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Original Article

The relationship of haplotype in lactotransferrin and its expression levels in Chinese Han ovarian cancer

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Chromosomal DNA sequence polymorphisms may contribute to individuality, confer risk for diseases, and most commonly are used as genetic markers in association study. The iron-binding protein lactoferrin inhibits bacterial growth by sequestering essential iron and also exhibits antitumor, anti-inflammatory, and immunoregulatory activities. The gene coding for lactotransferrin (LTF) is polymorphic, with the occurrence of several common alleles in the general population. This genetically determined variation can affect LTF functions. In this study, we determined the distribution of *LTF* gene polymorphisms (rs1126477, rs1126478, rs2073495, and rs9110) in the Chinese Han population and investigated whether these polymorphisms were associated with increased risk of ovarian carcinoma in the Chinese. It was found that the rs1126477 was correlated significantly with ovarian cancer. The frequency of A allele of rs1126477 was significantly higher in 700 ovarian cancer patients compared with that in the control group of 700 cases ($P < 0.01$, $\chi^2 = 6.79$). The frequency of AA genotype was significantly higher in ovarian cancer patients compared with that in the control group ($P < 0.05$, $\chi^2 = 6.49$). AA genotype is the risk factor of ovarian cancer. The odds ratio (OR) was 2.24 and the 95% confidence interval (CI) was 1.08–4.59, respectively. The 'A-G-C-C' haplotype constructed with rs1126477, rs1126478, rs2073495, and rs9110 was the risk factor to be ovarian cancer. The expression of LTF gene was lower in individuals with 'A-G-C-C' haplotype compared with that in individuals without 'A-G-C-C' haplotype. These findings suggested that rs1126477 could play important roles in ovarian carcinoma physiological processes in the Chinese.

Keywords ovarian carcinoma; genetic variation; haplotype diversity; lactotransferrin; gene expression

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Introduction

It is desirable to identify disease loci in the human genome based on DNA sequence polymorphism information by using various approaches including linkage-based association studies [1]. Decades ago, microsatellite markers of low densities were used in linkage analyses, and now single-nucleotide polymorphisms (SNPs) of high densities are used in association studies [2–5].

Despite estimates of >100,000 newly diagnosed cases of ovarian cancer and about 80,000 related deaths each year in China [6,7], the etiology of ovarian cancer remains poorly understood. Known risk factors include increased risk with family history and use of fertility drugs, and decreased risk with oral contraceptive use, parity, and long duration of breast feeding [8]. The search for additional loci includes thoughtful screening of candidate genes in key biological pathways, an approach that has been successful in identifying new risk alleles for a variety of cancers [9–12].

Inflammation has been implicated in ovarian carcinogenesis because of its role in ovulation and post-ovulatory repair. During ovulation the ovarian epithelial surface is damaged, requiring a repair process involving the recruitment of leukocytes and inflammatory cytokines, release of nitrous oxide, DNA repair, and tissue restructuring [13,14].

Lactotransferrin (LTF) is an iron-binding glycoprotein secreted by many types of cells and acts as one of the innate immune defenders against microbial pathogens [15]. Recently, LTF has been found to have anti-tumor activity by regulating tumorigenesis [16–18]. The lactoferrin protein possesses antimicrobial and antiviral activities. It is also involved in the modulation of the immune response. In the normal healthy individual, lactoferrin plays a role in the front-line host defense against infection and in immune and inflammatory responses [19]. Since LTF is involved in many biological processes, whether genomic variations, such as SNPs, have an effect on the structure and function

of lactoferrin protein and whether these variations contribute to the different susceptibility of individuals in response to environmental insults are interesting health-related issues [19].

There are no previous reports to examine the association of the *LTF* gene polymorphisms in patients with ovarian carcinoma. In this study, we investigated the relevance of selected SNPs in the human lactoferrin gene on the susceptibility to ovarian cancer. We hypothesized that inherited variation in the genes of LTF was associated with ovarian cancer risk. To examine this hypothesis, we assessed informative SNPs in two case–control study populations. We used MassARRAY technology to determine the distribution of *LTF* genotypes in the Chinese Han population and reveal the relationship between these polymorphisms and ovarian carcinoma.

Materials and Methods

Participants

The 700 participants, who were women over age 20 years with histologically confirmed epithelial ovarian cancer living in Hunan province within 1 year of diagnosis, were recruited at Xiangya Hospital, Central South University (Changsha, China). Consent forms were obtained from individual patients, and experimental protocols were approved by the institutional review board of Xiangya Hospital. Controls (700 normal people) without ovarian cancer were recruited from women for general medical examinations and frequency matched to cases on age and region of residence. All subjects enrolled in the study were Chinese. There was no significantly difference in distribution between ovarian cancer patients and controls (**Table 1**).

Table 1 Characteristics of ovarian cancer cases and controls

Characteristic	Controls ($n = 700$), N (%)	Ovarian Carcinoma ($n = 700$), N (%)
Age ≤ 30	75 (10.64)	70 (10.00)
$30 <$ age ≤ 40	149 (21.28)	137 (19.57)
$40 <$ age ≤ 50	342 (48.94)	335 (47.86)
Age > 50	134 (19.14)	158 (22.57)
Mean	48.7	49.3
Median	48.4	49.0
SD	12.4	12.9
χ^2	1.48	
P value	0.35	

Data and biospecimen collection

Information on known and suspected risk factors was collected through in-person interviews. The extra vial of blood was drawn from Xiangya Hospital participants during their scheduled medical visit. DNA was extracted from 10 to 15 ml fresh peripheral blood using BloodGen Maxi Kit (Takara, Dalian, China). Genomic DNA concentrations were adjusted to 50 ng/μl before genotyping. Samples were bar coded to ensure accurate and reliable sample processing and storage. Five ovarian cancer samples and five non-tumor ovarian epithelial tissues were collected (for detailed information please see **Supplementary Table 1**), and each biopsy sample was divided into two sections. One was submitted to routine histological diagnosis, and the remaining section was stored at −80°C in RNALater Reagent (Qiagen, Carlsbad, USA).

SNP selection and genotyping

Four SNPs of *LTF* gene are located in Chromosome 3. These SNPs located from 46480801 to 46501268 on chromosome and affect the amino acid residue change. The detailed information is shown in **Table 2**. Genotyping of 1400 genomic samples was performed at BGI company (Shenzhen, China) using MassARRAY technology for automated genotype clustering and calling separately for genomic according to a standard protocol (www.genomics.org.cn).

RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR) analysis

Total RNA was extracted from the biopsy samples with RNeasy® kit (Qiagen) according to the manufacturer's

Table 2 Information of SNPs in chromosome, mRNA, and protein

SNP	Chr[a]	Chr position	mRNA	mRNA position	Allele change	Protein	Protein position	Residue change
rs1126477	3	46501268	NM_002343.2	123	GCC⇒ACC	NP_002334.2	29	A [Ala] ⇒T [Thr]
rs1126478	3	46501213	NM_002343.2	178	AAA⇒AGA	NP_002334.2	47	K [Lys] ⇒R [Arg]
rs2073495	3	46480958	NM_002343.2	1775	GAG⇒GAC	NP_002334.2	579	E [Glu] ⇒D [Asp]
rs9110	3	46480801	NM_002343.2	1932	TTG⇒CTG	NP_002334.2	632	L [Leu] ⇒L [Leu]

[a]Chr: chromosome.

Table 3 Association of LTF SNP alleles with ovarian cancer risk

SNP	Allele	Cases (frequency), $N = 700$	Controls (frequency), $N = 700$	OR (95% CI)	χ^2	*P* value
rs1126477	A	734 (52.4%)	385 (27.5%)	2.90 (1.28–6.55)	6.79	0.009
	G	666 (47.6%)	1015 (72.5%)			
rs1126478	A	301 (21.5%)	288 (20.6%)	1.61 (0.63–4.19)	0.99	0.318
	G	1099 (78.5%)	1112 (79.4%)			
rs2073495	C	798 (57.0%)	693 (49.5%)	2.24 (1.04–4.82)	4.41	0.035
	G	602 (43.0%)	707 (50.5%)			
rs9110	C	928 (66.3%)	804 (57.4%)	2.18 (1.00–4.75)	3.94	0.047
	T	472 (33.7%)	596 (42.6%)			

recommendations. The total RNA samples (1 μg) were used to generate cDNA. RT was carried out as described previously [20]. After the RT reaction, the PCR reaction was preceded by 94°C for 5 min, then 30 cycles for *LTF* of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min followed by 72°C for 7 min. All RT-PCR reactions were repeated at least three times at different numbers of the extension cycle to avoid false results of the PCR. *GAPDH* was used as an endogenous control for normalization. The sequences of the primers used for RT-PCR were as follows: *LTF* forward, 5′-tcttcctcgtcctgctgttc-3′, reverse, 5′-tgagttcgtggctgtctttc-3′; *GAPDH* forward, 5′-accacagtccat gccatcac-3′, reverse, 5′-tccaccaccctgttgctgta-3′. The expression of mRNA was assessed by evaluated threshold cycle (CT) values. The CT values were normalized with the expression levels of *GAPDH* and the relative amount of mRNA specific to each of the target genes was calculated using the $2^{-\Delta\Delta CT}$ method [21].

Western blot analysis

Proteins of biopsy samples were prepared with the lysis buffer [1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 2 mM ethylenediaminetetraacetic acid, 10% glycerol plus complete protease inhibitor mixture (Roche Diagnostics, Indianapolis, USA) with NaCl adjusted to 400 mM]. The protein concentrations were determined using the bicinchoninic acid (Pierce Chemical, Rockford, USA) protein assay method. Extracts containing 50 μg proteins were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electroblotted onto nitrocellulose membranes (Hyclone Laboratories, Logan, USA). The membranes were blocked with Tris-buffered saline/Tween 20 (25 mM Tris-HCl, 150 mM NaCl, pH7.5, and 0.05% Tween 20) containing 5% non-fat milk followed by overnight incubation at 4°C with primary antibodies (Goat anti-LTF Antibody, MyBioSource company, San Diego, USA, 1:500). After three times of wash, secondary antibody (anti-horseradish peroxidase antibodies, Santa Cruz Biotechnology, Santa Cruz, USA, 1:2000) were added, and incubated for 1 h. Then anti-β-actin antibody (Santa Cruz Biotechnology, 1:3000) was used as a loading control.

Statistical analysis

Distribution of age was compared across case status using χ^2 tests. SNP associations for ovarian cancer risk were assessed using SHEsis (http://analysis.bio-x.cn/myAnalysis.php) [22,23]. Haplotyper (http://www.people.fas.harvard.edu/~junliu/Haplo/click.html) and PHASE (http://www.stat.washington.edu/stephens/phase/download.2.0.2.html) softwares were used for haplotype inference [24]. Testing for association was completed using the freely available program SNPGWA (www.phs.wfubmc.edu/web/publicbios/secgene/downloads.cfm) [25–27]. Each SNP was tested for departure from Hardy–Weinberg equilibrium.

Results

Association of *LTF* SNP alleles, genotypes with ovarian cancer risk

The allele frequencies for *LTF* gene polymorphisms are summarized in **Table 3**. The allele frequency distributions were in accordance with Hardy–Weinberg equilibrium expectations for both the control group and ovarian cancer patients ($P > 0.05$). The rs1126477 correlated significantly with ovarian cancer. The frequency of A allele of rs1126477 was significantly higher in ovarian cancer patients compared with that in the control group ($P < 0.01$, $\chi^2 = 6.79$). The population with A allele had more ovarian cancer risk than the population without A allele [odds ratio (OR) = 2.90, 95% confidence interval (CI): 1.28–6.55]. There was the same distribution of rs2073495 and rs9110. No significant differences in the distribution of alleles were observed between the control group and ovarian cancer patients in the rs1126478 polymorphism.

Table 4 Association of LTF genotypes with ovarian cancer risk

SNP	Genoype	Cases	Controls (frequency)	OR (95% CI)	χ^2	*P* value
rs1126477	AA	0.286	0.050	2.24 (1.08–4.59)	6.49	0.039
	AG	0.476	0.450			
	GG	0.238	0.500			
rs1126478	AA	0.091	0.059	1.45 (0.88–2.06)	1.07	0.584
	AG	0.409	0.294			
	GG	0.500	0.647			
rs2073495	CC	0.500	0.250	1.84 (0.98–4.80)	3.82	0.148
	CG	0.341	0.450			
	GG	0.159	0.300			
rs9110	CC	0.488	0.263	2.03 (1.02–4.35)	3.25	0.197
	CT	0.349	0.421			
	TT	0.163	0.316			

Table 5 Risk of ovarian cancer associated with common LTF haplotypes

Haplotype[a]	Case (frequency)	Control (frequency)	χ^2	*P* value	OR (95% CI)
A-A-G-T	0.007	0.036	3.026	0.082	1.130 (0.561–3.785)
A-G-C-C	0.476	0.120	12.463	0.000	6.515 (2.098–20.236)
A-G-G-T	0.043	0.184	6.118	0.013	0.199 (0.050–0.795)
G-A-C-C	0.191	0.152	0.323	0.569	1.376 (0.456–4.155)
G-A-G-T	0.100	0.005	2.597	0.107	20.644 (1.658–36.419)
G-G-C-C	0.005	0.130	11.322	0.001	0.235 (0.092–0.833)
G-G-G-T	0.179	0.374	4.981	0.025	0.363 (0.146–0.900)

[a]Haplotypes defined by SNP alleles in chromosomal order from 5′ to 3′ across the *LTF* gene region (rs1126477, rs1126478, rs2073495, and rs9110)

Further, we analyzed the relationship between genotype distribution and ovarian cancer. The genotype frequency distributions were in accordance with Hardy–Weinberg equilibrium expectations for both the control group and ovarian cancer patients ($P > 0.05$). No significant differences in the distribution of genotypes were observed between the control group and ovarian cancer patients in the rs1126478, rs2073495, and rs9110 polymorphism ($P > 0.05$). The rs1126477 polymorphism correlated significantly with ovarian cancer. The frequency of AA genotype was significantly higher in ovarian cancer patients compared with that in the control group ($P < 0.05$, $\chi^2 = 6.49$). AA genotype was the risk factor of ovarian cancer. The OR ratio was 2.24 and 95% CI was 1.08–4.59 (**Table 4**).

Risk of ovarian cancer associated with common LTF haplotypes

To find the haplotype of risk or the protective factors for ovarian cancer, we analyzed the distribution of haplotype between controls and ovarian cancer patients. Ovarian cancer patients had a higher rate of 'A-G-C-C' haplotype (constructed with rs1126477, rs1126478, rs2073495, and rs9110) compared with controls ($P < 0.001$, $\chi^2 = 12.463$). The population with 'A-G-C-C' haplotype had 6.515 fold more risk to be ovarian cancer, and the 95% CI was 2.098–20.236. Other four haplotypes, 'A-A-G-T', 'A-G-G-T', 'G-G-C-C', and 'G-G-G-T', had same tendency. These haplotypes were higher in the controls compared with that in the ovarian cancer ($P < 0.05$). So these four haplotypes were the protective factors. The population with these haplotypes had lower risk than the others. No significant differences in the distribution of 'G-A-C-C' and 'G-A-G-T' haplotype were observed between the control group and ovarian cancer patients ($P > 0.05$) (**Table 5**).

The relationship of 'A-G-C-C' haplotype with *LTF* mRNA and protein expression levels

To reveal the relationship between haplotypes and LTF expression levels, we chose five samples with 'A-G-C-C'

Table 6 The relevance of 'A-G-C-C' haplotype and LTF mRNA expression levels

Individuals (with or without A-G-C-C haplotype[a])	*N*	(Mean ± SD)				Flod[b]
		LTF C_T	*GAPDH* C_T	ΔC_T	$\Delta\Delta C_T$	
With 'A-G-C-C'	5	25.49 ± 2.51	19.31 ± 1.34	6.18 ± 0.82	1.72 ± 0.82	0.35
Without 'A-G-C-C'	5	22.94 ± 1.93	18.48 ± 1.71	4.46 ± 1.10		

[a]Haplotypes defined by SNP alleles in chromosomal order from 5′ to 3′ across the *LTF* gene region (rs1126477, rs1126478, rs2073495, and rs9110).
[b]The expression level of LTF in individuals with 'A-G-C-C' haplotype compared with that in those without 'A-G-C-C' haplotype.

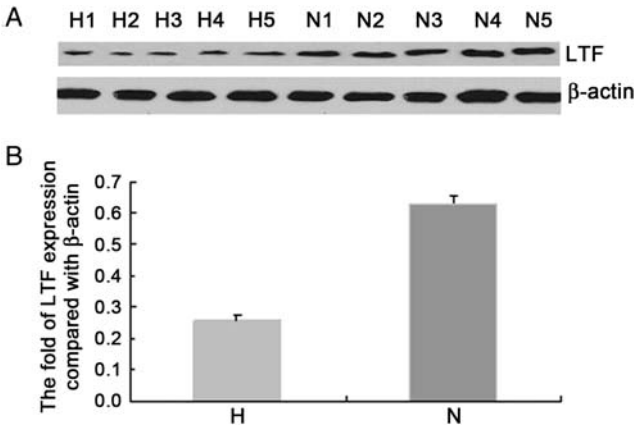

Figure 1 The relevance of 'A-G-C-C' haplotype and LTF protein expression levels (A) Protein levels were detected by western blot analysis. Individual of H1–H5 means group with 'A-G-C-C' haplotype, including three ovarian cancer tissues (H1–H3) and two non-tumor ovarian epithelial tissues (H4 and H5). Individual of N1–N5 means group without 'A-G-C-C' haplotype, including two ovarian cancer tissues (N1 and N2) and three non-tumor ovarian epithelial tissues (N3–N5). (B) The densitometric analysis of the western blot results. H means group with 'A-G-C-C' haplotype and N means group without 'A-G-C-C' haplotype.

haplotype and five samples without 'A-G-C-C' haplotype to perform real-time quantitative RT-PCR and western blot assay. The mRNA expression level of the *LTF* gene was normalized to the expression of internal control gene (*GAPDH*). The expression of the *LTF* gene was lower (35%) in individuals with 'A-G-C-C' haplotype compared with that in those without 'A-G-C-C' haplotype (**Table 6**). The protein level was analyzed by western blot. The expression level of LTF was lower in the samples with 'A-G-C-C' haplotype than that in those without 'A-G-C-C' haplotype (**Fig. 1**)., The average LTF expression level in the with 'A-G-C-C' haplotype group was 41% of that in the without 'A-G-C-C', when normalized to β-actin, which is consistent with the results of real-time quantitative RT-PCR.

Discussion

The iron-binding protein lactoferrin is a ubiquitous and abundant constituent of human exocrine secretions. Lactoferrin inhibits bacterial growth by sequestering essential iron, and also exhibits non-iron-dependent antibacterial, antifungal, antiviral, antitumor, anti-inflammatory, and immunoregulatory activities. Lys/Arg polymorphism (rs1126478) at position 29 in the N-terminal region of human lactoferrin was functionally different, which may contribute to the pathogenesis of localized juvenile periodontitis [28–30]. Moreno-Navarrete *et al*. assessed the association of circulating lactoferrin concentration and two LTF gene polymorphisms (rs1126477 and rs1126478) with dyslipidemia in men depends on glucose-tolerance status, and found that circulating lactoferrin concentration was inversely associated with fasting triglyceride concentration, body mass index, waist-to-hip ratio, and fasting glucose concentration, and directly correlated with HDL cholesterol concentration. Control AG heterozygotes for rs1126477 had significantly decreased fasting triglyceride concentrations. Similarly, control individuals who were G carriers for rs1126478 had significantly lower fasting triglyceride concentrations and significantly higher HDL cholesterol concentrations than AA homozygotes [31].

In this study, we investigated the distribution of *LTF* gene polymorphisms (rs1126477, rs1126478, rs2073495, and rs9110) in Chinese Han population, and found that rs1126477, rs2073495, and rs9110 correlated significantly with ovarian cancer. The frequency of A allele of rs1126477 was significantly higher in ovarian cancer patients compared with that in the control group ($P < 0.01$, $\chi^2 = 6.79$). The population with A allele had more ovarian cancer risk than the controls (OR = 2.90, 95% CI: 1.28–6.55). But no significant differences in the distribution of alleles were observed between the control group and ovarian cancer patients in the rs1126478 polymorphism. Next, our results of genotype showed that the frequency of AA genotype of rs1126477 was significantly higher in ovarian cancer patients compared with that in the control group ($P < 0.05$, $\chi^2 = 6.49$). AA genotype was the risk factor of ovarian cancer. The OR ratio was 2.24 and the 95% CI was 1.08–4.59. The 'A-G-C-C' haplotype constructed with rs1126477, rs1126478, rs2073495, and rs9110 was the risk factor to be ovarian cancer. Other four haplotypes, 'A-A-G-T', 'A-G-G-T', 'G-G-C-C', and 'G-G-G-T' were the protective factors.

To reveal the haplotypes of LTF and its expression levels, we tested the mRNA and protein expression levels in samples with or without 'A-G-C-C' haplotype. The results showed that the LTF expression was lower in individuals with 'A-G-C-C' haplotype than that in the group without 'A-G-C-C' haplotype at mRNA and protein levels, which hinted that haplotype may affect the LTF expression. Similar results were confirmed by other labs. Radovich *et al*. [32] have found that haplotypes in *VEGF* genes could affect the gene expression. Our findings suggested that rs1126477 and 'A-G-C-C' haplotype could play important roles in ovarian carcinoma physiological processes in Chinese Han population. These potentially functional polymorphisms might have contributed to the observed genetic selection of particular polymorphisms. Further studies are needed to investigate the biological role of these lactoferrin polymorphisms in normal ovary epithelium tissue and the pathological implication in the development of ovarian carcinoma.

Supplementary Data

Supplementary data are available at *ABBS* online.

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