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Polymorphism in heme oxygenase-1 (HO-1) promoter and alcohol are related to the risk of esophageal squamous cell carcinoma on Chinese males

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Chronic alcohol drinking is a strong risk factor for esophageal squamous cell carcinoma (ESCC). In this study, the correlation between the *HO-1* gene promoter polymorphism and alcohol, along with the risk of ESCC on Chinese males, was analyzed.The case-control study was performed in 143 ESCC patients and 264 cancer-free controls. All subjects were males. Allelotypic frequencies of (GT)*n* repeat were examed by PCR-based genotyping and DNA sequencing. The frequencies of L-allele and L-allele carriers (S/L and L/L genotypes) was significantly higher in ESCC patients than in controls ($p = 0.001$ and 0.004), The adjusted ORs for ESCC with S/L and L/L genotypes vs S/S genotype was 2.212 (95% CI 1.297-3.775, $p = 0.004$). The adjusted ORs for light, moderate and heavy drinking was 1.467, 5.215 and 9.525 respectively among L-allele carriers (S/L and L/L genotypes) and 1.389, 2.096 and 3.039 respectively for the S/S genotype. Length of a (GT)*n* repeat in the *HO-1* gene promoter may be associated with the development of ESCC in Chinese male drinkers. Reducing alcohol intake might be most protective among L-allele carriers of this polymorphism.

Keywords: esophageal squamous cell carcinoma; heme oxygenase-1 promoter polymorphism; alcohol drinking

Esophageal cancer is the sixth most common cause of cancer death among men on worldwide and the fourth incident and the fourth most common cause of cancer death among men in China [1]. The risk of esophageal cancer and its histology vary widely among geographic areas. In China, Japan and other East Asian countries, more than 90% of cases are squamous cell carcinoma. Meanwhile, China is a large country with great powers in production and consumption of liquor.

The epidemiological evidences shown that chronic alcohol consumption is a strong risk factor for cancer in the upper aerodigestive tract [2, 3]. In an epidemiological study of the American Cancer Society (ACS) on more than 750000 individuals, Bofetta and Garfinkel (1990) [4] found an increased risk for esophageal cancer already at a dose of 12 g alcohol daily (RR = 1.37) rising to an RR of 5.8 following 72 g alcohol daily. A follow-up study of the ACS came to the same conclusions [5]. Although many theories abound to explain the alcohol- esophageal cancer connection, alcohol metabolism is emerging as one of the main culprits. Ethanol metabolism is directly involved in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). These formed an environment favorable to oxidatives stress [6], thus acquired suitable conditions for the development of pathologies directly related with oxidative stress such as cancer, or the alcoholic liver disease [7].

Heme oxygenase (HO) is a rate-limited enzyme that degrades heme to produce biliverdin, carbon monoxide and free iron [8]. Three kinds of HO isoforms have been identified in human. *Heme oxygenase-1* (*HO-1*), the inducible isoforms of heme oxygenase, provides cellular protection against heme- and non-heme-mediated oxidant injury [9, 10]. An exogenous administration of *HO-1* in the rat lung by gene transfer was shown to protect against injury caused by hypoxia [11]. High *HO-1* expression levels have been detected in malignant tumors [12, 13]. Meanwhile, higher resistance to apoptosis was shown in gastric cancer cells with elevated *HO-1* [14]. *HO-1* gene, which encodes an oxidative response protein, plays a role in cytoprotection. A (GT)*n* dinucleotide repeat in *HO-1* promoter is polymorphic and modulates the transcriptional activity of the gene. A *HO-1* gene promoter polymorphism was

Abbreviations: CI, confidence interval; ESCC, esophageal squamous cell carcinoma; HO, heme oxygenase; ROS, reactive oxygen species; OR, odds ratio

reported to be associated with the risk of lung adenocarcinoma, especially in male smokers [15], oral squamous cancer [16] and gastric adenocarcinoma [17].

But the association between *HO-1* promoter polymorphism and ESCC risk has not been established, especially in Chinese male alcohol drinkers. So in the present study, we examined the contribution of (GT)*n* repeat in the *HO-1* gene in the development of ESCC in Chinese male drinkers, by reason that there was association between ROS induced by alcohol and the pathogenesis of ESCC. We screened allelic frequencies of the (GT)*n* repeats in the *HO-1* gene promoter and examined the association between the development of esophageal squamous cell carcinoma in those alcohol drinkers and length of the (GT)*n* repeats. Here we expect to find a biological marker of ESCC susceptibility by detecting the *HO-1* promoter polymorphism to predict the ESCC risk, furthermore, to establish a foundation for gene prediction and diagnosis of ESCC in very early stage.

Materials and methods

Samples. From April 2006 to October 2007, a total of 143 patients with ESCC in our institute were enrolled in this study. Cases were newly diagnosed as having esophageal cancers. A control group was formed with 263 subjects who presented for physical checkups and had no previous operation history. Those with autoimmune disorders, blood diseases and previous malignancies were excluded from the control group. All cases met the following inclusion criteria: (a) ages between 40 and 79 years and (b) Chinese males, and if they were drinkers, they were supposed to drink once a week. The study was approved by an Ethics reviewing Committee, and informed consent was obtained from each subject.

Questionnaires. Face to face interviews were conducted in-hospital for all participants by trained interviewers, using a structured questionnaire. Information was collected on drinking, smoking, other lifestyle factors and medical history; those with ESCC were instructed to report on their habits before they got sick. Weekly alcohol intake was converted into the number of units per week by dividing the total ethanol consumption in grams by 22 g per unit (1 u = 22 g) [18]. The subjects were classified as never/rare drinkers, ex-drinkers, or current drinkers who consumed 1-8.9 units/week (light drinkers), 9-17.9 units/week (moderate drinkers), or 18+ units/week (heavy drinkers) [19]. Smoking status was classified as smoker, ex-smoker or never smoker and the level of exposure was expressed in pack-years.

Genomic DNA was extracted from leukocytes using the Blood Genemic DNA purification Kit (Gentra CO., Minneapolis, Minnesota, USA) by conventional procedures.

Heme oxygenase-1 genotyping. HO-1 (GT)*n* repeat length polymorphism was determined by PCR-based genotyping. The 5'-flanking region containing (GT)*n* repeats of the HO-1 gene was amplified by PCR with a FAM(5'-carboxyl-fluorescein)-labeled sense primer, 5'- AGAGCCTGCAGCTTCTCAGA-3'; an unlabeled antisense primer 5'-ACAAAGTCTGGCCATAG-3' [20]. The amplification reaction mixture (25 μl) contained 100 ng genomic DNA, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer, 2 U Prozyme DNA polymerase (ABI Enterprise, California, USA) and 1×PCR buffer. The PCR reaction was carried out in three steps: firstly, 5 min at 94˚C; then 30 cycles of 30 sec at 94˚C, 45 sec at 56˚C and 45 sec at 72˚C; lastly, 10 min at 72˚C. 1 μL of the PCR product was mixed with 9 μL of HiDi-Formamide and 0.2 μL of the Genscan 500 LIZ size standard in 96-well plates. After a denaturation and cooling step, the fragments were analyzed on the ABI3100 sequencing system (Applied Biosystems). Each repeat number was calculated with 2 cloned alleles as size markers. For data analysis, we applied GeneMapper version 3.5 software (Applied Biosystems).

Statistical analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Accordance with the Hardy-Weinberg equilibrium, which indicates an absence of discrepancies between genotype and allele frequencies, was checked for controls with the χ^2 test. The values for age are reported as mean±SD. Statistical analysis of age was performed by the unpaired *t*-test (Table 1). We used χ^2 statistics to examine the differences in smoking history and alcohol history, Odds ratio (ORs) and their 95% confidence intervals (CIs) were calculated to assess the relative risk conferred by particular *HO-1* (GT)*n* allele and genotype, and adjusted for age, smoking history and alcohol drinking using unconditonal logistic regression analyses (Table 2). We also used unconditional logistic regression analysis to examine the risk of ESCC for L-allele carriers in Chinese male drinkers. Differences between the variants were considered significant when $p < 0.05$.

Table 1 Characteristics of cases and controls

Characteristic	Cases (%) (n=143)	Controls (%) (n=264)	*P* value
age (years)			
40~49	15 (10.5)	49 (18.6)	
50~59	50 (35.0)	103 (39.0)	0.042
60~69	46 (32.2)	74 (28.0)	
70~79	32 (22.3)	38 (14.4)	
Mean±SD	61.27±10.42	58.05±10.00	0.002
Drinking			
Never/rare	9 (6.3)	49 (18.6)	
Light	30 (21.0)	107 (40.5)	
Moderate	44 (30.8)	59 (22.3)	0.000
Heavy	52 (36.4)	40 (15.2)	
Ex-drinker	8 (5.6)	9 (3.4)	
Smoking (pack-years)			
Never/rare	22 (15.4)	119 (45.1)	
Former	27 (18.9)	28 (10.6)	
Current	94 (67.8)	117 (44.3)	0.000
≤50	36 (25.2)	60 (22.7)	
> 50	58 (40.6)	57 (21.6)	

Table 2 *HO-1* **(GT)$_n$ allele and genotype frequencies distribution in cases and controls**

	Allele		Genotype			
	S (%)	L (%)	S/S (%)	S/L (%)	L/L (%)	S/L and L/L (%)
Cases (n=143)	127(44.4)	159(55.6)	29 (20.3)	69(48.3)	45(31.4)	114(79.7)
Controls(n=264)	297(56.3)	231(43.7)	90(34.1)	117(44.3)	57(21.6)	174(65.9)
Crude OR	1.00	1.61	1.00	1.83	2.45	2.03
95% CI	Reference	1.21-2.15	Reference	1.10-3.06	1.38-4.34	1.26-3.29
P value	0.001		0.008	0.021	0.002	0.004
Adjusted ORa			1.00	2.04	2.58	2.21
95%CI			Reference	1.16-3.60	1.35-4.91	1.30-3.78
P value			0.010	0.014	0.004	0.004

[a]ORs adjusted for age, smoking status and alcohol consumption

Results

Table 1 summarized subject characteristics. The esophageal cancer patients were slightly older than the cancer-free control subjects, the mean ages of cases and controls were 61.27±10.42 and 58.05±10.00 years old respectively ($p = 0.042$). Although the age of control group did not match the age of the patient group, no significant age-related effect of *HO-1* promoter polymorphism was found after analyses in our control group. After adjustment for age, we oberserved that alcohol was more frequently and heavily consumed by cases ($p <0.001$) (30.8% for cases and 22.3% for controls in moderate drinking; 36.4% for cases and 15.2% for controls in heavy drinking). Heavy smokers (>50 pack-years) constituted 40.6% of cases and 21.6% of the controls ($p<0.001$).

The numbers of (GT)n repeats in *HO-1* gene were distributed between 12 and 40 in the subjects studied (Fig. 1). The distribution of the numbers of (GT)n repeats was bimodal, with two main peak located at 23 and 30 GT repeats. Therefore, we divided the alleles into two subclasses, as previously reported [21–25]: class S (<25 repeats) and class L (≥ 25).

As shown in table 2, distributions of 286 alleles were 127 (44.4%) for class S and 159 (55.6%) for class L in the ESCC patients group, respectively. Likewise, distribution of 528 alleles were 297 (56.3%) for class S and 231 (43.7%) for class L in the control subjects, respectively. The allele frequency revealed that a higher frequency of L-allele and a lower frequency of S-allele occurred in ESCC patients than in controls (OR = 1.61, 95% CI 1.21-2.15, $p = 0.001$).

The genotype distribution among cases and controls were in accordance with the Hardy-Weinberg equilibrium law. The genotype distribution among cases was: S/S, 20.3%; S/L, 48.3%; L/L, 31.4%. The genotype distribution among controls was: S/S, 34.1%; S/L, 44.3%; L/L, 21.6%. The genotypic distribution revealed a higher frequency of L-allele carriers (S/L and L/L) in ESCC patients than in controls (OR = 2.03, 95% CI 1.26-3.29, $p = 0.004$). The age, smoking status and alcohol consumption adjusted OR was 2.21 (95% CI 1.30-3.78, $p = 0.004$).

After adjusting for age and smoking status, we estimated the risk for esophageal cancer in five drinking categories (never/rare, ex-drinkers, light, moderate, and heavy) by *HO-1* (GT)n genotype (Table 3). The ORs for L-allele carriers (S/L and L/L genotype) compared with S/S genotype in heavy and moderate drinkers was 3.44 (95% CI 1.36-8.70, $p = 0.009$) and 3.06 (95% CI 1.25-7.50, $p = 0.014$) respectively in ESCC patients than in controls. When subjects were analyzed according to alcohol consumption, the adjusted ORs for S/L and L/L compared with S/S were higher for heavy and moderate drinkers (OR in heavy drinkers for S/L and L/L 3.24, 95% CI 1.14-9.23, $p = 0.028$; and OR in moderate drinkers for S/L and L/L 3.53, 95% CI 1.28-9.73, $p = 0.015$) than light/never/ex-drinkers (OR 1.11, 95% CI 0.42-2.94, $p = 0.827$/ OR 1.15, 95% CI 0.15-8.60, $p = 0.892$/ OR NC.

Table 4 gave the ORs for lifestyle factors according to *HO-1* (GT)n genotype. After adjustments for age, smoking status, we

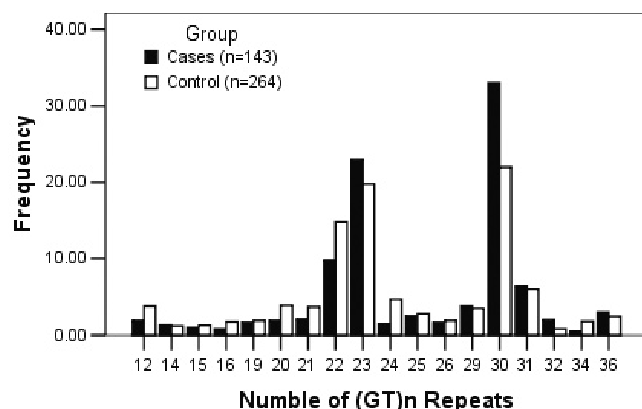

Figure 1 Allelic distribution of HO-1 (GT)n polymorphism in cases and controls

Table 3 Adjusted odds ratios and 95% CI for *HO-1* (GT)*n* L allele carriers relative to the S/S genotype according to alcohol consumption

Subjects	Genotype	Cases(%) (n=143)	Controls(%) (n=264)	Crude OR (95% CI)	*P* value	Adjusted OR[a] (95% CI)	*P* value
Heavy drinker							
	S/S	10(7.0)	18(6.8)	1.00	0.025	1.00	0.088
	S/L	24(16.8)	15(5.7)	2.88(1.05-7.88)	0.039	3.15(1.02-9.77)	0.047
	L/L	18(12.6)	7(2.7)	4.63(1.44-14.86)	0.010	3.40 (0.93-12.47)	0.065
	S/L+L/L	42(29.4)	22(8.4)	3.44(1.36-8.70)	0.009	3.24(1.14-9.23)	0.028
Moderate drinker							
	S/S	9(6.3)	26(9.8)	1.00	0.047	1.00	0.039
	S/L	22(15.4)	22(8.3)	2.89(1.11-7.55)	0.031	3.08(1.05-9.03)	0.041
	L/L	13(9.1)	11(4.2)	3.41(1.13-10.30)	0.029	4.68(1.34-16.32)	0.015
	S/L+L/L	35(24.5)	33(12.5)	3.06(1.25-7.50)	0.014	3.53(1.28-9.73)	0.015
Light drinkers							
	S/S	8(5.6)	32(12.1)	1.00	0.555	1.00	0.438
	S/L	13(9.1)	53(20.1)	0.98(0.37-2.63)	0.970	0.89(0.31-2.52)	0.819
	L/L	9(6.3)	22(8.3)	1.64(0.55-4.90)	0.379	1.75(0.54-5.69)	0.355
	S/L+L/L	22(15.4)	75(28.4)	1.17 (0.47-2.91)	0.730	1.11(0.42-2.94)	0.827
Neve							
	S/S	2(1.4)	13(4.9)	1.00	0.894	1.00	0.928
	S/L	5(3.5)	23(8.7)	1.41(0.24-8.34)	0.703	1.30(0.16-10.69)	0.809
	L/L	2(1.4)	13(4.9)	1.00(0.12-8.21)	1.000	0.89(0.08-10.40)	0.928
	S/L+L/L	7(4.9)	36(13.6)	1.26(0.23-6.88)	0.786	1.15(0.15-8.60)	0.892
Ex-drinkers							
	S/S	0(0.0)	1(0.4)	NC[b]			
	S/L+L/L	8(5.6)	8(3.0)				

[a]ORs adjusted for age, smoking status and alcohol consumption
[b]NC, not calculated

Table 4 Adjusted ORs and 95% CI for smoking and alcohol consumption according to the *HO-1* (GT)*n* genotype

	Cases (n=143)		Controls (n=264)		Adjusted OR (95% CI)		
	S/S	S/L+L/L	S/S	S/L+L/L	S/S	S/L+L/L	All
Drinking							
Never/rare	2(6.9)	7(6.1)	13(14.4)	36(20.7)	1.00	1.00	1.00
Light-drinking	8(27.6)	22(19.3)	32(35.6)	75(43.1)	1.40(0.25-7.68)	1.47(0.57-3.77)	1.45(0.64-3.31)
Moderate-drinking	9(31.0)	35(30.7)	26(28.9)	33(19.0)	2.10(0.39-11.24)	5.22(2.03-13.41)	3.89(1.72-8.82)
Heavy-drinking	10(34.5)	42(36.8)	18(2.0)	22(12.6)	3.04(0.56-16.59)	9.53(3.63-24.98)	6.70(2.93-15.33)
Ex-drinker	0(0.00)	8(7.0)	1(1.1)	8(46.0)	NC	5.27(1.46-18.99)	4.92(1.48-16.39)
Smoking history							
Non-smoking	4(13.8)	18(15.8)	37(41.1)	82(47.1)	1.00	1.00	1.00
Former	6(20.7)	21(18.4)	8(8.9)	20(11.5)	7.86(1.73-35.73)	4.85(2.17-10.80)	5.41(2.67-10.96)
Current	19(65.5)	75(65.8)	45(50.0)	72(41.4)	3.61(1.12-11.69)	4.63(2.52-8.48)	4.19(2.46-7.14)
Pack years<50	8(27.6)	28(24.6)	21(23.3)	39(22.4)	3.29(0.87-12.40)	3.24(1.60-6.57)	3.17(1.71-5.89)
Pack years≥50	11(37.9)	47(41.2)	24(26.7)	33(19.0)	3.90(1.09-13.87)	6.25(1.00-1.05)	5.24(2.91-9.43)

[a]ORs adjusted for age, smoking status and alcohol consumption

estimated the risk for ESCC in five drinking categories (never/rare, light, moderate, heavy, and ex-drinkers) by *HO-1* (GT)*n* genotype. We used overall never/rare drinkers as the reference category (due to the small numbers of cases, n = 9).The risk for ESCC in light, moderate and heavy drinkers with L-allele carriers (S/L and L/L genotypes) (OR = 1.47, 5.22 and 9.53, respectively) exceeded that risk in those with S/S genotype (OR = 1.39, 2.10 and 3.04, respectively). Thus, in comparison with the S/S genotype group, a significant increased risk for esophageal cancer was associated with L-allele carriers (S/L

and L/L genotypes) in all drinking categories from light to heavy. But the impact of smoking in ESCC patients with the S/L and L/L genotypes did not showed difference than with the S/S genotype.

Discussion

This genetic correlation analysis demonstrated for the first time that the $(GT)_n$ microsatellite polymorphism was correlated with the ESCC development. In our case-control study, we found *HO-1* L allele to have a statistically significant interaction with moderate and heavy drinking with respect to risk of esophageal carcinoma. In addition, we confirmed the increased risk with *HO-1* L allele or L allele carriers and moderate or heavy drinking in ESCC development, and the protective function of the S-(GT)*n* allele which decreased the ESCC risk.

We showed that the proportion of both the L allelic frequencies and the L genotypic frequencies (S/L and L/L) was higher in ESCC patients than in control in Chinese males, but a lower frequencies of S allele and S/S genotype. There was no significant difference in S/L genotype between ESCC patients and controls. Furthermore, in Chinese male drinkers, the proportion of L allele carriers was significantly higher in ESCC patients than that in controls. Although smoking is also a high-risk factor of ESCC, in this research we did not find the interaction between smoking and *HO-1* L phenotype. It was suggested from these findings that the microsatellite polymorphism in the *HO-1* gene promoter may be associated with the development of ESCC in Chinese male alcohol drinkers. Presented study represents the first report on the relationship between *HO-1* promoter microsatellite polymorphism and carcinoma in Chinese people. Our finding, was consistent with those previous reports which focused on the association between susceptibility to the development of lung adenocarcinoma and the *HO-1* gene promoter polymorphism in Japanese male smokers [15] as well as the correlation between polymorphism in heme oxygenase-1 (*HO-1*) promoter and the risk of oral squamous cell carcinoma occurring on male areca chewers in Taiwan [16].

Polymorphism of (GT)*n* repeat allele in *HO-1* promoter is a potent risk factor for many kinds of diseases, such as pulmonary disease, cardiovascular disease, renal transplantation, obstetrics, neurological disease and hematological disorders [26]. Longer (GT)*n* repeats allele in *HO-1* promoter was associated with the lower transcription and expression activity of *HO-1*, which will subsequently increase the incidence of emphysema in smokers [23] and the risks of coronary heart disease in people of high risk categories such as smoking, hyperlipemia, diabetes, hypertension [20, 21]. Although the specific molecular mechanism remains unclear, a large (GT)*n* dinucleotide repeats in the 5'-flanking region of human *HO-1* gene form a purine–pyrimidine alternating sequence, possessing Z-conformation potential [26] which negatively affects transcriptional activity in the genes. As a result, Z-conformation in (GT)*n* repeats on the human *HO-1* gene promoter is identified as a basis for distinct transcriptional activities of *HO-1* promoter with diverse (GT)*n* repeats. All those changes of conformations could possibly interfere some important regulation elements in *HO-1* promoter, such as combination sites for NF-κB, so that influence the activity of *HO-1* promoter [22, 26].

Alcohol abusage emerges as a major risk factor for ESCC, usually with a monotonic and strong dose-response relationship [27–29]. Meanwhile, ethanol is a strong source of oxidation stress and long-term excessive intake would induce imbalance between oxidation and anti-oxidation. In anti-oxidation system of human body, *HO-1* is a stress-responsive protein and a novel protective factor with potent anti-inflammatory, antioxidant and anti-proliferative effects, acting as a critical mechanism for anti-oxidation stress [30, 31]. Therefore depression on expression levels or activities of *HO-1* is probably contributed to the pathology process induced by oxidation stress. An exogenous administration of fluorescein promoter into cells by transient transfectin has been progressed in order to research on the effects of distinct (GT)*n* repeats of *HO-1* promoter on transcription activities. It has been revealed that construction with lengths of < 25 repeats showed an increased *HO-1* basal promoter activity compared to > 25 repeats [21], or increased transcriptional up-regulation in response to various stimuli like H_2O_2 [23]. It has been revealed that a team has established lymphoblastoid cell lines from each subject with different (GT)*n* repeats and examined the *HO-1* mRNA expression, HO enzyme activity, and anti-apoptotic effect against oxidative injury in order to study the biology correlation in Japan [25]. Those results demonstrated firstly that the shorter polymorphism in the *HO-1* gene promoter has a higher regulatory effect on the inductivity of *HO-1* mRNA and HO activity and on the strength of the anti-apoptotic effects of *HO-1* than the higher polymorphism. Current study showed that drinkers with no-L allelotype had higher susceptibility to oxidative injury compared to drinkers with L allelotype. Higher (GT)*n* repeats length polymorphisms may be associated with an inhibitive effect on anti-oxidation through depressing *HO-1* expressions, and might subsequently induce esophageal squamous cell carcinoma involvement.

Of course, there were still some limitations in our research. In current study, we emphasized that *HO-1* played a solo role in development of tumor, however the tumor progression is a result of multiple factors, for example the *MTHFR C677T* and SHMT(1) C1420T polymorphism had the interaction with the incident and prevalent esophageal cancer cases [32]. And whether the $(GT)_n$ microsatellite polymorphism was correlated with ESCC genesis by modulating the individual *HO-1* expression level either, the $(GT)_n$ microsatellite polymorphism was connected with the T(-413)A SNP, so which one here played the dominant role in modulating the *HO-1* function? We have already been working on these questions, and the answer is still pending.

In summary, the presented study represent firdt reporty demonstrating that the microsatellite polymorphism in the *HO-1* gene is associated with the development of ESCC in Chinese male drinkers. The risk of ESCC for L allele carriers vs non-L allele carriers was much higher on Chinese male drinkers. The results showed that the $(GT)_n$ microsatellite polymorphism can serve as a novel genetic marker of ESCC, which may have guided significance for ESCC clinical prevention and treatment. Of course, these meaningful conclusions still need the confirmation of correlation research from different genetic backgrounds or prospective correlation research.

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