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RESEARCH ARTICLE

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Identification of sequence polymorphisms in the mitochondrial cytochrome c oxidase genes as risk factors for hepatocellular carcinoma

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Background: Single nucleotide polymorphisms (SNPs) accumulated in the mitochondrial DNA (mtDNA) is susceptible to the tumor formation. We discovered previously that SNPs in the mitochondrial displacement loop (D-loop) was associated with the risk of hepatocellular carcinoma (HCC).

Methods: The cytochrome c oxidase (*COX*) genes of mtDNA were sequenced between 107 HCC patients and 100 matched healthy controls. The χ^2 test was used to analyze single SNPs' statistical difference between HCC patients and healthy controls.

Results: In this study, cancer risk-associated SNPs in the *COX* genes of mtDNA coding region were assessed in HCC patients and health controls. The nucleotide position at site 9545A/G (*P=.036)* was identified its association for HCC with the 9545G allele susceptible to cancer risk.

Conclusions: The SNPs in the *COX* genes may help us to evaluate the cancer risk of HCC.

KEYWORDS
cytochrome c oxidase genes, hepatocellular carcinoma, mitochondrial DNA, single nucleotide polymorphisms

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and is responsible for more than half a million deaths each year, which makes it the third leading cause of cancer deaths worldwide.[1] In China, the incidence of HCC is increasing and now accounts for 55% of all HCC cases in the world.[2,3] This disease is strongly associated with several risk factors, including chronic hepatitis B virus (HBV), chronic hepatitis C virus (HCV) infection, and alcohol abuse.[4] HBV infection is a challenging health issue in China, where about 93 million peoples are HBV carries and 30 million have chronic B hepatitis.[5] Alcohol abuse is also on the rise in China with about 6.6% of males and 0.1% of females diagnosed with alcohol dependence.[6] Many of these patients develop liver disease such as alcoholic hepatitis and cirrhosis thereby to subsequent carcinogenesis.

The human mitochondrial genome is a 16-kb closed circular duplex molecule that contains 37 genes, including two ribosomal RNAs and a complete set of 22 tRNAs. Because of its lack of the protection of histone protein, inefficient repaired mechanism, and generation of reactive oxygen species (ROS), mtDNA has high susceptibility to mutations and polymorphisms.[7-9]

Oxygen species production could cause oxidative damage that lead to mutations and polymorphisms in the mitochondrial genes as well as affect the process of oxidative phosphorylation (OXPHOS), which might ultimately initiate carcinogenesis.[7,10-13] However, the mechanism of dysfunction of OXPHOS in HCC remains unclear. It is reported that abnormal level of ROS could induce HCC by affecting the process of oxidative phosphorylation (OXPHOS) and downregulation of the tumor suppressor genes in vitro.[14]

We found that the highly polymorphic sequence in D-loop that might be related to the breast cancer risk, esophageal squamous cell carcinoma, non-Hodgkin lymphoma, kidney cancer, and lung cancer.[15-20] But few studies focused on the relationships between coding region of mtDNA and HCC. *COX* genes codes three subunits of

TABLE 1 Primer pairs used to amplify the COX region

Gene ID	Forward primer Reverse primer
COX1 (5530-6050)	5′-GCTACTCCTACCTATCTCCC -3′
	5′- TGTGGTCGTTACCTAGAAGG-3′
COX1 (6040-6530)	5′- CTATTATTCGGCGCATGAGC -3′
	5′- TTGAGGTTGCGGTCTGTTAG -3′
COX1 (6550-7130)	5′- CCTATCTCTCCCAGTCCTAG -3′
	5′- GGATTTTGGCGTAGGTTTGG -3′
COX2 (7120-7600)	5′- GCCATCATAGGAGGCTTCAT-3′
	5′- AGACCTACTTGCGCTGCATG -3′
COX2 (7640-8180)	5′- ACATGCAGCGCAAGTAGGTC-3′
	5′- AACTGTGGTTTGCTCCACAG -3′
COX2 (8200-8770)	5′- CACTTTCACCGCTACACGAC -3′
	5′- TCCGAGGAGGTTAGTTGTGG -3′
COX3 (8870-9320)	5′- CCACAACTAACCTAATCGGA-3′
	5′- AGCGTTATGGAGTGGAAGTG -3′
COX3 (9320-9810)	5′- TCTCAGCCCTCCTAATGACC-3′
	5′- TGACGTGAAGTCCGTGGAAG -3′
COX3 (9640-10090)	5′-GTCCCACTCCTAAACACATC -3′
	5′-GTAAGGCTAGGAGGGTGTTG -3′

respiratory complex IV, a key enzyme as the third and final enzyme of the electron transport chain of mitochondrial oxidative phosphorylation in aerobic metabolism.[21] Gene polymorphisms of *COX* genes (including *COXI*, *COXII*, and *COXIII*) contributed to the dysfunction of mitochondrial respiratory function and associated with susceptibility of prostate cancer.[22] In this study, we sequenced a region of approximately 3010 bp flanking the majority of the *COX* genes (including *COXI*, *COXII*, and *COXIII*) in mtDNA coding region from the blood DNA of HCC patients to identify cancer risk-associated single nucleotide polymorphisms (SNPs).

2 | MATERIALS AND METHODS

2.1 | Tissue specimens and DNA extraction

Blood samples were collected at the Fourth hospital of Hebei Medical University including 107 patients with HCC who underwent tumor resection in the Department of Hepatobiliary Surgery between Feb 2008 and Dec 2009. Blood samples were also collected from 100 healthy controls. mtDNA extraction was carried out by using the TIANamo Genomic DNAki (TIANGEN, Beijing, China). All procedures were supervised and approved by the hospital's Human Tissue Research Committee. All patients provided written informed consent for the collection of samples and subsequent analysis.

2.2 | Sequencing the mtDNA COXI, COX II, and COX III genes

The primer pairs used to amplify the *COXI* (5530~6050 bps), *COXI* (6040~6530 bps), *COXI* (6550~7130 bps), *COXII* (7120~7600 bps), *COXII* (7640~8180 bps), *COXII* (8200~8770 bps), *COXIII* (8870~9320 bps), *COXIII* (9320~9810 bps), and *COXIII* (9640~10090 bps) genes were listed in Table 1, PCR was performed with the PCR Green Master Mix (Thermo, Billerica, MA, USA) and PCR production was purified prior to sequencing. Cycle sequencing was carried out with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and separated using the ABI PRISM Genetic Analyzer 3100 (Applied Biosystems).

2.3 | Statistical analysis

The χ^2 test (*P* value of < .05 was considered statistically significant) was used to analyze single SNPs' statistical difference between different groups, such as the presence or absence of an individual SNP between HCC patients and healthy controls. Two groups Kruskal-Wallis H test (*P* value of <.0126 was considered statistically significant) was used to compare SNPs frequency (ratio between number of SNPs per person and the length of target genes) and assisted by Nemenyi grammar to do the comparison among groups. All of the statistical analysis was done with the SPSS version21 software package (SPSS Company, Chicago, IL, USA).

3 | RESULTS

A total of 107 HCC patients were enrolled in this study. The clinical characteristics of the HCC patients and healthy controls are listed in Table 2, the age and gender showed no statistical difference between the HCC and healthy control.

We firstly analyzed mitochondrial *COXI* (nucleotide 5904~7445), *COXII* (nucleotide 7586~8269), *COXIII* (nucleotide 9207~9990) sequences in 30 HCC and healthy controls. A total of eight SNPs were identified in the *COX* coding region (Table 3). Kruskal-Wallis H test assisted by Nemenyi grammar was used to evaluate SNPs frequency in different groups (the sum of SNPs per person/length of target genes). Higher SNPs frequency was found in the HCC comparing with that of normal controls. A tendency toward an increased SNP frequency was

TABLE 2 The age and gender of HCC and controls

Group	HCC cancer (n=107)[a]	Control (n=100)	t/χ^2	*P*-value
Age (y)	54.860±9.767 [b]	56.360±10.437	−1.068	.287
Gender (M/F)	94/13	91/9	0.540	.463

[a]Sample size.
[b]Mean±standard deviation.

TABLE 3 The associations of the eight SNPs with HCC risk

SNP	Genetype	Number (HCC)	Number (Control)	χ^2	*P* (value)
(6962)	G/G	28	30	2.069	.492
	A/A	2	0		
(7196)	C/C	25	27	0.577	.706
	A/A	5	3		
(7853)	G/G	28	29	0.351	1.000
	A/A	2	1		
(8414)	C/C	26	27	0.162	1.000
	T/T	4	3		
(8584)	G/G	23	29	5.192	.052
	A/A	7	1		
(8701)	A/A	15	12	0.606	.436
	G/G	15	18		
(9540)	T/T	13	13	0.000	1.000
	C/C	17	17		
(9545)	A/A	26	30	4.286	.112
	G/G	4	0		

TABLE 4 SNP site showing frequency difference between HCC and controls

SNP	Genetype	HCC (n=107) [a]	Control (n=100)	χ^2	*P*-value
9545	A/A	99	99	5.214	.036
	G/G	8	1		
8584	G/G	88	82	0.002	.962
	A/A	19	18		

[a]Sample size.

observed in HCC patients, but did not reach statistical significance (Data not shown).

The *P* value of two sites (8584G/A *P*=.052, 9545A/G *P*=.112) was found near to the *P* value of .05 by a case-control study with HCC patients and controls using χ^2 test, so we genotyped the two sites in more patients and controls (Table 4). As shown in Table 4, the 9545 nucleotide was identified for its association with HCC risk with G allele susceptible to cancinogenesis. These data demonstrated that 9545 allele was responsible for HCC susceptibility.

4 | DISCUSSION

The relationships between mitochondrial DNA variation and oncogenesis were demonstrated in many kinds of tumor.[23-26] We previously focused on the role of mitochondrial D-loop variation in tumor development.[27,28] In this study, we sequenced the whole *COXI*, *COXII*, and *COXIII* genes in the coding region of mitochondiral DNA and identified the association of 9545 nucleotide with HCC risk by χ^2 analysis.

At the evolution process, more somatic mutation occurred in mtDNA that was transferred to daughter cells as SNP, an increased proliferation rate of tumor cells and an decreased apoptotic ability of tumor cells might give rise to accumulation of mutations and polymorphisms,[13] the higher SNPs frequency accumulations might influence the mitochondrial function. Consisted with our results, the higher SNP frequency in the mitochondrial *COX* gene was found in prostate cancer and pilocytic astrocytoma,[22,29,30] which may induce increased ROS level and subsequent impaired OXPHOS. The decreased aerobic respiration in cancer cell cloud induces the abnormal mitochondrial function so as to initiate carcinogenesis.[12] A study on colon cancer also showed that decreased expression of cytochrome c oxidase subunit I, which was coded by *COXI* gene, was significantly correlated with apoptosis resistance and increases the level of ROS.[31] The amino acid substituted somatic mutation which have potential to cause mitochondrial dysfunction was found in the HCC patients, the *COXIII* could colocalize with HBx to upregulate the mitochondrial function and HBx-induced ROS generation thereby to initiate the oncogenesis of HBV-associated HCC.[32,33]

Polymorphisms in mtDNA coding region have recently been reported being association with human cancer.[29,31] One cancer risk-related SNP 9545A/G was found in HCC patients, however, it is a silent variant (9545A/G) which meant that it did not induce amino acid substitution. The site might effect tRNA or other cotranscription genes to modulate the function of mitochondria so as to contributed to the tumor occurrence and development. So the functional study of this site needs to be performed. The genes variation linked to the different haplotype that was divided by 9545 SNP also need further analysis.

In conclusion, analysis of the genetic polymorphisms in the mitochondrial coding region (*COXI*, *COXII*, and *COXIII*) may help identify patients at a higher risk for developing HCC. Further researches will be essential to clear and identify the role of these changes.

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CONFLICTS OF INTEREST

All authors disclose any financial, consulting, and personal relationships with other people or organizations that could influence their work.

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