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 chemo-resistant 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
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)**

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 MgCl₂, 1 U Taq polymerase (Cinagen, Iran), and 50 ng genomic...
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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.

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
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**Table 3. Methylation expression level**

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

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

**Table 4. XRCC5 and XRCC7**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Glioma vs. Normal</th>
<th>Radiotherapy vs. Normal</th>
<th>Radiotherapy vs. Glioma</th>
</tr>
</thead>
<tbody>
<tr>
<td>XRCC5</td>
<td>P 0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Z -11.352</td>
<td>-10.783</td>
<td>-3.503</td>
</tr>
<tr>
<td>XRCC7</td>
<td>P 0.000</td>
<td>0.000</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td>Z -6.313</td>
<td>-7.315</td>
<td>-1.555</td>
</tr>
</tbody>
</table>

(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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