC7 compared to glioma group. No significant difference for the methylation of the other three genes, LIG4, XRCC4 and XRCC6 were detected among three groups. In conclusion: our findings indicate that DNA methylation modification plays an important role to regulate the gene expression of XRCC5 and XRCC7, from the results that the gene methylation level of the glioma group is higher than that of the normal group. Increased methylation of XRCC5 and XRCC7 in blood samples of glioma patients and patients with radiotherapy and chemotherapy suggests that blood-based methylation level of XRCC5 and XRCC7 could be a potential indicator for evaluating of the effect of radiotherapy and chemotherapy for glioma patient.

Keywords: Methylation, glioma, non-homologous end-joining, DNA repair gene

Introduction

Non-homologous end joining (NHEJ), one of the two major double-strand breaks (DSBs) repair systems, is the predominant type of DSBs repair induced by ionizing radiation and genomic instability in mammalian cells [1-3]. NHEJ is a multi-enzymatic pathway initially involving damage recognition by binding of the Ku70/80 hetero-dimer (coded by XRCC5 and XRCC6) followed by attachment of the DNA-PK catalytic subunit (DNA-PKcs) coded by XRCC7. This complex then recruits XRCC4 and DNA ligase IV (LIG4) to complete the broken strands rejoining [4-7]. Recurrent or chemoresistant tumor cells have a stronger ability to repair the DNA damage. Many studies have demonstrated that the tSNPs, methylation status and gene expression of DNA repair genes could affect the repair capacity of tumor cells.

Although the etiology of recurrent glioma remains unclear, exposure to ionizing radiation has been identified as the key risk factor. In our previous studies [8, 9], we have showed that the glioma risk was statistically significantly associated with two LIG4 SNPs, three XRCC5 tSNPs and one XRCC6 tSNP in the single-locus analysis with the study population of 771 glioma patients and 752 healthy controls. The MDR analysis suggested that the association was even stronger when gene-gene interactions within the five DSBs repair genes in NHEJ pathway were considered. The repair genes involved in NHEJ pathway, LIG4, XRCC4, XRCC5, XRCC6 and XRCC7, are related to the risk of glioma. We had found six tSNPs and four haplotypes of NHEJ pathway genes that was significantly correlated with risk of gliomas [8, 9]. Our results suggested that the NHEJ genes could also play an important role in the progression of gliomas except for MGMT, another DNA repair gene involved in alkyl repair, which is well known for the relativity with Temozolomide (TMZ). In this study, we further evaluated the methylation levels of five DNA repair genes in the blood

Table 1. Clinical data of the study subjects

Group	Average age	Total (n=291)	Population, n		Grade, n	
			Male	Female	I~II	III~IV
Glioma	46.5	114	66	48	41	73
Normal	41.5	96	57	39		
Radiotherapy	48.4	81	47	34	20	61

Table 2. MSP primers

Gene	Forward (5'-3')	Reverse (5'-3')
Primers for methylation		
LIG4	TTCGAGATAAAATTTATTGGC	TACCGATTCCGCCAACTA
XRCC4	TAAGCGGAAATTCGAGAAGC	AAACCCAACCGAACGACTAA
XRCC5	TTGTCGTATTAGCGTTTTAGGC	CCGAAACTCTAAACATACGCA
XRCC6	AGTGTATTGGGTTATTCGAGC	CCGAATCTCTATCCGATAAAA
XRCC7	GAGGTGGAATTTTCGTTTTTAC	ATACTCCGAACACTATATCGTCC
Nest primers for methylation		
LIG4	TTGTCGAGGTTAATAGGAGTC	CGAAACCTTACAATCACCG
XRCC4		TAAAACTCCCAAATCGCGAT
XRCC5	GGAGAGAATGTGCGTATGTTC	
XRCC6		AACTTAAACCACGACTCACG
XRCC7	TATTTTAGGCGAGGTTGGTATC	CTACCGCTTAATACGCACAA
Nest primers for unmethylation		
LIG4	TGTTGTTGAGGTTAATAGGAGTT	CCAAAACCTTACAATCACCAA
XRCC4		TAAAACTCCCAAATCACAATTAA
XRCC5	AATGGAGAGAATGTGTGTATGTTT	
XRCC6		AACTTAAACCACAACTCACAAA
XRCC7	GTTTATTTTAGGTGAGGTTGGTATT	ACACTACCACTTAATACACACAA
Primers for unmethylation		
LIG4	TTTTTTGAGATAAAATTTATTGGT	TACCAATTCCACCAACTACTC
XRCC4	AAATAAGTGGAAATTTGAGAAGT	AACAAACCCAACCAAACAACTAA
XRCC5	AATTTGTTGTATTAGTGTTTTAGGT	CCCCAAAACTCTAAACATACACAA
XRCC6	AGGAGTGTATTGGGTTATTTGAGT	CCACCAAATCTCTATCCAATAAAA
XRCC7	TGGAGGTGGAATTTTTGTTTTTAT	AAATACTCCAAACACTATATCATCC

Note: reverse primer was used as corresponding non nested primer for that has only one nested primer.

specimen of glioma patients to reveal the possible mechanism of decreasing gene expression. Meanwhile, there is considerable interest in identifying those repair genes which could have relationship with tumor cells sensitivity to DNA damaging agents such as ionizing radiation and alkylating agents.

Patients and methods

Study subjects

A total of 291 subjects were included in this study. In glioma group, a total of 114 patients with histological diagnosis of glioma were included, with 41 low grade glioma (WHO I-II) and 73 high grade glioma (WHO III-IV). Sixty-six cases are male and 48 cases are female, with an average age of 46.5 years. In normal group, 96 healthy volunteers were included, with 57 males and 39 females, and an average age of 41.5 years. In radiotherapy group, 81 glioma patients underwent surgical resection with follow-up radiotherapy and chemotherapy, among which 47 are male and 34 are female, with an average age of 48.4 years. The clinical data of subjects were shown in **Table 1**. All participants have signed the consent form based on well understanding the protocol. All trial procedures were proved by the ethics committee of Huashan Hospital of Fudan University.

Blood sample collection

Two milliliter peripheral venous blood specimens were collected and stocked at -80°C until use. For radiotherapy group blood samples were collected before surgical procedure and after postoperatively radiotherapy and chemotherapy.

Methylation-specific PCR (MSP)

A nested MSP approach was performed on the sodium-bisulfate-treated DNA samples to amplify the promoter region of the five repair genes. For each gene (LIG4, XRCC4, XRCC5, XRCC6, and XRCC7), four pairs of PCR primers were designed using the primer express 2.0 software and synthesized by Invitrogen, two for methylated sequences and the other two for unmethylated sequences (**Table 2**).

For nested MSP, the first round PCR reaction was carried out in a 50 μL solution which included 50 μmol/L of each dNTP (dATP, dCTP, dGTP, and dTTP), 0.5 mmol/L $MgCl_2$, 1 U Taq polymerase (Cinagen, Iran), and 50 ng genomic

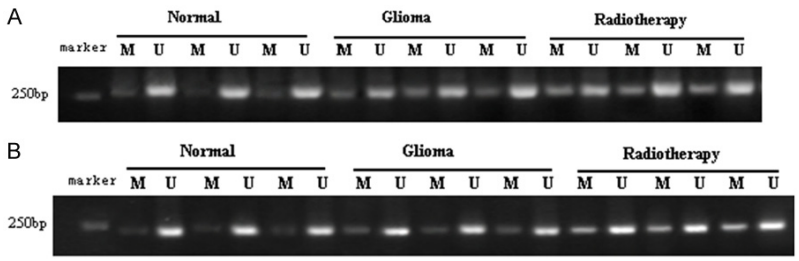

Figure 1. Selected cases of MGMT methylation status of XRCC5 (A) and XRCC7 (B) determined by MSP. Three cases were showed in normal group, glioma group and radiotherapy group, respectively. Molecular weight standard is 250 bp. M, methylation; U, unmethylation.

DNA, 0.75 ng of each. The PCR protocol began with sample denaturing at 95°C for 3 min, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 1 min, and one cycle of elongation at 72°C for 7 min. The PCR products were examined on 12.5% non-denaturing polyacrylamide gel stained with silver nitrate staining. The first round PCR products were diluted to 20 times for the second round nested PCR with nested primer. The final products were showed in agarose gel electrophoresis.

Statistical analysis

All data were analyzed using SPSS 13.0 version. The difference among the three groups and any other two groups were statistically analyzed using Kruskal-Wallis test and Mann-Whitney test. *P* value less than 0.05 was considered as a significant difference.

Results

Representative results of agarose gel electrophoresis for methylation of XRCC5 and XRCC7 in each group were presented in **Figure 1** and **Table 3**. For each group, we presented result of one case. We observed noticeable increased methylation levels in glioma subjects than normal controls. Methylation levels of XRCC5 and XRCC7 in patients from radiotherapy group were further higher than the glioma group. While there were no obvious difference in the methylation levels of LIG4, XRCC4 and XRCC6 among each group.

We further quantified the methylation levels of each gene among the groups. Quantitative results were presented in **Figure 2** and **Table 4**. The methylation levels of XRCC4 and XRCC6 showed no difference among group. Methylation of LIG4 showed a trend of increase but no statistical significance in glioma and radiotherapy group compared to the normal group. The methylation levels of XRCC5 and XRCC7 in glioma group were significantly higher than those of normal group.

Discussion

In this study, we evaluated the blood-based DNA methylation status of the five repair genes involved in NHEJ pathway. Results showed that the methylation level of all the five NHEJ repair genes in glioma group were higher than that of normal group. Among those genes, XRCC5 and XRCC7 showed significantly difference among three groups. It is worth note that the DNA methylation level has the trends that radiotherapy group > glioma group > normal group. It revealed that after radiotherapy and chemotherapy, the methylation level of XRCC5 and XRCC7 in glioma cells would be elevated, thus decrease the two genes expression level, which could diminish the repair ability of glioma cells and promote tumor cells to death. From this perspective, we could classify the XRCC5 and XRC7 as the benefit genes for radiotherapy and chemotherapy. The results were similar with our previous study of genetic susceptibility of glioma-one protective XRCC5 haplotype accounting for a 40% reduction in glioma risk [10], which demonstrated that XRCC5 might be a potential target gene in treatment of glioma.

It was interesting that although there was not statistically significant difference in LIG4, XRCC4 and XRCC6, a tendency that the methylation levels of these three genes were glioma group > radiotherapy group > normal group. The methylation levels of radiotherapy group were lower than that of glioma group, suggesting that the methylation of LIG4, XRCC4 and XRCC6 would be up-regulated after radiotherapy or chemotherapy. On the other hand, down-regulated the expression of LIG4, XRCC4 and XRCC6 but not of XRCC5 and XRCC7 could increase the radiosensitivity of non-small-cell lung cancer cells [11]. LIG4 was correlated with

Table 3. Methylation expression level

Gene	Normal (N=96)	Glioma (N=114)	Radiotherapy (N=81)	*P*
LIG4	5717.10 (2312.97-14366.57)	9162.57 (3656.61-20661.03)	6954.48 (4394.12-11732.98)	0.245
XRCC4	2104.31 (1232.81-3077.52)	2311.71 (1576.29-3210.28)	2035.65 (1444.77-2778.49)	0.186
XRCC5	591.69 (200.22-1267.39)	6488.69 (3771.88-11163.00)	9754.32 (5751.48-15389.83)	0.000
XRCC6	4199.77 (1043.63-10885.96)	4589.40 (1918.05-8014.96)	4539.12 (2511.14-6806.72)	0.986
XRCC7	1128.79 (473.48-2583.05)	3276.57 (1680.72-8330.96)	4319.74 (2414.82-9513.87)	0.000

Note: Inter Quantile Range (IQR), (*Kruskal-Wallis test, $P<0.005$).

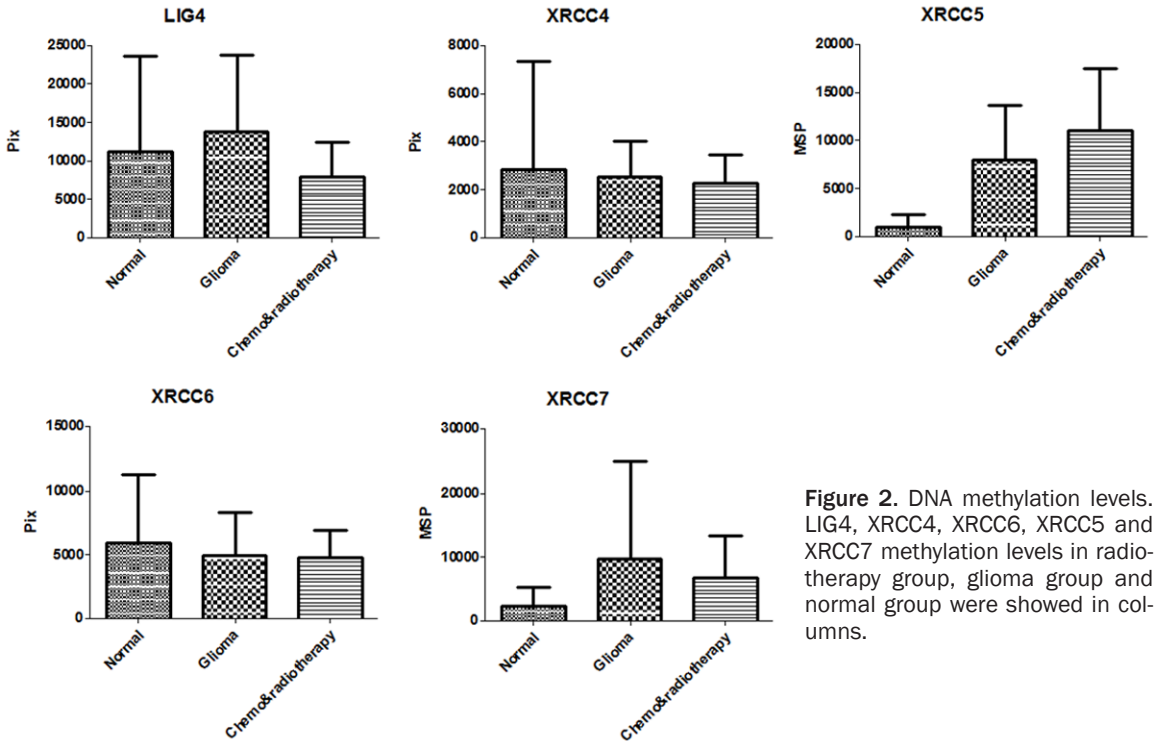

Figure 2. DNA methylation levels. LIG4, XRCC4, XRCC6, XRCC5 and XRCC7 methylation levels in radiotherapy group, glioma group and normal group were showed in columns.

Table 4. XRCC5 and XRCC7

Gene		Glioma vs. Normal	Radiotherapy vs. Normal	Radiotherapy vs. Glioma
XRCC5	P	0.000	0.000	0.000
	Z	-11.352	-10.783	-3.503
XRCC7	P	0.000	0.000	0.010
	Z	-6.313	-7.315	-1.555

(Mann-Whitney test, $P<0.050$).

TMZ and ACNU cell sensitivity, down regulation of LIG4 might provide a useful tool for cell sensitization during TMZ and ACNU chemotherapy [12, 13]. But the exact roles of LIG4, XRCC4 and XRCC6 in the radiosensitivity therapy need to be further elucidated.

In summary, our study explored possible mechanism of radio- and chemo-sensitivity and DNA repair genes which might suggest a better way in which radio-resistant tumor might be made more sensitive. Our results indicated that NHEJ repair genes might be potential targets for future treatment strategy of gliomas.

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Disclosure of conflict of interest

None.

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References

[1] Abner CW and McKinnon PJ. The DNA double-strand break response in the nervous system. DNA Repair (Amst) 2004; 3: 1141-1147.

[2] Jackson SP. Sensing and repairing DNA double-strand breaks. Carcinogenesis 2002; 23: 687-696.

[3] Iliakis G, Wang H, Perrault AR, Boecker W, Rosidi B, Windhofer F, Wu W, Guan J, Terzoudi G and Pantelias G. Mechanisms of DNA double strand break repair and chromosome aberration formation. Cytogenet Genome Res 2004; 104: 14-20.

[4] Anderson JA, Harper JV, Cucinotta FA and O'Neill P. Participation of DNA-PKcs in DSB repair after exposure to high- and low-LET radiation. Radiat Res 2010; 174: 195-205.

[5] Budman J, Kim SA and Chu G. Processing of DNA for nonhomologous end-joining is controlled by kinase activity and XRCC4/ligase IV. J Biol Chem 2007; 282: 11950-11959.

[6] Lieber MR. The mechanism of human nonhomologous DNA end joining. J Biol Chem 2008; 283: 1-5.

[7] Weterings E and Chen DJ. The endless tale of non-homologous end-joining. Cell Res 2008; 18: 114-124.

[8] Liu Y, Zhou K, Zhang H, Shugart YY, Chen L, Xu Z, Zhong Y, Liu H, Jin L, Wei Q, Huang F, Lu D and Zhou L. Polymorphisms of LIG4 and XRCC4 involved in the NHEJ pathway interact to modify risk of glioma. Hum Mutat 2008; 29: 381-389.

[9] Liu Y, Zhang H, Zhou K, Chen L, Xu Z, Zhong Y, Liu H, Li R, Shugart YY, Wei Q, Jin L, Huang F, Lu D and Zhou L. Tagging SNPs in non-homologous end-joining pathway genes and risk of glioma. Carcinogenesis 2007; 28: 1906-1913.

[10] Halazonetis TD, Gorgoulis VG and Bartek J. An oncogene-induced DNA damage model for cancer development. Science 2008; 319: 1352-1355.

[11] Nishikawa T, Munshi A, Story MD, Ismail S, Stevens C, Chada S and Meyn RE. Adenoviral-mediated mda-7 expression suppresses DNA repair capacity and radiosensitizes non-small-cell lung cancer cells. Oncogene 2004; 23: 7125-7131.

[12] Kondo N, Takahashi A, Mori E, Ohnishi K, McKinnon PJ, Sakaki T, Nakase H and Ohnishi T. DNA ligase IV as a new molecular target for temozolomide. Biochem Biophys Res Commun 2009; 387: 656-660.

[13] Kondo N, Takahashi A, Mori E, Noda T, Su X, Ohnishi K, McKinnon PJ, Sakaki T, Nakase H, Ono K and Ohnishi T. DNA ligase IV is a potential molecular target in ACNU sensitivity. Cancer Sci 2010; 101: 1881-1885.