ASSOCIATION OF KLOTHO GENE POLYMORPHISM WITH CEREBRAL INFARCTION

VEZA POLIMORFIZMA KLOTHO GENA SA CEREBRALNIM INFARKTOM

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Summary

Background: We aimed to investigate the expression of Klotho gene in peripheral blood of patients with cerebral infarction (CI) and the association of its polymorphisms with the occurrence of CI.

Methods: A total of 60 CI patients (CI group) and 20 healthy people receiving physical examination (control group) were enrolled as the research subjects. The expression of Klotho gene in CI group and control group was determined using enzyme-linked immunosorbent assay kit. Single nucleotide polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of the Klotho gene were typed via conformational difference gel electrophoresis. Besides, whether the distribution frequencies of Klotho genotypes conformed to Hardy-Weinberg equilibrium was evaluated by chi-square test. Meanwhile, the associations of Klotho alleles and gene polymorphisms with CI occurrence were analyzed.

Results: The protein expression level of Klotho in the peripheral blood was remarkably lower in patients in CI group than that in control group (P<0.05). Hardy-Weinberg equilibrium analysis revealed that Klotho gene polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of the Klotho gene were typed via conformational difference gel electrophoresis. Besides, whether the distribution frequencies of Klotho genotypes conformed to Hardy-Weinberg equilibrium was evaluated by chi-square test. Meanwhile, the associations of Klotho alleles and gene polymorphisms with CI occurrence were analyzed.

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Kratak sadržaj

Uvod: Cilj nam je bio da istražimo ekspresiju Klotho gena u perifernoj krvi pacijenata sa cerebralnim infarktom (CI) i povezanost njegovih polimorfizama sa pojavom CI.

Metode: Ukupno 60 pacijenata sa CI (CI grupa) i 20 zdravih osoba na fizikalnom pregledu (kontrolna grupa) upisano je kao subjekti istraživanja. Ekspresija Klotho gena u CI grupi i kontrolnoj grupi je određena korišćenjem kompleta za enzymski imunosorbentni test. Polimorfizmi pojedinačnih nukleotida (rs192031, rs200131 i rs102312) u promotorskom regionu Klotho gena su tipizovani putem konformacione razlike u gel elektroforezi. Osim toga, da li su frekvencije distribucije Klotho genotipova u skladu sa Hardy-Weinbergovom ravnovesom procenjeno je hi-kvadrat testom. U međuvremenu, analizirane su asocijacije Klotho alela i polimorfizama gena sa pojavom CI.

Rezultati: Nivo ekspresije proteina Klotho-a u perifernoj krvi bio je značajno niži kod pacijenata u CI grupi nego u kontrolnoj grupi (P<0.05). Hardy-Weinbergovom analizom određena je da se polimorfizmi Klotho gena (rs192031, rs200131 i rs102312) podudaraju sa distribucijom genetičke ravnoteže (P>0.05). Analiza asocijacije zasnovana na genima pokazala je da su samo rs192031 polimorfizam i aleli u korelaciji sa pojavom CI (P<0.05). Sistolni krvni pritisak i cholesterol lipoproteina visoke gustine bili su značajno viši kod pacijenata sa CI sa TT genotipom polimorfizma Klotho gena rs192031 od onih u kontrolnoj grupi (P<0.05). Šta više, nije bilo asocijacije rs200131 i rs102312 polimorfizama i alela sa pojavom CI (P>0.05).
Furthermore, there were no associations of rs200131 and rs102312 polymorphisms and alleles with the occurrence of CI (P>0.05).

**Conclusions:** The expression level of Klotho is evidently reduced in the peripheral blood of CI patients. Rs192031 in the promoter region of the Klotho gene is associated with the occurrence of CI, while rs200131 and rs102312 have no relations with CI.

**Keywords:** Klotho, cerebral infarction, polymorphism

**Introduction**

Cerebral infarction (CI) is an ischemic-hypoxic necrosis induced by insufficient blood supply to local brain tissues. It is characterized by high incidence and disability rates. Various factors can lead to the occurrence of CI, the main cause of which is cerebral atherosclerosis (1). The nature of atherosclerosis lies in the chronic activation of endothelial cells caused by inflammatory and fibro proliferative reactions, which can induce vascular stenosis and insufficient blood supply to the brain, and the secondary rupture of fibrous cap in atherosclerotic plaque eventually leads to CI (2, 3). The correlation of genetic factors (especially gene polymorphisms) with acute atherosclerotic CI has become a research hotspot in recent years.

Cathepsin S (CTSS), a cysteine protease of the papain superfamily, plays a vital role in the degradation and reconstruction of extracellular matrix, antigen presentation, inflammation, immunity and angiogenesis (4). A study revealed that single nucleotide polymorphisms (SNPs) (rs774320676 and rs928508030) of the CTSS gene are related to the risk of acute atherosclerotic CI. The T allele of rs774320676 and the G allele of rs928508030 of CTSS are genetic susceptibility genes for acute atherosclerotic CI (5).

Klotho, as an anti-aging gene, is able to reduce oxidative stress, thus protecting the cardio-cerebrovascular system (6). A study indicated that the elevated plasma Klotho concentration in patients with acute ischemic stroke is correlated with a good functional prognosis (7). Basic experiment illuminated that Klotho is an inducer of metabolic coupling between neurons and astrocytes. Klotho can be released by the neuronal glutamatergic activity and insulin regulation, thereby stimulating the formation and release of lactic acid in astrocytes (8). Traditional Chinese medicine ligustilide is capable of ameliorating cerebral ischemia-reperfusion injury in mice by up-regulating Klotho expression (9). However, the association of Klotho gene polymorphism with CI has not been reported yet.

The distribution of polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of the Klotho gene was determined in CI patients in this study, so as to provide a certain reference for further research of the genetic pathogenesis of CI.

**Materials and Methods**

**Subjects**

A total of 60 CI patients admitted to our hospital from January 2016 to January 2019 were enrolled as the research subjects, including 31 males and 29 females aged (57.41±2.34) years old. About 4 mL of venous blood was extracted, anticoagulated with sodium citrate, and stored in a refrigerator at -20 °C. Meanwhile, 20 healthy people receiving physical examination were selected as the controls, with 10 males and 10 females aged (57.13±2.19) years old. This study was approved by the Ethics Committee of Linyi Central Hospital, and all the participants signed the informed consent. The patients in the CI group met the diagnostic criteria of the Chinese Guidelines for Diagnosis and Treatment of Acute Ischemic Stroke 2019, without transient ischemic attack, and the diagnosis was confirmed by imaging examinations. The subjects in the control group were healthy people who underwent routine physical examination in our hospital, without a history of cardiovascular and cerebrovascular diseases and related family history.

**Detection of serum Klotho protein**

About 4 mL of venous blood was collected and anticoagulated with sodium citrate to detect serum Klotho protein using an enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the instructions.

**Deoxyribonucleic acid (DNA) extraction**

A total of 4 mL of patient’s EDTA-anticoagulated blood was taken to extract genomic DNA according to the instructions of the DNA Extraction Kit (Guge Bio-Technology Co., Ltd., Wuhan, China). Subsequently, the quality of 2 μL of sample was measured in 1.5% agarose gel electrophoresis. Meanwhile, the concentration of the extracted DNA was determined using an ultraviolet spectrophotometer.

**Polymerase chain reaction (PCR) amplification**

Primers were designed to amplify Klotho gene polymorphismsrs192031, rs200131 and rs102312.
Table I Primer sequences and product sizes of different polymorphisms in the promoter region of the Klotho gene.

| Polymorphism | Primer sequence (5’-3’) | Product (bp) |
|--------------|-------------------------|--------------|
| rs192031     | Forward: AGCTGATGGCTATCGTAGCAGGCReverse: TGGGCTAGCTAGCTAGCG    | 223          |
| rs200131     | Forward: AAGTCGATCGTTAGGGCAARReverse: GTGACTTAGGCAATGAAA      | 302          |
| rs102312     | Forward: AGGCAAATTCTGATCTAGCTAGReverse: TGCTGTAGCTAGCTG      | 381          |

Table II Probe sequences and product sizes of ligase reaction for different polymorphisms of the Klotho gene.

| Polymorphism | Probe | Probe sequence (5’-3’) | Product (bp) |
|--------------|-------|------------------------|--------------|
| rs192031     | rs192031 | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 124          |
|              | rs192031- | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 124          |
|              | Ars192031-T | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 124          |
| rs200131     | rs192031 | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 115          |
|              | rs192031-C | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 115          |
|              | rs192031-T | P-ACGTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 115          |
| rs102312     | rs102312-Ar | P-ACGTAGCTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 108          |
|              | rs102312-C | P-ACGTAGCTAGCTAGCTAGTTTTTTTTTTTTTTTTTTTTTTTTT-FAM | 108          |

Primer sequences of each polymorphism were shown in Table I. The reaction system of PCR (20 μL) included 2.0 μL of DNA template, 10.0 μL of 2× mix, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 7.2 μL of ddH2O. PCR amplification was performed under the following conditions: 95 °C for 120 s, 35 cycles of 94 °C for 30 s, 57 °C for 90 s and 72 °C for 60 s, followed by extension at 72 °C for 10 min. Subsequently, agarose gel electrophoresis was utilized to detect the amplification of gene fragments.

Ligase detection reaction

The upstream and downstream probes used in this reaction were designed and synthesized by BGI. The upstream probe was modified by phosphorylation at the 5’-terminal region to prepare a probe mixture with a concentration of 12.5 pmol/μL. Ligase detection reaction system (3.05 μL) was composed of 0.05 μL of ligase, 1 μL of buffer, 1 μL of PCR product, and 1 μL of probe mixture. PCR amplification was carried out under the following conditions: 95 °C for 120 s, 94 °C for 15 s and 50 °C for 25 s, 30 cycles in total. After that, the concentration was measured using the ultraviolet spectrophotometer. Subsequently, BGI was commissioned to sequence and analyze the target gene. All data were analyzed using Gene Mapper (Table II).

Statistical analysis

Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA) was applied for statistical analysis. Enumeration data were expressed by frequency and percentage, and measurement data were presented as mean±standard deviation. The genotype frequency in the sample was calculated and tested using the Hardy-Weinberg equilibrium formula. The chi-square test was used for multiple comparisons of enumeration data. Besides, t-test and analysis of variance were utilized for measurement data. P<0.05 indicated that the difference was statistically significant.

Results

Comparisons of clinical baseline data between CI group and control group

As shown in Table III, blood glucose and systolic blood pressure were obviously increased, but high-density lipoprotein cholesterol (HDL-C) significantly declined in CI group compared with those in control group (P<0.05).

Comparison of Klotho protein levels in peripheral blood between CI group and control group

As shown in Figure 1, the expression level of Klotho in the peripheral blood of patients was markedly reduced in CI group in comparison with that
Table III: Comparisons of clinical data between CI group and control group.

| Index               | Control group | CI group | P   |
|---------------------|---------------|----------|-----|
| Triglyceride (mmol/L) | 1.75±0.28     | 1.81±0.21 | 0.112 |
| Total cholesterol (mmol/L) | 4.88±0.48     | 4.91±0.25 | 0.341 |
| HDL-C (mmol/L)      | 1.28±0.11     | 1.14±0.49 | 0.003 |
| LDL-C (mmol/L)      | 2.99±0.28     | 3.02±0.12 | 0.288 |
| Blood glucose (mg/dL) | 5.22±1.28     | 6.29±0.94 | 0.001 |
| Systolic blood pressure (mmHg) | 131.92±4.02   | 145.03±6.82 | 0.000 |
| Diastolic blood pressure (mmHg) | 80.02±3.02    | 84.29±4.02 | 0.018 |

Figure 1: Comparison of Klotho protein levels in peripheral blood between CI group and control group. CI: cerebral infarction group. Control: healthy control group. *P<0.05: a statistical difference vs. control group.

Table IV: Results of linkage equilibrium test for the Klotho gene polymorphisms between groups.

| Polymorphism | \( r^2 \) CI | \( r^2 \) Control | \( r^2 \) P |
|--------------|---------------|-----------------|--------|
| rs192031     | -             | 0.012           | 0.108  |
| rs200131     | 0.012         | -               | 0.221  |
| rs102312     | 0.108         | 0.221           | -      |

Table V: Distribution of different genotypes of Klotho gene polymorphisms and CI.

| Group | rs192031 | rs200131 | rs102312 |
|-------|----------|----------|----------|
|       | AA       | AT       | TT       | CC       | CT       | TT       | AA       | AC       | CC       |
| CI    | 10.1%    | 50.9%    | 39.0%    | 20.1%    | 48.0%    | 30.0%    | 20.1%    | 50.9%    | 29.0%    |
| Control | 24.0% | 51.2% | 24.8% | 22.8% | 46.0% | 31.2% | 19.3% | 51.2% | 29.5% |
| C2    | 1.661    | 0.499    | 0.717    |          |          |          |          |          |          |
| P     | 0.052    | 0.221    | 0.610    |          |          |          |          |          |          |

in control group (P<0.05), indicating that Klotho might be involved in the occurrence and development of CI.

Analysis results of Klotho gene polymorphism rs192031, rs200131 and rs102312

The Klotho gene polymorphisms rs192031, rs200131 and rs102312 in CI group and control group were cleaved by BSTU I restriction enzyme, manifesting that polymorphism rs192031 had two alleles (A and T) and three genotypes (AA, AT and TT), rs200131 had two alleles (C and T) and three genotypes (CC, CT and TT), and rs102312 had two alleles (A and C) and three genotypes (AA, AC and CC).

Results of Hardy-Weinberg equilibrium test

The linkage disequilibrium test results of different Klotho gene polymorphisms were tested using the Hardy-Weinberg equilibrium formula. As shown in Table IV, \( r^2 < 0.33 \) was detected between groups of polymorphisms, indicating the conformity of polymorphisms with the equilibrium test between groups.

Associations of Klotho gene polymorphisms with CI

The genotype frequencies of each gene polymorphism in the two groups were shown in Table V. The polymorphism rs192031 was remarkably related to the occurrence of CI (P<0.05), while rs200131 and rs102312 were not correlated with CI (P>0.05).
Association of Klotho alleles with CI

According to the genotype frequencies of each polymorphism in the two groups (Table VI), the polymorphism rs192031 was obviously associated with occurrence of CI (P<0.05), while rs200131 and rs102312 had no relations with CI (P>0.05).

Correlations of TT genotype of Klotho gene polymorphism rs192031 with clinical parameters of CI

The further research revealed that the systolic blood pressure and HDL-C were notably higher in CI patients with TT genotype of Klotho gene polymorphism rs192031 than those in control group (P<0.05) (Table VII).

Discussion

CI is a cerebrovascular disease caused by cerebral blood supply disorders, which is characterized by high morbidity and high mortality, seriously threatening people’s lives (10). CI can be caused by multiple factors, especially atherosclerosis. Atherosclerotic CI is a multi-source disease resulting from the combined action of genetic and environmental factors. Atherosclerosis can lead to stenosis, occlusion and thrombosis of the blood vessel cavity, or the shedding of unstable plaques can result in CI (11). Therefore, further elucidating the genetic mechanism of CI occurrence is of important significance for the early prevention and precise treatment of CI.

Previous studies have shown that the polymorphisms of multiple genes are potentially correlated with the occurrence and prognosis of CI. The allele frequency of APOE 4 is notably higher in CI patients than that in healthy controls (12). Tissue inhibitors of metalloproteinases (TIMPs), as endogenous inhibitors of matrix metalloproteinases, participate in the normal cellular processes and the occurrence and progression of atherosclerosis. A study indicated that there is a strong linkage disequilibrium between 1296T/C and -915A/G of TIMP gene, and people with TC+ CC genotype are 1.8 times more likely to suffer mixed carotid plaque than those with TT genotype (13). The certain association of Klotho gene polymorphisms with the occurrence and progression of CI was revealed in this study.

In animal models, a broad phenotype similar to the aging phenotype will be caused by suppressing the Klotho gene, including atherosclerosis, ectopic calcification, infertility, skin atrophy and severe hypoglycemia, while the overexpression of Klotho gene increases the overall life span of guinea pigs by 20-30% (14). The human Klotho gene is located on chromosome 13 and can be expressed as a secretory Klotho protein by variable cleavage of the third exon. The anchored Klotho protein is mainly present in the distal convoluted tubules of the kidney and the choroid plexus of the brain, but it can be processed
and released into the blood after translation, dissociating outside the cells and playing a hormone-like role (15). Klotho gene has 6 SNPs in exon 2 and flanking regions, and such polymorphisms are closely related to the occurrence and progression of cardio-cerebrovascular diseases (16). The content of serum Klotho is higher in patients with a history of myocardial infarction but no history of coronary artery disease or stroke, but the haplotype of Klotho is not correlated with the above variables (17). Klotho gene polymorphism may be a genetic risk factor for atherosclerotic coronary artery disease rather than vasospasm angina pectoris in the Japanese population. Specifically, a higher ratio of A genotype of the Klotho gene polymorphism G-395A was observed in patients with coronary heart disease than that in healthy controls (18). The correlation between polymorphisms (rs192031, rs200131 and rs102312) in the promoter region of Klotho and CI occurrence in the Han population was analyzed in this study. The protein was extracted from the peripheral blood of healthy people undergoing physical examination and CI patients. First, the results of ELISA revealed that the expression of Klotho protein was lower in the peripheral blood of patients in CI group (P<0.05).

Subsequently, the target polymorphisms were genotyped to record the distribution of genotype and allele frequencies in different groups. The results indicated that there were significant correlations between the Klotho gene polymorphism rs192031 and its genotypes and the occurrence of CI (P<0.05). People with AT genotype were more likely to suffer CI than those with AA or TT genotype. The polymorphisms rs200131 and rs102312 and their genotypes had no remarkable associations with CI occurrence (P>0.05).

Conclusions

In conclusion, this study illuminates for the first time that the Klotho gene polymorphism rs192031 was potentially associated with the occurrence of CI, while polymorphisms rs200131 and rs102312 and their genotypes were not related to the onset of CI.

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Conflict of interest statement

The authors reported no conflict of interest regarding the publication of this article.

References

1. Rabinstein AA, Friedman JA, Weigand SD, McClelland RL, Fulgham JR, Manno EM, et al. Predictors of cerebral infarction in aneurysmal subarachnoid hemorrhage. Stroke 2004; 35(8): 1862–6.
2. Fletcher AP, Alkaersig N, Lewis M, Tulevski V, Davies A, Brooks JE, et al. A pilot study of urokinase therapy in cerebral infarction. Stroke 1976; 7(2): 135–42.
3. Zhang Y, Liu G, Wang Y, Su Y, Leak RK, Cao G. Procalcitonin as a Biomarker for Malignant Cerebral Edema in Massive Cerebral Infarction. Sci Rep 2018; 8(1): 993.
4. Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, et al. Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem 1996; 271(21): 12511–6.
5. Luo L, Zhu M, Zhou J. Association between CTSS gene polymorphism and the risk of acute atherosclerotic cerebral infarction in Chinese population: a case-control study. Biosci Rep 2018; 38(6): BSR20180586.
6. Li Y, Chen F, Wei A, Bi F, Zhu X, Yin S, et al. Klotho recovery by genistein via promoter histone acetylation and DNA demethylation mitigates renal fibrosis in mice. J Mol Med (Berl) 2019; 97(4): 541–52.
7. Jerin A, Mosa OF, Kalisnjak JM, Zibert J, Skitek M. Serum Klotho as a marker for early diagnosis of acute kidney injury after cardiac surgery. J Med Biochem 2020; 39(2): 135–39.
8. Mazucanti CH, Kawamoto EM, Mattson MP, Scavone C, Camandola S. Activity-dependent neuronal Klotho enhances astrocytic aerobic glycolysis. J Cereb Blood Flow Metab 2019; 39(8): 1544–56.
9. Long FY, Shi MQ, Zhou HJ, Liu DL, Sang N, Du JR. Klotho upregulation contributes to the neuroprotection of ligustilide against cerebral ischemic injury in mice. Eur J Pharmacol 2018; 820: 198–205.
10. Brott T, Tomsick T, Feinberg W, Johnson C, Biller J, Broderick J, et al. Baseline silent cerebral infarction in the Asymptomatic Carotid Atherosclerosis Study. Stroke 1994; 25(6): 1122–9.
11. Kim J, Song TJ, Song D, Lee HS, Nam CM, Nam HS, et al. Serum alkaline phosphatase and phosphate in cerebral atherosclerosis and functional outcomes after cerebral infarction. Stroke 2013; 44(12): 3547–9.
12. Jin ZQ, Fan YS, Ding J, Chen M, Fan W, Zhang GJ, et al. Association of apolipoprotein E 4 polymorphism with cerebral infarction in Chinese Han population. Acta Pharmacol Sin 2004; 25(3): 352–6.
13. Zheng Z, He X, Zhu M, Jin X, Li C, Zhu F, et al. Tissue inhibitor of the metalloproteinases-3 gene polymorphisms and carotid plaque susceptibility in the Han Chinese population. Int J Neurosci 2018; 128(10): 920–7.
14. Yamamoto M, Clark JD, Pastor JV, Gurnani P, Nandi A, Kurosu H, et al. Regulation of oxidative stress by the anti-aging hormone klotho. J Biol Chem 2005; 280(45): 38029–54.
15. Nagai T, Yamada K, Kim HC, Kim YS, Noda Y, Imura A, et al. Cognition impairment in the genetic model of aging klotho gene mutant mice: a role of oxidative stress. Faseb J 2003; 17(1): 50–2.

16. Shimada T, Takeshita Y, Murohara T, Sasaki K, Egami K, Shintani S, et al. Angiogenesis and vasculogenesis are impaired in the precocious-aging klotho mouse. Circulation 2004; 110(9): 1148–55.

17. Paula RS, Souza VC, Machado-Silva W, Almeida BR, Daros AC, Gomes L, et al. Serum Klotho (but not haplotypes) associate with the post-myocardial infarction status of older adults. Clinics (Sao Paulo) 2016; 71(12): 725–32.

18. Jo SH, Kim SG, Choi YJ, Joo NR, Cho GY, Choi SR, et al. KLOTHO gene polymorphism is associated with coronary artery stenosis but not with coronary calcification in a Korean population. Int Heart J 2009; 50(1): 23–32.

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