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## CLINICAL STUDY

# Single nucleotide polymorphisms in the D-loop region of mitochondrial DNA is associated with the kidney survival time in chronic kidney disease patients

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**Abstract**

**Background:** The mitochondrial displacement loop (D-loop) is known to accumulate mutations and SNPs at a higher frequency than other regions of mitochondrial DNA (mtDNA). We had identified chronic kidney disease (CKD) risk-associated SNPs in the D-loop of CKD patients previously. In this study, we investigated the association of SNPs in the D-loop of mtDNA with the kidney survival of CKD. **Methods:** The D-loop region of mtDNA was sequenced for 119 CKD patients from the inpatient of the Fourth Hospital of Hebei Medical University. The Kaplan–Meier method was used to identify disease outcome-associated SNPs in the D-loop of CKD patients. The Cox proportional hazards model was used to identify risk factors for the kidney survival of CKD. **Results:** In the present study, we identified 20 SNPs with a frequency higher than 5% and assessed the relationship of these SNPs with kidney survival time in CKD patients, a SNP of 146 was identified by log-rank test for statistically significant prediction of the kidney survival time. In an overall multivariate analysis, allele 146 was identified as an independent predictor of kidney survival time in CKD patients. The survival time of kidney in the CKD patients with 146C was significantly shorter than that of kidney in CKD patients with 146T (relative risk, 2.336; 95% CI, 1.319–3.923;  $p = 0.001$ ). **Conclusion:** SNPs in the D-loop can predict the kidney survival of CKD patients. Analysis of genetic polymorphisms in the mitochondrial D-loop can help to identify CKD patient subgroup at high risk of a poor disease outcome.

**Keywords**

Chronic kidney disease, D-loop, mtDNA, SNP

**History**

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**Introduction**

Chronic kidney disease (CKD) is becoming a major public health concern. An estimated 23 million people in the United States (11.5% of the adult population) have CKD and are at increased risk for cardiovascular events and progression to kidney failure.<sup>1,2</sup> CKD is also highly prevalent in developing countries.<sup>3,4</sup> Recently, based on a report published on the Lancet in 2012, China had a higher incidence and prevalence rate of CKD patients, estimating the overall prevalence of CKD to be 10.8%.<sup>5</sup> Although there are proven therapies to improve outcomes in patients with CKD, these therapies may also cause harm and add cost. Clinical decision making for CKD is still challenging due to the variability in rates of disease progression and the competing risk of cardiovascular mortality.<sup>6,7</sup> The survival period of kidney was equivalent to the time from diagnosed CKD to start dialysis treatment or receive a renal transplant.<sup>8</sup> Currently, there are no widely

accepted predictive instruments for CKD progression, it is vital to develop new approaches for the kidney survival time prediction in the patients with CKD.

CKD can result from a variety of ecologically distinct causes, including infectious glomerulonephritis, renal vascular, genetic alterations, autoimmune disease and oxidative stress.<sup>9</sup> Increased oxidative stress has been observed in CKD patients before the hemodialysis.<sup>10</sup> During the pathogenesis of CKD, ROS play important role in regulating the cell signaling pathways of nuclear factor kappa B (NF- $\kappa$ B), which can promote renal cell apoptosis and senescence and decrease regenerative ability of cells in the kidney.<sup>11</sup> Mitochondria are major intracellular sources of ROS and highly susceptible to oxidative damage.<sup>12,13</sup> Hence, we aimed to investigate the possible relationship between the kidney survival time and mitochondrial DNA (mtDNA) mutations.

The human mitochondrial genome is 16 kb circular double-stranded DNA molecule. It contains 12 coding genes for engaged in respiration and oxidative phosphorylation, two rRNAs, and a set of 22 tRNAs that are essential for the protein synthesis in mitochondria.<sup>14</sup> mtDNA is believed to be more susceptible to DNA damage and acquires mutations at a

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higher rate than nuclear DNA, due to high levels of ROS, a lack of protective histones and a limited capacity for DNA repair in the mitochondria.<sup>15,16</sup> mtDNA contains a non-coding region that includes a unique displacement loop (D-loop) that controls replication and transcription of mtDNA, because it contains the initial site of heavy chain replication and the promoters for heavy and light chain transcription. The D-loop is highly polymorphic, and some polymorphisms are associated with aging,<sup>17</sup> coronary artery disease,<sup>18</sup> and cancers.<sup>19,20</sup> However, few of these studies focused on the association of SNPs in the mtDNA D-loop region with the kidney survival time in CKD patients.

The D-Loop contains a length of 1122 bps (nucleotide 16,024–16,569 and 1–576) refer to mitochondria database <http://www.mitomap.org>. We identified 20 SNPs with a frequency higher than 5% and 6 SNPs (73A\G, 146T\C, 150C\T, 194C\T, 195T\C and 310T\C) associated with the risk of CKD.<sup>21</sup> In this study, we assessed the prediction power of these SNPs on the kidney survival time in CKD patients.

## Methods

### Tissue specimens and DNA extraction

This study was a prospective trial. Blood samples were obtained from 119 CKD patients from the inpatient of the Fourth Hospital of Hebei Medical University between 2002 and 2008. The presence of CKD was defined in accordance with the 2002 National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K-DOQI) classification<sup>22</sup> on the basis of an estimated glomerular filtration rate (eGFR) of less than 60 mL/min/1.73 m<sup>2</sup> for at least 90-days. Laboratories in China report eGFR using the four-variable modified diet in renal disease formula.<sup>23</sup> The etiologies of CKD in these patients were all chronic glomerulonephritis, including membranous nephropathy ( $n = 60$ , 50.4%), non-membranous nephrology ( $n = 59$ , 49.6%). The survival period of kidney was equivalent to the time from diagnosed CKD to ESRD. The ESRD was defined as starting dialysis treatment or receiving a renal transplant.<sup>8</sup> Total DNA was extracted using a Wizard Genomic DNA extraction kit (Promega, Madison, WI) and stored at  $-20^{\circ}\text{C}$ . Phone calls and follow-up visits to the CKD patients were used to obtain information on patients' kidney survival status. The study was approved by the Human Tissue Research Committee of the Fourth Hospital of Hebei Medical University. All patients provided written informed consent for the collection of samples and subsequent analysis.

### PCR amplification and sequence analysis

The forward primer 5'-CCCCATGCTTACAAGCAAGT-3' (nucleotide 16,190-16,209) and reverse primer 5'-GCTTTGAGGAGGTAAGCTAC-3' (nucleotide 602-583) were used for amplification of a 982-bp product from the mtDNA D-loop region. PCR was performed according to the protocol of PCR Master Mix Kit (Promega) and purified prior to sequencing. The PCR condition consisted of one incubation of 2 min at 95 °C, followed by 35 cycles of a 30 s denaturation at 95 °C, a 30 s an annealing at 55 °C, and a 45 s extension at 72 °C, and a final extension at 72 °C for 5 min. Cycle sequencing was performed with the Dye Terminator Cycle

Sequencing Ready Reaction Kit (Applied Biosystem, Foster City, CA) and the products were then separated on the ABIPRISM Genetic Analyzer 3100 (Applied Biosystem). Polymorphisms were confirmed by repeated analyses from both strands.

### Statistical analysis

The kidney survival curve was calculated using the Kaplan–Meier method, and compared with the log-rank test. Multivariate survival analysis was performed using a Cox proportional hazards model. The  $\chi^2$  test was used to analyze dichotomous values, such as the presence or absence of an individual SNP in CKD patients. All statistical analyses were performed using the SPSS17.0 software (SPSS Company, Chicago, IL). For all the statistical tests,  $p < 0.05$  was considered statistically significant.

## Results

A total of 119 CKD patients were enrolled in this study. At baseline, the mean age of 119 CKD patients was  $55.6 \pm 14.2$  years, and 56.3% were male (Table 1). The mean eGFR was  $81.2 \pm 12.4$  mL/min/1.73 m<sup>2</sup>, with 79.8% ( $n = 95$ ) of them having an eGFR  $< 60$  mL/min/1.73 m<sup>2</sup>. All participants had an eGFR  $> 30$  mL/min/1.73 m<sup>2</sup>. A review was conducted every six months over a five-year period. Seventeen of these patients were lost to follow-up. Those patients lost to follow up during this time period were as follows: three patients in Year 1; four patients in Year 2; four patients in Year 3; five patients in Year 4; one patient in Year 5. The remaining 102 patients were assessed. The relationship between the data collected during the 5-year follow-up and patients' clinical characteristics were analyzed using the Kaplan–Meier method and the log-rank test. Age, body mass index (BMI) and mean article pressure (MAP) were not statistically significant predictors of the kidney survival time in CKD patients. Sex, smoking classification and eGFR of the starting point were correlated with the kidney survival time of the progressive kidney disease (Table 2). The survival rates of kidney in

Table 1. Baseline characteristics of the CKD participants.

| Variables                                    | All participants |
|--|------------------|
| Number of participants                       | 119              |
| Male (%)                                     | 56.3             |
| Female (%)                                   | 43.7             |
| Age (years)                                  | $55.6 \pm 14.2$  |
| Current smoking (%)                          | 32.7             |
| Yes  | 32.7             |
| No   | 67.3             |
| BMI (kg/m <sup>2</sup> )                     | $21.8 \pm 2.7$   |
| Hypertension (%)                             | 68.9             |
| Systolic BP (mmHg)                           | $156.3 \pm 24.8$ |
| Diastolic BP (mmHg)                          | $87.5 \pm 12.6$  |
| MAP(mmHg)                                    | $112.6 \pm 14.7$ |
| Etiologies of CKD                            |                  |
| Membranous nephrology (%)                    | 50.4             |
| Non-membranous nephrology (%)                | 49.6             |
| eGFR (mL/min/1.73 m <sup>2</sup> )           | $81.2 \pm 12.4$  |
| eGFR $< 60$ (mL/min/1.73 m <sup>2</sup> , %) | 79.8             |

Note: BMI, body mass index; MAP, mean arterial pressure; BP, blood pressure.

female CKD patients were higher than those of kidney in male CKD patients. Smoking habit showed an association with survival rates of kidney in CKD patients when the survival time of kidney in smoking CKD patients was lower than those of kidney in non-smoking CKD patients. The survival rates of kidney in CKD patients with eGFR  $<60$  mL/min/1.73 m<sup>2</sup> was lower than those of kidney in CKD patients with eGFR  $\geq 60$  mL/min/1.73 m<sup>2</sup>. These data demonstrate that gender, smoking habit and the eGFR of baseline were the risk factors for the survival time of kidney in the CKD patients.

The correlation between the mtDNA genotype and the kidney survival was compared. SNPs with a minor allele frequency of  $<5\%$  were excluded; thus, a remaining 20 SNPs were used for further analysis. CKD patients were divided into two groups on the basis of their genotype at each SNP site, the kidney survival curve was plotted using the Kaplan–Meier method for all CKD patients at these sites. A dramatic difference in kidney survival rate was found for allele 146 (Figure 1). The rare allele 146C was associated with a significantly shorter ( $p=0.001$ ) length of kidney survival (Figure 1). The allele 146 also belong to CKD risk associated SNPs we identified before. We used the multivariate analysis by the Cox proportional hazards model with the prediction factors, including one SNP, gender, smoking and eGFR. As shown in Table 3, the 146 alleles were identified as independent predictors for the kidney survival time of progressive kidney disease. The survival time of kidney in the CKD patients with the rare allele 146C genotype was significantly shorter than that of kidney in CKD patients with the frequent allele 146T (relative risk, 2.336; 95% CI, 1.319–3.923;  $p=0.001$ ) at the 146 site. The eGFR was also an independent predictor for the kidney survival time of progressive kidney disease. The gender and smoking were excluded in the multivariate analysis by the Cox proportional

Table 2. Univariate analysis of clinical characteristics associated with the kidney survival time in chronic kidney disease patients.

| Characteristics                    | No. cases | 5 years survival rate of kidney(%) |                            | $\chi^2$ | <i>p</i> Value |
|------------------------------------|-----------|------------------------------------|----------------------------|----------|----------------|
|                                    |           | No. cases                          | survival rate of kidney(%) |          |                |
| Age (years)                        |           |                                    |                            | 0.044    | 0.834          |
| $\leq 65$                          | 69        |                                    | 26.1                       |          |                |
| $>65$                              | 33        |                                    | 24.2                       |          |                |
| Gender                             |           |                                    |                            | 5.935    | 0.015          |
| Male                               | 54        |                                    | 16.7                       |          |                |
| Female                             | 48        |                                    | 35.4                       |          |                |
| Smoking                            |           |                                    |                            | 4.620    | 0.032          |
| Yes                                | 42        |                                    | 14.3                       |          |                |
| No                                 | 60        |                                    | 33.3                       |          |                |
| BMI (kg/m <sup>2</sup> )           |           |                                    |                            | 1.117    | 0.291          |
| $<25$                              | 79        |                                    | 30.4                       |          |                |
| $\geq 25$                          | 23        |                                    | 8.7                        |          |                |
| MAP (mmHg)                         |           |                                    |                            | 0.013    | 0.909          |
| $\leq 105$                         | 38        |                                    | 28.9                       |          |                |
| $>105$                             | 64        |                                    | 23.4                       |          |                |
| Etiologies of CKD                  |           |                                    |                            | 1.115    | 0.291          |
| Membranous nephrology              | 56        |                                    | 23.2                       |          |                |
| Non-membranous nephrology          | 46        |                                    | 28.3                       |          |                |
| eGFR (mL/min/1.73 m <sup>2</sup> ) |           |                                    |                            | 32.370   | $<0.001$       |
| $<60$                              | 81        |                                    | 12.3                       |          |                |
| $\geq 60$                          | 21        |                                    | 76.2                       |          |                |

Note: BMI, body mass index; MAP, mean arterial pressure.

hazards model. We also further analyzed the relationship of ESRD risk factors between 146C and 146T case, the result showed that there was no difference between 146C and 146T in age, gender, smoking, BMI, MAP, etiologies of CKD and eGFR (Table 4). These data demonstrated the strong prediction power of nucleotide 146 on the kidney survival time of progressive kidney disease.

## Discussion

Selected SNPs in the mtDNA D-loop region have been examined for the ability to predict disease risk in CKD.<sup>21</sup> In our previous case-control study published on Renal Failure,<sup>21</sup> we found that the SNPs in the mitochondrial DNA D-loop region were associated with the risk of CKD. The present study has extended those analyses to determine the kidney survival-associated SNPs in a continuous sequence of mtDNA between nucleotides 16,190 and 583. One SNP, 146T/C, was identified by the log-rank test for the association with the kidney survival. Multivariate survival analysis identified 146T/C as an independent prediction marker for the kidney survival time. We identified six SNPs as the CKD risk SNPs including 73G, 146C, 150T, 194T, 195C and 310C.<sup>21</sup> It is interesting that the allele 146 also belong to these CKD risk SNPs. Nucleotide 146 is located in hypervariable segment 2 (HV2). The functional significance of HV segments, mutational hotspots at which germ line and mtDNA mutations

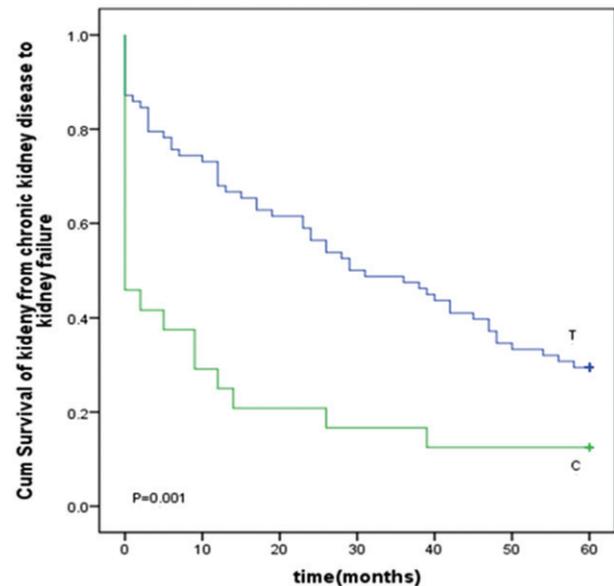


Figure 1. Survival curve of kidney according to the nucleotide at position 146 in D-loop of the chronic kidney disease patients.

Table 3. Multivariate analysis of prognostic factors associated with the kidney survival time in chronic kidney disease patients by the Cox proportional hazards model.

| Factors | Relative risk | 95% C.I.    | <i>p</i> Value |
|---------|---------------|-------------|----------------|
| Gender  | 0.358         | 0.165–0.776 | 0.009          |
| Smoking | 1.970         | 0.919–4.225 | 0.041          |
| eGFR    | 0.091         | 0.035–0.234 | $<0.001$       |
| 146T/C  | 2.762         | 1.599–4.770 | $<0.001$       |

Table 4. The relationship of clinical characteristics with the frequency of allele 146.

| Characteristics                    | 146T | 146C | $\chi^2$ | p Value |
|------------------------------------|------|------|----------|---------|
| Age (years)                        |      |      | 2.606    | 0.106   |
| ≤65                                | 56   | 13   |          |         |
| >65                                | 22   | 11   |          |         |
| Gender                             |      |      | 0.366    | 0.545   |
| Male                               | 40   | 14   |          |         |
| Female                             | 38   | 10   |          |         |
| Smoking                            |      |      | 0.281    | 0.596   |
| Yes                                | 31   | 11   |          |         |
| No                                 | 47   | 13   |          |         |
| BMI (kg/m <sup>2</sup> )           |      |      | 0.622    | 0.430   |
| <25                                | 59   | 20   |          |         |
| ≥25                                | 19   | 4    |          |         |
| MAP (mmHg)                         |      |      | 3.840    | 0.051   |
| ≤105                               | 25   | 13   |          |         |
| >105                               | 53   | 11   |          |         |
| Etiologies of CKD                  |      |      | 1.373    | 0.241   |
| Membranous nephrology              | 38   | 18   |          |         |
| Non-membranous nephrology          | 36   | 10   |          |         |
| eGFR (mL/min/1.73 m <sup>2</sup> ) |      |      | 1.256    | 0.262   |
| <60                                | 60   | 21   |          |         |
| ≥60                                | 18   | 3    |          |         |

preferentially occur,<sup>24</sup> is not known, but our data suggest that these SNPs are potential genetic markers for the kidney survival time. Minor alleles of 146C are associated with a dramatically shorter period of kidney survival and the kidney survival curve decreased rapidly in CKD patients with these alleles (Figure 1). The 146 site is hypervariable; Alzheimer's disease and Parkinson's disease have been identified as having mutations at this site.<sup>25,26</sup> Further study is needed to solve the mystery of this allele.

The D-loop region of mtDNA is important for the regulation of mitochondrial genome replication and expression. SNPs in the D-loop region might affect mtDNA replication and lead to electron transport chain alteration, which is responsible for the release of highly ROS and could contribute to the nuclear genome damage.<sup>27,28</sup> The ROS may promote kidney disease by activating the signaling pathways of NF- $\kappa$ B.<sup>11</sup> NF- $\kappa$ B is activated by ROS and upregulates of ROS-induced granulocyte macrophage-colony-stimulating factor (GM-CSF), which can enhance glomerular inflammation and induce mesangial proliferation.<sup>11</sup>

Consistent with earlier reports, our data demonstrated smoking and gender were associated with the CKD progression,<sup>29–33</sup> which confirmed the validity of our data. The mechanism explained the relationship between smoking and CKD progression is that smoking contribute to oxidative stress, which plays important role in the development and progression of CKD.<sup>34</sup> The relationship between gender and CKD progression suggests that sex hormones may be important determinants of the greater susceptibility of males to progressive kidney injury.

## Conclusion

In conclusion, SNPs in the mtDNA D-loop were found to be independent prognostic markers for the kidney survival time in CKD patients. The analysis of genetic polymorphisms in the D-loop might help to identify CKD patient subgroups at

high risk for a disease outcome, thereby helping to refine therapeutic decisions in CKD patients.

## Declaration of interest

All of the authors disclose any financial, consulting and personal relationships with other people or organizations that could influence the author's work.

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