Identification of sequence polymorphisms in the displacement loop region of mitochondrial DNA as a risk factor for renal cell carcinoma

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Abstract. The accumulation of single-nucleotide polymorphisms (SNPs) in the displacement loop (D-loop) of mitochondrial DNA (mtDNA) may be associated with an increased cancer risk. In this case-control study, the SNPs in the mitochondrial D-loop of renal cell carcinoma (RCC) patients were identified and their association with cancer risk was evaluated. The minor alleles of nucleotides 16293A/G, 262A/G and 488T/C were associated with an increased risk, whereas the minor alleles of nucleotides 16298T/C and 16319G/A were associated with a decreased risk for RCC. Moreover, the nucleotides 16293, 262, 16298 and 16319 were identified as specifically associated with the risk of clear cell RCC (ccRCC), whereas 262 and 488 were specifically associated with papillary RCC and renal oncocytoma. In conclusion, SNPs in mtDNA are potential modifiers of RCC. The analysis of genetic polymorphisms in the mitochondrial D-loop may help identify the patient subgroups at a high risk of developing RCC.

Introduction

Renal cell carcinoma (RCC) accounts for ~3% of all cases of adult malignancies worldwide, with >270,000 new cases (2.1% new cases of all cancers) and 100,000 deaths annually (1). The incidence of RCC has been on the increase worldwide (2). However, the mechanism involved in the carcinogenesis of RCC has not been elucidated. Previous studies demonstrated that genetic factors are important for the development of RCC (3,4), as is oxidative stress (5).

The human mitochondrial genome is a 16-kb circular double-stranded DNA molecule. It contains 12 coding genes involved in respiration and oxidative phosphorylation, 2 rRNAs and a set of 22 tRNAs that are essential for mitochondrial protein synthesis (6). Mitochondrial DNA (mtDNA) is considered to be more susceptible to DNA damage and acquires mutations at a higher rate compared to nuclear DNA, due to the presence of high levels of reactive oxygen species (ROS), the lack of protective histones and the limited capacity for DNA repair in the mitochondria (7,8). In addition, mtDNA contains a non-coding region that includes a unique displacement loop (D-loop) that controls replication and transcription of mtDNA, as it contains the initial site of heavy chain replication and the promoters for heavy and light chain transcription. In several types of cancer, somatic mutations and polymorphisms are located in an mtDNA non-coding region known as the D-loop (9,10). This region is crucial for the regulation of both the replication and expression of the mitochondrial genome as it contains the leading-strand origin of replication and the main promoter required for transcription (11).

Sequence changes in the D-loop have been extensively investigated in various types of cancer (9,10,12). A few single-nucleotide polymorphisms (SNPs) have been selected for predicting cancer risk; however, their predictive values have not yet been elucidated (13-16). The D-loop contains a length of 1,122 bps (nucleotides 16024-16569 and 1-576) according to the mitochondria database http://www.mitomap.org. In this study, a region of ~1 kb franking almost all of the D-loop was sequenced in the blood collected from RCC patients and healthy controls to determine the RCC risk-associated SNPs.

Materials and methods

Tissue specimens and DNA extraction. Blood samples were collected from 75 RCC patients who underwent nephrectomy in the Department of Urinary Surgery at the Fourth Hospital of Hebei University between 2002 and 2007. Blood samples were also collected from 68 healthy female controls. Total DNA

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was extracted using the Wizard Genomic DNA extraction kit (Promega, Madison, WI, USA) and stored at -20˚C. The study was approved by the Human Tissue Research Committee of the Fourth Hospital of Hebei Medical University. The patients provided written informed consent for the collection of samples and subsequent analysis.

Polymerase chain reaction (PCR) amplification and sequence analysis. Forward 5'-CCCCATGCTTACAAGCAAGT-3' (nucleotides 16190-16209) and reverse 5'-GCTTTGAGGAGG TAAGCTAC-3' (nucleotides 602-583) primers were used for the amplification of a 982-bp product from the mtDNA D-loop region. PCR was performed according to the protocol of the PCR Master Mix kit (Promega) and purified prior to sequencing. The PCR conditions were as follows: incubation for 2 min at 95˚C, followed by 35 cycles of a 30-sec denaturation at 95˚C, a 30-sec annealing at 55˚C, a 45-sec 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 Biosystems, Foster City, CA, USA) and the products were separated on the ABI PRISM Genetic Analyzer 3100 (Applied Biosystems). Polymorphisms were confirmed by repeated analyses from the two strands.

Statistical analysis. The χ^2 test was used to analyze dichotomous values, such as the presence or absence of any individual SNP in RCC patients and healthy controls. Statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). For all the statistical tests, $P<0.05$ was considered to indicate a statistically significant difference.

Results

Patients. A total of 75 patients, including 59 diagnosed with clear cell RCC (ccRCC), 13 with papillary RCC and 3 with renal oncocytoma, were enrolled in this study. The clinical characteristics of the RCC patients and healthy controls are listed in Table I.

SNP identification and analysis. SNPs were detected in 143 sites of the 982-bp mitochondrial D-loop region in the blood samples of the RCC patients and healthy controls, with no statistical reference on the distribution frequency of each SNP according to age and gender. The 14 SNPs (16293, 16298, 16304, 16319, 16362, 16519, 262, 263, 309-310, 315-316, 488, 489, 523 and 525) with a minor allele frequency >5% in the controls or RCC patients were used for cancer risk analysis via the χ^2 test.

When individual SNPs were compared between RCC patients and controls, a statistically significant increase in the SNP frequency for the 16293G, 262G and 488C alleles was observed in RCC patients ($P<0.05$), indicating that the patients who carry these alleles may be susceptible to RCC development (Table II). It was also observed that the SNP frequency for 16298C and 16319A was significantly decreased in the RCC patients compared to the healthy controls (Table II). The cancer risk-associated SNPs were further analyzed in RCC subgroups: the 16293, 16298, 16319 and 262 sites were associated with ccRCC risk, whereas the 262 and 488 sites were also associated with risk for papillary RCC and renal oncocytoma (Tables III-V).

Table I. Association of clinical characteristics of RCC patients and controls with cancer risk.

	RCC (n=75)	Control (n=68)
Age (years)		
≤55	33	28
>55	42	40
Gender		
Male	49	43
Female	26	25

RCC, renal cell carcinoma.

Discussion

An increase of mutations and SNPs in the mitochondrial D-loop region have been reported in a variety of cancers, including esophageal squamous cell carcinoma (17), hepatocellular carcinoma (18), oral squamous cell carcinoma (19) and lung cancer (20). In this case-control study, cancer risk-associated SNPs for RCC were investigated in a continuous sequence of mtDNA between nucleotides 16190 and 583, and 5 SNP sites, comprising 16293, 262, 488, 16298 and 16319, were identified. These SNPs may prove useful in future studies on SNP biological functions.

The mechanism through which SNPs in the D-loop transcription-regulatory region increase the risk of cancer has not been elucidated, although these genetic changes have been detected in several types of cancer. The D-loop region of mtDNA is important for the regulation of mitochondrial genome replication and expression. SNPs in this region may affect mtDNA replication and lead to alterations of the electron transport chain, which is responsible for the release of ROS and may contribute to nuclear genome damage as well as cancer initiation and progression (21,22). These SNPs may alter mitochondrial genome transcription, thus enhancing ROS generation (23). The ROS-mediated mechanism may subsequently promote tumor formation.

It has been reported that the 16293 site is associated with the risk of developing hepatocellular carcinoma (18) and the 16298 site is associated with resistance to small-cell lung carcinogenesis (20). The D-loop is the most variable region in mtDNA. The mutation rate at hypervariable (HV) regions in the D-loop region was estimated to be 100- to 200-fold that of nuclear DNA (24). In our study, all the cancer risk-associated SNPs were identified in the HV segment region of the D-loop, with nucleotides 16293, 16298 and 16319 belonging to HV-I, 262 to HV-II and 488 to HV-III. Cancer risk- and outcome-associated SNPs were identified in these regions in other types of cancer as well, including esophageal squamous cell carcinoma (17,27) and hepatocellular carcinoma (25,26). The HV segments are mutational hotspots at which germline and tumor mtDNA mutations preferentially occur (28). Their

Table II. SNP sites exhibiting frequency differences between RCC patients and controls.

Nucleotide	RCC (n=75)	Control (n=68)	χ^2	P-value
262A/G	50/25 (33.3%)	68/0 (0%)	27.469	0.000
488T/C	70/5 (6.7%)	68/0 (0%)	4.698	0.030
16293A/G	62/13 (17.3%)	66/2 (2.9%)	7.868	0.005
16298T/C	71/4 (5.3%)	54/14 (20.6%)	7.543	0.006
16319G/A	70/5 (6.7%)	55/13 (19.1%)	5.025	0.025

SNP, single-nucleotide polymorphism; RCC, renal cell carcinoma.

Table III. SNP sites exhibiting frequency differences between ccRCC patients and controls.

Nucleotide	ccRCC (n=59)	Control (n=68)	χ^2	P-value
262A/G	43/16 (27.1%)	68/0 (0%)	21.099	0.000
16293A/G	48/11 (18.6%)	66/2 (2.9%)	8.478	0.004
16298T/C	55/4 (6.8%)	54/14 (20.6%)	4.952	0.026
16319G/A	55/4 (6.8%)	55/13 (19.1%)	4.418	0.042

SNP, single-nucleotide polymorphism; ccRCC, clear cell renal cell carcinoma.

Table IV. SNP sites exhibiting frequency differences between papillary RCC patients and controls.

Nucleotide	Papillary RCC (n=13)	Control (n=68)	χ^2	P-value
262A/G	7/6 (46.2%)	68/0 (0%)	33.895	0.000
488T/C	11/2 (15.4%)	68/0 (0%)	10.726	0.001

SNP, single-nucleotide polymorphism; RCC, renal cell carcinoma.

Table V. SNP sites exhibiting frequency differences between renal oncocytoma patients and controls.

Nucleotide	Renal oncocytoma (n=3)	Control (n=68)	χ^2	P-value
262A/G	1/2 (66.7%)	68/0 (0%)	46.647	0.000
488T/C	2/1 (33.3%)	68/0 (0%)	22.990	0.000

SNP, single-nucleotide polymorphism.

functional significance has not been elucidated; however, our data suggest a role for these segments in the prediction of cancer risk.

SNPs in the mtDNA D-loop were identified as risk markers for RCC. The use of mtDNA SNPs for the prediction of RCC risk is a promising area for future research on cancer prevention. Our results have demonstrated the association of genetic variations of the exonuclear genome with cancer risk. The subsequent investigation for SNPs in the D-loop is more challenging compared to SNPs in individual genes, which may be assessed by expression levels, protein properties and interacting genes. Additional investigations are required, involving large-size samples, to determine the use of these minor alleles and validate the predictive values of SNPs identified in this pilot study.

In conclusion, analysis of the genetic polymorphisms in the mitochondrial D-loop may help identify patient subgroups at a higher risk for developing RCC, thereby helping to refine therapeutic decisions for these patients.

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