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Original Article

Association between *clusterin* polymorphisms and esophageal squamous cell carcinoma risk in Han Chinese population

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Abstract: Genetic susceptibility plays an essential role in an individual's risk of esophageal squamous cell carcinoma (ESCC). The aim of this study is to investigate the associations between *clusterin* (*CLU)* gene polymorphisms and ESCC risk. We undertook a case-control study to analyze three *CLU* polymorphisms (*gene rs9331888 C>G, rs17466684 A>G and rs1532278 T>C*) in an Han Chinese population, by extraction of genomic DNA from the peripheral blood of 642 patients with ESCC and 658 control participants, and performed *CLU* genotyping using DNA sequencing. The obtained results indicated that overall, no statistically significant association was observed in rs17466684 and rs1532278. However, gene rs9331888 C>G genotype was at increased risk of ESCCs (*P*=0.037; odds ratio (OR)=1.089, 95% CI: 1.006-1.175). Moreover, rs9331888 G/G genotype ESCCs were more significantly common in patients with tumor size of >5 cm than T allele ESCC and in cases of poor differentiation and lower advanced pathological stage. In conclusion, polymorphism in rs9331888 C>G was observed to be associated with susceptibility of ESCC. Nevertheless, further investigation with a larger sample size is needed to support our results.

Keywords: Allele, CLU, esophageal squamous cell carcinoma, polymorphism

Introduction

Esophageal squamous cell carcinoma (ESCC) is the eighth most common human cancer, but has a high mortality rate [1]; the 5-years survival rate for all stages combined is less than 20% [2]. Almost 50% of ESCC cases occur in China [3]. The development of ESCC is a complex process, which is related to the multiple environment factors, including diet [4], infection [3] and lifestyle factors, particularly tobacco smoking and alcohol [5]. However, the fact that a small portion of individuals, who did not expose to the above mentioned disease causes, develop ESCC suggests that genetic susceptibility maybe play an important role in an individual's risk of esophageal carcinoma [6, 7].

Clusterin (CLU), first discovered as serum apolipoprotein J with chaperoning properties for protein stabilization, is virtually expressed in all tissues, and found in all body fluids [8, 9]. Its multiple functions include roles in apoptosis, complement regulation, lipid transport, sperm maturation, endocrine secretion, membrane protection, promotion of cell interactions and as a chaperone [10-12]. There are two known CLU protein isoforms generated in human cells: a nuclear form of CLU protein (nCLU) is pro-apoptotic, and a secretory form (sCLU, cytoplasmic or ectocytic) is pro-survival [13, 14]. Interestingly, nCLU is often absent in advanced tumors or tumor cell lines, while upregulation of sCLU has been reported in various human malignancies, including bladder, kidney, prostate, breast, ovarian, cervix, liver, colon, and lung tumors [15-18]. Overexpression of cytoplasmic CLU was observed to correlate closely with tumor aggressiveness, chemotherapy/radiotherapy resistance, and/or poor patient prognosis in some of these cancers [15-20].

Single nucleotide polymorphisms (SNP) are the most common human genetic variation. Previous studies have demonstrated that some

Table 1. General characteristics for the ESCC cases and control population

	No. of cases (%) N=642	No. of controls (%) N=658	*P* value[c]
Age[a,b]			
≤45	56 (8.7)	64 (9.7)	0.767
45-69	463 (72.1)	464 (70.5)	
≥70	123 (19.2)	130 (19.8)	
Sex			
Female	194 (30.2)	207 (31.5)	0.628
Male	448 (69.8)	451 (68.5)	
Alcohol drinking			
No	257 (40.0)	271 (41.2)	0.672
Yes	385 (60.0)	387 (58.8)	
Cigarette smoking			
No	298 (46.4)	309 (47.0)	0.844
Yes	344 (53.6)	349 (53.0)	
Family history of cancer			
No	469 (73.1)	482 (73.3)	0.935
Yes	173 (26.9)	176 (26.7)	

[a]Age of diagnosis for cases. [b]Age of control population at the time of diagnosis for the matched case. [c]*P* value obtained by χ^2 (cases *vs.* control group).

variants of *CLU* may contribute to an individuals' susceptibility to cancer or Alzheimer's disease [21-23]. Recent study indicates that the minor allele carriers of *CLU* rs9331888 were more likely to have breast tumors with regional lymph node metastasis and stages II-IV than the major homozygotes [21]. However, no study has yet looked at the link between SNP polymorphism of *CLU* and the risk of ESCC. Therefore we hypothesize that the *CLU* allele also plays a role in ESCC susceptibility. Thus, in this study, in order to clarify association between *CLU* SNPs rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphisms and ESCC risks, we have performed a hospital-based case-control study on Han Chinese population.

Materials and methods

Subjects

A total of 642 cases of patients with ESCC and 658 healthy controls were qualified for this study. We performed a hospital-based case-control study. All samples had been collected before any kind of therapeutic measures were taken at the Department of Gastroenterology, Affiliated Hospital of Binzhou Medical College, between January 2009 and May 2014. The samples of the patients were collected after the diagnosis had been confirmed by esophageal endoscopy. All patients were submitted to surgery or preoperative chemoradiotherapy. Written informed consent was obtained from all participants. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Binzhou Medical University in accordance with the Declaration of Helsinki (2000). The ESCC patients were staged according to the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis (TNM) staging system [24].

DNA extraction

Genomic DNA from the whole blood was extracted using a QIAamp Blood kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA concentration and purity of each sample were measured by ultraviolet spectrophotometer (Eppendorf, Hamburg, Germany). DNA samples were routinely stored at -20°C.

Genotyping

Analysis of the rs9331888, rs17466684 and rs1532278 SNPs of *CLU* gene was performed using multiplex polymerase chain reaction (PCR) with an ABI premix (Applied Biosystems, Carlsbad, USA). Primers for PCR and single base extension were designed using the Assay Designer software package (Sequenom, San Diego, CA, USA). In each 25 μl reaction, 1 μl genomic DNA (100 ng/μl) was amplified by 1.25 U Taq DNA polymerase (Takara, Dalian, China) with 2 μl of 2.5 mM dNTPs and 0.5 μl of each primer. The PCR conditions were performed as previously described [25]. PCR products were analyzed on a 3% ethidium bromide added agarose gel, and photographs were taken under ultraviolet light transilluminator. Subsequently, PCR product was sequenced in an ABI PRISM 3100 sequencer using BigDye Terminator v3.1 Cycle Sequencing method (Applied Biosystems, Carlsbad, USA) as recommended by the manufacturer. Candidate SNP regions were detected and typed with the aid of

Table 2. Association between *CLU* gene rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphisms and ESCCs

Genotype	Cases[a], n (%) (N=642)	Controls[a], n (%) (N=658)	*P* value	Crude OR (95% CI)	Adjusted OR (95% CI)[b]
rs9331888 (C>G)					
CC	187 (29.1)	215 (32.7)		1 (Reference)	1 (Reference)
CG	331 (51.6)	346 (52.6)	0.450	0.909 (0.711-1.168)	0.912 (0.862-1.105)
GG	124 (19.3)	97 (14.7)	0.022	1.219 (1.023-1.451)	1.223 (1.078-1.459)
C allele	705 (54.9)	776 (59.0)			
G allele	579 (45.1)	540 (41.0)	0.037	1.089 (1.006-1.175)	
rs17466684 (A>G)					
AA	183 (28.5)	191 (29.0)		1 (Reference)	1 (Reference)
AG	297 (46.3)	306 (46.5)	0.922	1.006 (0.887-1.142)	1.017 (0.924-1.445)
GG	162 (25.2)	161 (24.5)	0.747	1.025 (0.884-1.188)	1.028 (0.897-1.242)
A allele	663 (51.6)	688 (52.3)			
G allele	621 (48.4)	628 (47.7)	0.742	1.013 (0.939-1.093)	
rs1532278 (T>C)					
TT	213 (33.2)	222 (33.7)		1 (Reference)	1 (Reference)
TC	294 (45.8)	297 (45.2)	0.805	1.016 (0.899-1.147)	1.035 (0.946-1.345)
CC	135 (21.0)	139 (21.1)	0.937	1.006 (0.867-1.167)	1.012 (0.986-1.715)
T allele	720 (56.1)	741 (56.3)			
C allele	564 (43.9)	575 (43.7)	0.905	1.005 (0.931-1.085)	

[a]The χ^2 for HWE of *CLU* gene rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphisms in case and control group is 1.09 and 4.94, 3.49 and 3.04, and 3.18 and 4.50 respectively (all *P*>0.05). [b]ORs were adjusted for gender, age (≤45, 45-69 and ≥70 years), smoking status, alcohol consumption and family history (FH) of cancer.

DNA Star Software (DNASTAR, Madison, WI, USA).

Statistical analysis

Statistical calculations were performed using the SPSS Statistics 13.0 for Windows software package (SPSS Inc., Chicago, Ill). Frequency and susceptibility to ESCC associated with each mutation were compared using the χ^2 test. The *P* values obtained were 2-tailed, and the association of significance was assumed to be less than 0.05. Hardy-Weinberg equilibrium (HWE) was checked for the rs9331888, rs17466684 and rs1532278 variants in ESCC and control subjects by Fisher's exact test. *P*>0.05 was considered not to deviate from HWE. The crude and adjusted odds ratio (OR) and the corresponding 95% confidence intervals (CI) were calculated using unconditional multiple logistic regression.

Results

Characteristics of subjects

This study comprised 642 patients and 658 controls. All the cases and controls were selected from the general Han Chinese population of China. **Table 1** shows the main characteristics of case-control populations. The differences in distributions of age, gender, alcohol consumption, smoking habits, and family history (FH) of cancer between case and control groups are not statistically significant. The cases and controls were well matched by age (mean ± SD, 63.18 ± 3.39 years in cases and 63.82 ± 5.27 years in controls) and gender (the same proportion for males and females), which suggests that frequency matching was adequate. The frequency of males was significantly higher, being in accordance with a worldwide estimation for ESCC [1].

CLU gene rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphisms in ESCC

The gene polymorphisms of *CLU* rs9331888, rs17466684 and rs1532278 SNPs were successfully amplified in all of ESCCs and control cases. The genotypic distributions of the three gene polymorphisms in cases and controls were in HWE (all *P*>0.05) (**Table 2**). Overall, no statistically significant association was observ-

Table 3. Clinicopathological relevance of *CLU* gene rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphisms in ESCC

Parameters	rs9331888 (C>G)		*P* value	rs17466684 (A>G)		*P* value	rs1532278 (T>C)		*P* value
	CC+CG (%)	GG (%)		AA+AG (%)	GG (%)		TT+TC (%)	CC (%)	
Gender									
Female	149 (23.2)	45 (7.0)	0.101	143 (22.3)	51 (7.9)	0.685	153 (23.8)	41 (6.4)	0.965
Male	369 (57.5)	79 (12.3)		337 (52.5)	111 (17.3)		354 (55.2)	94 (14.6)	
Age									
<55 years	247 (38.5)	61 (9.5)	0.762	234 (36.4)	74 (11.6)	0.499	250 (38.9)	58 (9.0)	0.190
≥55 years	271 (42.2)	63 (9.8)		246 (38.3)	88 (13.7)		257 (40.1)	77 (12.0)	
Size									
≤5 cm	269 (41.9)	47 (7.3)	0.005	237 (36.9)	79 (12.3)	0.893	252 (39.3)	64 (10.0)	0.635
>5 cm	249 (38.8)	77 (12.0)		243 (37.9)	83 (12.9)		255 (39.7)	71 (11.0)	
Differentiation									
Good	141 (22.0)	23 (3.6)	0.009	116 (18.1)	48 (7.5)	0.381	132 (20.6)	32 (5.0)	0.839
Moderate	186 (29.0)	37 (5.8)		169 (26.3)	54 (8.4)		174 (27.1)	49 (7.6)	
Poor	191 (29.6)	64 (10.0)		195 (30.4)	60 (9.3)		201 (31.3)	54 (8.4)	
T stage									
1+2	287 (44.7)	53 (8.3)	0.011	252 (39.3)	88 (13.7)	0.688	269 (41.9)	71 (11.0)	0.923
3+4	231 (36.0)	71 (11.0)		228 (35.5)	74 (11.5)		238 (37.1)	64 (10.0)	
Lymph node metastasis									
Present	285 (44.4)	68 (10.6)	0.971	262 (40.8)	91 (14.2)	0.725	276 (43.0)	77 (12.0)	0.590
Absent	233 (36.3)	56 (8.7)		218 (34.0)	71 (11.0)		231 (36.0)	58 (9.0)	
TNM pathological stage									
I and II	265 (41.3)	50 (7.8)	0.030	239 (37.3)	76 (11.8)	0.526	249 (38.8)	66 (10.3)	0.963
III and IV	253 (39.4)	74 (11.5)		241 (37.5)	86 (13.4)		258 (40.2)	69 (10.7)	

ed in *CLU* SNP rs17466684 and rs1532278. Individuals with *CLU* rs9331888G/G genotype were more susceptible to ESCCs compared to major homozygotes (*P*=0.022, OR=1.219). Moreover, the variant allele frequency G of *CLU* rs9331888 (C>G) was significantly higher in cases than in controls (22.3% versus 20.8%), this result also showed statistically significance (*P*=0.037).

Relationship between CLU gene rs9331888 (C>G), rs17466684 (A>G) and rs1532278 (T>C) polymorphism and known clinicopathological variables

Table 3 shows the association of *CLU* gene rs9331888, rs17466684 and rs1532278 polymorphisms with clinicopathological characteristics, including gender, age at diagnosis, tumor size, differentiation, T stage, lymph node metastasis, and pathological stage of the cancer.

For all three SNPs, the polymorphisms were not related to the gender and age of the patients (*P*>0.05). Our data indicated that *CLU* gene rs9331888 polymorphism may be a susceptible genotype for ESCC development and may increase the risk of ESCC among Han Chinese population. Moreover, rs9331888 (C>G) SNP was observed to be associated with increased risk of having larger tumor, poorly differentiated tumor, advanced T stage and pathological stage (*P*=0.005, *P*=0.009, *P*=0.011 and *P*=0.030, respectively). However, the rs17466684 and rs1532278 polymorphism in *CLU* gene may be not association with ESCC susceptibility.

Although for the *CLU* gene rs9331888 (C>G) SNP, the polymorphism was not related to the presence of lymph nodal metastases, however, the rs9331888 GG genotype was more common in ESCC of higher advanced pathological staging (stages I & II versus III & IV, *P*=0.030).

Discussion

Clusterin is a lipoprotein expressed in most mammalian tissues with higher levels in brain, ovary, testis and liver [26]. It has been suggested to be involved in a variety of physiological processes, such as, ongoing synapse turnover [27], apoptosis [28], cytoprotection at fluid-tissue boundaries, membrane recycling during development and in response to injury and reg-

ulation of complement-mediated membrane attack complex [29, 30]. Previous studies revealed that CLU is associated with tumorigenesis, therapeutic resistance, and poor prognosis in numerous human cancers [15-18, 31]. The use of antisense oligodeoxynucleotide or siRNA targeting the *CLU* gene enhanced apoptosis induced by either radiation or chemotherapeutic agents, further supporting the importance of CLU expression in tumor progression [32-34]. Moreover, He and his colleagues demonstrated that high CLU epithelial expression might be a promising predictor of ESCC resistance to chemoradiotherapy [35].

In one previous study, Abraham and his colleagues undertook a case-control study to analyze associations between *CLU* polymorphisms (rs9331888 C>G, rs17466684 A>G and rs1532278 T>C) and Fuchs' endothelial dystrophy (FED, a degenerative disease of the corneal endothelium) risk [36]. The results indicated that no statistically significant association was observed in rs17466684 A>G and rs1532278 T>C SNP. Nevertheless, rs9331888 C>G genotype was demonstrated to be associated with susceptibility of Fuchs' endothelial dystrophy. Therefore, authors hypothezsized that *CLU* rs9331888 C>G may affect its secretion and expose corneal endothelial cells to physiological stresses such as aging and apoptosis, which may be important in FED pathogenesis. In our work, we also did not find the association of *CLU* rs9331888 C>G and rs17466684 A>G *SNP* with ESCC susceptibility. In another study, Hong has found that, in the *CLU* rs9331888 SNP, the minor allele G carriers were more likely to have tumors with regional lymph node metastasis (OR 1.52, 95% CI 1.09-2.12) and stages II-IV (OR 1.40, 95% CI 1.05-1.87) in breast cancer [21].

The associated SNP (rs9331888) is located in the five prime untranslated regions (5'-UTR) of *CLU* gene. Thus, this polymorphism does not modify the encoded protein directly. However, 5'-UTR variants may potentially influence the protein expression levels and therefore the disease susceptibility [37]. In Lambert's study, three SNPs (rs2279590, rs11136000, rs933-1888) within *CLU* gene showed statistically significant association with Alzheimer's disease (AD) (OR: 0.86, 0.86 and 1.12, respectively) [38]. In this study, our data for rs9331888 C>G variant showed that the G allele distribution was significantly associated with ESCCs compared with controls (P=0.037). The homozygous GG variant in our study was observed to be significantly associated with increased risk of tumor size, differentiation, T stage, and pathological stage. However, further experimental research is necessary to define the direct functional association between *CLU* rs9331888 polymorphism and the occurrence of ESCC.

In conclusion, this is the first experience suggesting that a *CLU* polymorphism has significant impact on clinical outcome in ESCC. Despite this, our investigation has some limitations that should be pointed out: it is a retrospective study, conducted on a small population. Thus, our exploratory data need to be confirmed by larger prospective independent series in order to overcome possible bias inherent to retrospective evaluations. In particular, to explore the predictive value of this SNP, an adequately powered prospective randomized trial should be carried out.

Disclosure of conflict of interest

None.

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