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RESEARCH

Association of mtDNA D-Loop Polymorphisms with Risk of Gastric Cancer in Chinese Population

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Abstract The aim of present study was to evaluate the association of common polymorphisms detected in mitochondrial DNA (mtDNA) D-loop region (mononucleotide repetitive D310, single nucleotide polymorphism (SNP) D16521) with susceptibility to gastric cancer (GC) in northwestern Chinese population. A total of 180 GC patients and 218 healthy controls were investigated by using PCR- denaturing high performance liquid chromatography (DHPLC) assay. Genotype and allele distributions and haplotype construction were analyzed in case–control study. We found D310 and D16521 heteroplasmy were significantly different between GC cases and controls ($p < 0.05$), and D16521 homoplasmy showed association with histological grade of GC ($p < 0.05$). Haplotype 7C/T, 8C/C and 9C/C had significant association with GC risk implied from analysis of D310 and D16521. Taken together, these findings suggested that mtDNA D-Loop polymorphisms and haplotypes may contribute to genetic susceptibility to GC in Chinese population.

Keywords Gastric cancer · MtDNA · D-loop · Single nucleotide polymorphisms

Abbreviations

GC	Gastric cancer
MtDNA	Mitochondrial DNA
SNP	Single nucleotide polymorphism
D310	Polytract of cytosines at nucleotide 310 in the mitochondrial genome
DHPLC	Denaturing high performance liquid chromatography

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Introduction

The mortality of gastric cancer (GC) in China is among the highest in the world, especially in northwest China [1]. Almost two thirds of the cases occur in developing countries and 42% in China alone [2]. Gastric cancer is considered as a complicated heterogeneous disease of multi-pathogen including genetic and environmental factors [3, 4]. Recently, involvement of mitochondria in cell apoptosis has stimulated interest in examining the potential

role of mtDNA mutation [5, 6]. Mitochondrial DNA alterations (mutations, deletions and instability) have been implicated as new molecular markers for detecting a variety of cancers in tissue samples and biofluids [7, 8].

D-loop is the only non-coding mtDNA region which contains crucial elements for replication and transcription, so the sequence alterations in this region may contribute to altered replication or transcription properties [9, 10]. Some studies described mutations frequently occurred in the D-loop region of mtDNA in human gastric cancer [11–13]. The C-tract D310 (between nucleotide 303 and 315) located in the hypervariable region II (HVII) (HVI, 16024~16383; HVII, 57~372) [14] of D-loop has emerged as a target of increased polymorphism in the general population and mutation in tumors [15–18]. Meanwhile, there are sequences in the D-loop region that are better conserved than other sequences of some coding protein genes [19].Of particular interest is D16521, which is located between HVI and HVII. D310 and D16521 are both in mtDNA D-loop but different fragment with distinct mutation rates [20].

Increasing evidence supported the existence of the relationship between variability of genes related mitochondrion and differences in energy metabolism, and it may result in pathophenotypic consequences [21, 22]. The aim of present study is to investigate whether the polymorphisms of the genes, which related mitochondrion, are associated with the occurrence of gastric cancer in Chinese population.

Materials and Methods

Samples Collection and DNA Extraction

A total of 180 GC cases and 218 healthy controls were obtained with consent from subjects in four hospitals in Wuwei city of Gansu province in north-western China. All of the GC cases were diagnosed histopathologically and none of the patients received any other treatment such as chemotherapy before operation. The controls were cancer-free healthy subjects matched with age, sex and ethnics from checking-up in the same hospital, yet not with a family history of GC. All the samples were collected according to a protocol approved by the medical ethics committee for conducting human research in the four hospitals. The case group comprised 47 females and 133 males aged from 32 to 82 years (mean±SD, 57.84±10.577). The control group comprised 49 females and 169 males aged from 38 to 87 years (mean±SD, 57.58±10.478).

Genomic DNA was extracted from EDTA anticoagulated peripheral venous blood by proteinase K digestion followed by phenol/chloroform extraction under the standard condition.

PCR Amplification

Primers for the two loci (D310 and D16521) were designed as shown in Table 1. All the two fragments were amplified under the standard PCR conditions. The different PCR annealing temperatures and fragment characters were also presented in Table 1. PCR products were visualized by 1.5%~2.0% agarose gels electrophoresis on stained with ethidium bromide.

Mutation Detection by DHPLC

The Genotyping of those two polymorphisms were conducted by WAVE 3500 apparatus (Transgenomic Inc, Omaha, USA). Denatured and re-annealed PCR fragments (6 μl; heated at 95°C for 5 min and cooled to 56°C at a rate of 0.1°C/s) were directly injected to the plate. The melting temperatures of amplicons were given in Table 1. Oven temperature for optimal heteroduplex separation under partial DNA denaturation was determined for each PCR fragment using DHPLC Melt Program. The gradient for the elution of each fragment (buffer A: 0.1 M triethylammonium acetate; buffer B: 0.1 M triethylammonium acetate, 25% acetonitrile) was regulated automatically by the Navigator software. Samples in question were mixed with sequence-known wild-type sample (ratio 1:1) to screen corresponding mutations, and their elution profiles were compared with standard reference profile. Data was collected and analyzed by the WAVEMAKER 4.1 software. The results were further confirmed by direct- sequencing (percentage of sequenced samples >10%) and DHPLC reanalysis by mixing mutation samples. The DNA sequences were compared with the published Human mtDNA

Table 1 Primer sequences and annealing temperature used to PCR and DHPLC analysis temperatures

Loci	Region	Fragment length(bp)	Forward primer (5′-3′)	Reverse primer (5′-3′)	PCR annealing temperature (°C)	DHPLC oven temperature (°C)
D310	D-loop/HVII	337	tttggtattttcgtctggg	taggctggtgttagggttc	56	57.6
D16521	D-loop/16320-67np[a]	319	agccatttaccgtacatagc	ccagacgaaaataccaaatg	55	59

[a] np (nucleotide position)

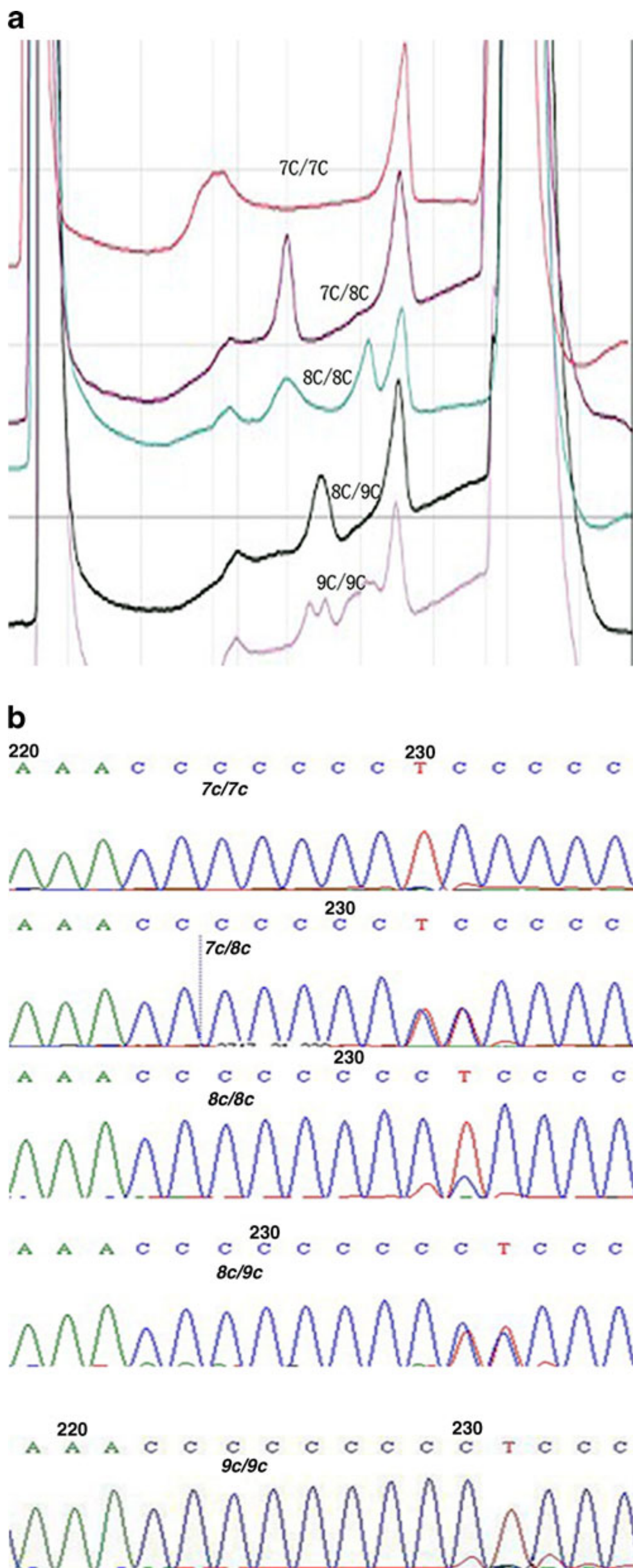

Fig. 1 DHPLC analysis for D310 PCR products. **a** DHPLC analysis of the D310 PCR product: C-tract alterations with the distinct elution profiles: 7C/7C, 7C/8C, 8C/8C, 8C/9C, 9C/9C; **b** Direct-sequencing analysis of the D310 PCR product

Revised Cambridge Reference Sequence (rCRS) [23] using Mutation Surveyor Version 2.2 (Soft Genetics LLC, State College, USA).

Statistical Analysis

The association of the two polymorphisms with GC risk between cases and controls were assessed using chi-square test performed with SPSS for Windows (version 13.0; SPSS Inc, Illinois, USA). The comparisons were also conducted according to different groups of gender, age and histological grade of GC [24]. A value of $P<0.05$ (two tails) was regarded as significant. The magnitude of association was estimated as odds ratio (OR), using 95% confidence intervals (95%CI). Haplotype constructions were analyzed using SHEsis software [25].

Results

D310 Polymorphism in mtDNA D-Loop

The analysis for D310 the first poly*C* stretch alterations consisted of single base deletion/expansion, as well as exceeding 1 bp. Those samples deemed to be positive for an alteration must exhibit distinct elution profiles in DHPLC assay (Fig. 1a) and reconfirmed by direct-sequencing (Fig. 1b).

There was a significant difference in D310 polymorphism between cases and controls ($P<0.05$). Heteroplasmy *7C/8C* was more frequent in patients (59.3%) than in controls (40.7%) (Table 2). In contrast, heteroplasmy *8C/*

Table 2 D310 genotype distributions between GC patients and controls

Genotype		Case	Control	Total
7C/7C	Count	91	115	206
	% within genotype	44.2%	55.8%	100.0%
	% of total	22.9%	28.9%	51.8%
7C/8C	Count	64	44	108
	% within genotype	59.3%	40.7%	100.0%
	% of total	16.1%	11.1%	27.1%
8C/8C	Count	8	6	14
	% within genotype	57.1%	42.9%	100.0%
	% of total	2.0%	1.5%	3.5%
8C/9C	Count	15	44	59
	% within genotype	25.4%	74.6%	100.0%
	% of total	3.8%	11.1%	14.8%
9C/9C	Count	2	9	11
	% within genotype	18.2%	81.8%	100.0%
	% of total	0.5%	2.3%	2.8%

Table 3 D310 and D16521 polymorphisms between cases and controls

Locus	Genotype/allele	Case (freq%)	Control (freq%)	χ2	*P* value	OR	95%CI
D310	7C/7C	91(55.8)	115(69.7)			1.000	
	7C/8C	64(39.3)	44(26.7)	6.450	***0.011***	1.838	[1.146–2.947]
	8C/8C	8(4.9)	6 (3.6)	0.891	0.345	1.685	[0.564–5.303]
	7C/8C+8C/8C	72(44.2)	50(30.3)	6.750	***0.009***	1.820	[1.156–2.864]
	7C	246(75.5)	274(83.0)			1.000	
	8C	80(24.5)	56(17.0)	5.719	***0.017***	1.591	[1.086–2.332]
	8C/8C	8(32.0)	6(10.2)			1.000	
	8C/9C	15(60.0)	44(74.5)	5.275	***0.022***	0.256	[0.076–0.875]
	9C/9C	2 (8.0)	9 (15.3)	3.896	0.099	0.167	[0.026–1.073]
	8C/9C+9C/9C	17(68.0)	53(89.8)	6.030	***0.014***	0.241	[0.073–0.792]
	8C	31(62.0)	56(47.5)			1.000	
	9C	19(38.0)	62(52.5)	2.975	0.085	0.554	[0.282–1.088]
D16521	T/T	30(16.7)	22(10.1)			1.000	
	C/T	68(37.8)	103(47.2)	5.202	0.023	0.484	[0.258–0.909]
	C/C	82(45.5)	93(42.7)	1.883	0.170	0.647	[0.346–1.208]
	C/T+C/C	150(83.3)	196(89.9)	3.752	0.053[a]	0.561	[0.311–1.012]
	T	128(35.6)	147 (33.7)			1.000	
	C	232(64.4)	289(66.3)	0.295	0.587	0.922	[0.688–1.236]

[a] Genotype (C/T+C/C) of D16521 had only a marginal impact, just failing to reach statistical significance (*p*=0.053)

9C was less frequent in patients (25.4%) than in controls (74.6%). Similar result was also found in homoplasmy *9C/9C*: 18.2% in patients and 81.8% in controls. Taken *7C/7C* and *8C/8C* as reference group respectively, statistical differences of *7C/8C* and *8C/9C* were noted in Table 3 (*p*=0.011, OR=1.838, 95%CI [1.146–2.947]; *p*=0.022, OR=0.256, 95%CI [0.076–0.875]). We also found evident discrepancies in dominant-trait genotype comparison of *8C* and *9C*, respectively (*p*=0.009, OR=1.820, 95%CI [1.156–2.864]; *p*=0.014, OR=0.241, 95%CI [0.073–0.792]). In addition, *8C* (*p*=0.017, OR=1.591, 95%CI [1.086–2.332]) was likely to be a more susceptible risk of GC compared with *7C* (Table 3). It appeared that *7C/8C* had a higher risk of GC but *8C/9C* might protect against GC, and this association was more obvious in dominant-trait genotype comparison.

There were great associations between D310 first poly*C* stretch alterations and GC risk both in males and females (*p*=0.013, *p*=0.012 respectively). This relation was also implied from analysis for subjects over 55 years (*p*=0.009) (according to the age distribution of subjects). We did not find any correlation between the distribution of D310 abnormalities and histological grade of the GC cases.

D16521 Polymorphism in mtDNA D-Loop

The results showed *C* allele took two-fold than *T* allele. Taken *T/T* as wild-type, we found *C/T* was significantly different between patients and controls (*p*=0.023, OR=0.484, 95%CI [0.258–0.909]) (Table 3, Fig. 2). Besides, genotype *C/T* and *C/C* had statistically significant relationship with GC risk in female subjects but not males (*p*=0.014; *p*=0.019). And the association was more significant in dominant-trait genotype comparison in females (*p*=0.011, OR=0.157, 95%CI [0.032–0.763]). In addition, D16521*C* allele appeared to be less susceptible to GC than *T* allele only in subjects over 55 years (*p*=0.000, OR=0.438, 95%CI [0.293–0.655]). Genotype *C/C* and comparison in dominant-trait genotype also had lower risk of GC within age group over 55 years (*p*=0.002; *p*=0.022). Furthermore, *C/C* showed associations with the extent of glandular differentiation of the GC cases in moderately differentiated adenocarcinoma and poorly differentiated adenocarcinoma (*p*=0.009, OR=0.087, 95%CI [0.010–0.732]; *p*=0.009, OR=0.083, 95%CI [0.010–0.697]) (Table 4).

Haplotype Analysis

The main haplotype distributions of D310 and D16521 polymorphisms in mtDNA D-Loop were summarized in Table 5. In this study, significant differences were observed between cases and controls for three haplotypes: *7C/T*, *8C/C*, and *9C/C* (*p*=0.016, OR=1.500, 95% CI [1.076~2.091]; *p*=0.008, OR=1.683, 95% CI [1.141~2.484]; *p*=0.0002, OR=0.335, 95% CI [0.181~0.618]).

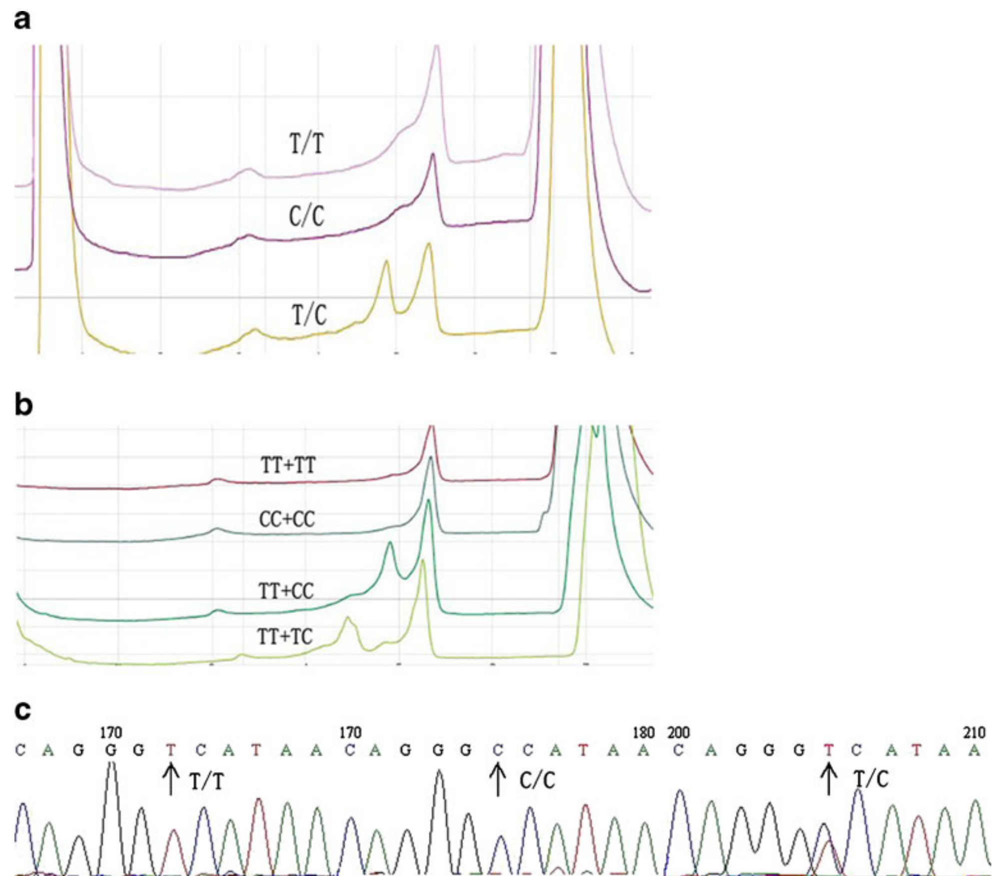

Fig. 2 DHPLC analysis for D16521 PCR products. **a** DHPLC analysis of the single D16521 PCR product were as follows: T/T, C/C, T/C; **b** DHPLC reanalysis of the mixed D16521 PCR products were as follows: TT + TT, CC + CC, TT + CC, TT + TC; **c** Directsequencing analysis of D16521

Discussion

We analyzed 180 GC cases for D310 C-tract deletion/expansion and SNP D16521 in mtDNA D-Loop region. A total of 89 abnormalities in D310 were found (49%), which was consistent with the reported frequencies 16%~62.5% in the whole mtDNA region of gastric cancers [11, 15, 26, 27]. The frequencies difference may account for populations analyzed from different areas.

D310 was highly polymorphic ranging between *7C* and *9C* with the most common sequence represented by *7C*, and the frequencies of D310 and D16521 are higher in early stages of GC compared with advanced stage (Table 6), especially in D16521, which supported the report from Sanchez-Cespedes M [14]. The distributions of D310 genotype and allele were significantly different in case and control groups. The *9C* alteration might protect against GC compared with *8C*, which also can be implied from haplotype analysis for D310&D16521: haplotype *9C/C* (p= 0.0002, Table 5).

D16521 polymorphisms analysis showed heteroplasmy *C/T* had association with GC. It is known that D16521 conserves among species with intense selective constraints since it is mapped in the D-loop central domain [28]. It may partially explain the reason why D16521 polymorphism is associated with GC in female but not in male.

Although these alterations occur frequently in GC patients, the causative role of D-loop mutations in GC is still uncertain. One rational explanation is D-Loop contains the major mtDNA elements for replication and transcription promoters. During tumor clone expansion, continuous replication of mtDNA needs repeated melting and reannealing, which would make this region unstable and mutation formation [29]. An alternative explanation is D-loop mutations might alter mtDNA transcription and lead to a respiratory chain alteration which is responsible for high reactive oxygen species levels release and therefore contribute to nuclear genome damage and also to cancer initiation and promotion[30].

The difference in the frequency of heteroplasmy across different age groups was statistically significant in GC, which suggests that heteroplasmy increase with age [31]. And the different frequencies of alterations in the two distinct mtDNA D-Loop regions may reflect the importance of mutations at different nucleotide positions in tumor transformation. Consequently, the high frequency of mtDNA D-loop alterations presence in GC could perhaps be exploited as clinical markers for early cancer detection [32, 33].

Table 4 D16521 polymorphisms with different grading of GC

Extent of glandular differentiation	Genotype			C/C&T/T [a]		
	T/T	C/T	C/C	Fisher's P	OR	95%CI
Well-differentiated adenocarcinoma	1(3.7)	4(14.8)	22(81.5)		1.000	
Moderately-differentiated adenocarcinoma	11(22.0)	18(36.0)	21(42.0)	0.009	0.087	[0.010–0.732]
Moderately-poorly adenocarcinoma	4(11.4)	17(48.6)	14(40.0)	0.150	0.159	[0.016–1.573]
Poorly-differentiated adenocarcinoma	12(21.4)	22(39.3)	22(39.3)	0.009	0.083	[0.010–0.697]
Mucinous adenocarcinoma	2(16.7)	6(50.0)	4(33.3)	0.100	0.091	[0.007–1.257]
Signet-ring [b] cell carcinoma	0	1(50.0)	1(50.0)	_	_	_
Adenosquamous [b] carcinoma	0	1(100.0)	0	_	_	_

[a] ORs determined by comparing each histological category with Well-differentiated adenocarcinoma

[b] Genotype T/T missing in signet-ring cell carcinoma and adenosquamous carcinoma has been dropped

Table 5 Main haplotype frequencies of D310/D16521 genetic polymorphisms between GC patients and controls

loci	Haplotype	Case (freq%)	Control (freq%)	$\chi 2$	Fisher's P	OR [95%CI]
D310/D16521	7C/C	149.53(0.415)	188.48(0.432)	0.232	0.630249	0.933 [0.703~1.238]
	7C/T	96.47(0.268)	85.52(0.196)	5.770	0.016336	1.500 [1.076~2.091]
	8C/C	68.42(0.190)	53.34(0.122)	6.980	0.008268	1.683 [1.141~2.484]
	8C/T	26.58(0.074)	46.66(0.107)	2.601	0.106869	0.665 [0.404~1.095]
	9C/C	14.05(0.039)	47.18(0.108)	13.291	0.000269	0.335 [0.181~0.618]
	9C/T	4.95(0.014)	14.82(0.034)	3.336	0.067841	0.396 [0.142~1.107]

Table 6 Alterations in D310 and D16521 in relation to clinicopathological variables of GC patients

Characters	D310				D16521			
	Alterations	Freq%	Hetero-plasmy	Freq%	Alterations	Freq%	Hetero-plasmy	Freq%
Age(years)								
≤55	30	42.9	28	40	58	82.9	29	41.4
>55	58	53.2	51	53.2	92	83.6	53	48.2
Gender								
Male	61	45.9	53	39.8	113	85.0	49	36.8
Female	28	59.6	26	55.3	37	78.7	19	40.4
Extent of glandular differentiation								
Well-differentiated adenocarcinoma	14	53.8	12	46.2	26	96.3	4	14.8
Moderately-differentiated adenocarcinoma	28	56.0	27	54.0	39	78.0	18	36.0
Moderately-poorly differentiated adenocarcinoma	18	51.4	17	48.6	31	88.6	17	48.6
Poorly-differentiated adenocarcinoma	24	42.9	19	33.9	44	78.6	22	39.3
Mucinous adenocarcinoma	6	50.0	5	41.7	10	83.3	6	50.0
Signet-ring cell carcinoma	1	50.0	1	50.0	2	100	1	50.0
Adenosquamous carcinoma	0	0	0	0.0	1	100	1	100.0

However, the results to date suggested the mtDNA mutations in peripheral blood may have limited clinical utility in the detection of carcinoma. Gastric tumor with heterogeneity may confound genetic analysis. So DHPLC could be used to detect these heteroplasmic species, therefore can be used as a rapid screening test for mtDNA mutations [34].

Till now, there was few report about association of mtDNA D-loop alterations with GC risk in Chinese population. This study may provide some genetic information for understanding it. The effects of these gene polymorphisms on the occurrence and development of GC are still need further investigation for the clinically useful genetic marker.

Conclusion

Taken together, these findings suggested that mtDNA D-Loop polymorphisms and haplotypes may contribute to genetic susceptibility to GC in Chinese population.

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Conflicts of interest No conflicts of interest were declared in relation to this article.

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