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Epistasis of oxidative stress-related enzyme genes on modulating the risks in oral cavity cancer

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ABSTRACT

Background: The aim of this study was to evaluate the risks of the polymorphisms of oxidant stress-related enzymes on patients with oral cavity cancer by genotyping of manganese superoxide dismutase (*MnSOD* [1183 T>C]), myeloperoxidase (*MPO* [−463G>A]), catalase (*CAT* [−15A>T]) and glutathione peroxidases 1 (*GPx1* [Pro198Leu]).
Methods: A case–control study was conducted on 122 biopsy-proven oral cavity cancer patients with, at least, one of the past habits of cigarette smoking, alcohol drinking or betel-quid chewing, and 122 approximately age- and habit-matched controls.
Results: The independent risks of the polymorphisms for each enzyme on carcinogenicity were non-significant. The 2-order gene–gene interactions of the polymorphisms, assessed by using a logistic regression model, on risk did not show significant changes, neither. However, the epistasis, assessed by multifactor dimensionality reduction (MDR) for three-order (*CAT*, *MnSOD*, and *MPO*) and four-order was significant. Additionally, the fact that the levels of O_2^-, GSSG and total GSH in the patients were significantly different according to certain genotypes which revealed that the polymorphisms of these enzymes could affect these parameters to some extent.
Conclusions: The results suggested that the genetic-effects of the polymorphisms of these enzymes could slightly modify the risk in oral cavity cancer development individually, but significantly when they functioned together.

1. Introduction

Oral cavity cancer including oral, oro-pharyngeal, and hypo-pharyngeal cancer is a prevalent cancer in South-East Asia area, and it is one of the 10 most common cancers worldwide [1]. Cigarette smoking and alcohol drinking as major risk factors for development of these cancers had been widely established in Western countries [2,3]. However, studies from Taiwan, Pakistan, India and other Far Eastern countries consistently show that betel-quid chewing, along with cigarette smoking and alcohol drinking, is the major etiologic factor in these countries [4–6].

Cigarette smoking, alcohol drinking, and betel-quid chewing had been reported to induce oxidative stress or damage in the human body [7–10]. Moreover, such oxidative imbalance resulted from excess reactive oxygen species (ROS) such as superoxide anion radicals played an important role in carcinogenesis [11]. Many previous studies had also revealed oral and laryngeal cancer patients suffered from oxidative stress or damage [12–14]. Nevertheless, in order to lessen the damage of oxidative stress, different antioxidant systems including oxidative stress-related enzymes such as SOD, MPO, CAT, and GPx1 had been developed in the human body.

In humans, MnSOD is the key antioxidant enzyme involved in the detoxification of superoxide radicals, which catalyze the conversion of $O_2^{\cdot -}$ to H_2O_2. In the polymorphism of *MnSOD* (1183 T>C), an valine (CTT) to alanine (GCT) substitution at position-9 in the signal peptide of human MnSOD has been shown to change the structural conformation of the mitochondrial targeting sequence of the enzyme. This substitution may lead to misdirected intracellular trafficking, followed by changes in the MnSOD activity in the mitochondria [15,16]. In the anti-oxidative system of an organism, following the action of MnSOD, the catalase enzyme is an endogenous antioxidant enzyme that neutralizes reactive oxygen species by converting H_2O_2

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into H_2O and O_2. A catalase-15 A>T polymorphism has been identified in the promoter region just proximal to the start site of the human catalase gene, and it is plausible that the endogenous variability associated with this polymorphism plays a role in the host response to oxidative stress. However, H_2O_2, if not neutralized by CAT or GPx1, may contribute to further generation of reactive oxygen species (ROS) by a reaction between H_2O_2 and chloride to generate hypochlorous acid (HCl), a potent oxidizing agent, catalyzed by myeloperoxidase (MPO). The *MPO* (*G-463-A*) A variant allele confers lower transcriptional activation than the G common allele *in vitro* due to the disruption of the binding site [17]. Thus, the A allele presumed association with lower levels of ROS. GPx 1 is the main glutathione peroxidase in the mammalian liver. It has a genetic polymorphism encoded for proline (Pro) or leucine (Leu) at codon 198 of human GPx1. In addition, selenium at concentrations anticipated in the human serum increased the activity of the Pro-GPx1 variant more than the Leu-GPx1 variant, and the less active Leu-GPx1 variant was associated with breast cancer [18].

Although many carcinogens have been identified in cigarettes and betel-quid, oxidative stress induced by cigarette smoking, alcohol drinking, and betel-quid chewing could contribute to the risk on the development of oral cavity cancer. Therefore, we proposed the hypothesis that different polymorphisms of these enzymes could modify the risk in the carcinogenesis of the oral cavity. Since the importance of genetic expression could depend on environmental exposure, we launched this case–control study to evaluate whether the polymorphisms of *MnSOD*, *CAT*, *MPO*, and *GPx1* could influence the risk on those who kept at least one of the habits of cigarette smoking, alcohol drinking, and betel-quid chewing. In addition, we also evaluated the associations between the epistasis (gene–gene interactions), different combinations of polymorphisms of the enzymes mentioned above, and the risk of the development of oral cavity cancer.

2. Materials and methods

2.1. Study subjects

The study was approved by the Ethics Committee of Kaohsiung Medical University Hospital. The study population consisted of a consecutive series of patients with oral cavity cancer, including oral, oro-pharyngeal, and hypo-pharyngeal cancer, and non-cancer control subjects admitted to Kaohsiung Medical University Hospital, Kaohsiung, Taiwan between September 2005 and March 2008. Since most of the cases are males in our country, men who kept the habits of cigarette smoking, alcohol drinking, and betel-quid chewing and were diagnosed as oral cavity cancer with a pathological confirmation and from whom a blood sample was available were selected as cases ($n=122$). Male control subjects ($n=122$) with the same habits and without a present or previous history of any cancer were simultaneously recruited within the same hospital for their annual general health check-ups. In addition, people with benign tumors or other oxidative stress-related disease such as heart disease, diabetes mellitus, etc. were excluded from both groups. Approximately 1% of the cases and 5% of the approached control subjects were excluded from the final study groups because of their refusal to participate, lack of blood collection, or failure to isolate DNA from the blood samples, etc. Informed consent and simple demographic characteristic information including ages and educational levels were obtained from all participants by questionnaires at the time of blood withdrawals. The basic information of the study subjects were listed in Table 1.

2.2. Reagents and chemicals

All the chemicals and reagents used in the study were of analytical grade. GSH and GSSG were obtained from Sigma (St. Louis, MO). Boric acid was obtained from Mallinckrodt Baker, Inc (Phillipsburg NJ).

Table 1
General characteristics of the patients with oral cavity cancer and the controls.

Variables	Patients ($n=122$)	Controls ($n=122$)
Clinical profiles		
Ages (yr, mean ± SD)	53 ± 11	51 ± 4
Cancer sites		
Oral	74 (61%)	
Oro-pharynx	26 (21%)	
Hypo-pharynx	22 (18%)	
Pathological diagnosis		
Squamous cell carcinoma	122 (100%)	
Clinical stages		
Stage I	12 (8.7%)	
Stage II	28 (20.3%)	
Stage III	27 (19.6%)	
Stage IV	42 (30.4%)	
Unknown	13 (21.0%)	

2.3. Sample collection and preparation

Fasting blood samples were collected from the study subjects and then put into heparin tubes for the assay of $O_2^{\cdot-}$ generation immediately. In addition, EDTA blood samples were obtained for the assay of GSH and GSSG. After the separation of plasma, the erythrocytes were washed 3 times with normal saline and aliquots of washed RBC were prepared for the GSH status assay. The genomic DNA was extracted from a buffy coat by using a salting out method.

2.4. Measurement of $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ generation was measured by lucigenin-based chemiluminescence (LBCL). Briefly, 0.5 ml of whole blood was mixed with 1 ml of 2 mmol//l lucigenin solution. After being gently mixed, the reaction mixture was put in the flat-bottom reaction cuvette into the appropriate position in a black-box unit of the ultraweak CL analyzer. The ultraweak photon was measured by using a BJL ultraweak CL analyzer with a high-sensitivity detector (3.3×10^{-15} W/(cm^2 count) from Jye Horn Co. (Taipei, Taiwan). Photon emission from leukocytes was counted at 10 s intervals at 37 °C for a total of 600 s under an atmospheric condition. Numbers of leucocytes were counted by an auto-hemacytometer (Beckman Coulter, Miami FL).

2.5. GSH status analysis

GSH status analysis was assayed according to the method of Lin et al. [19] Briefly, 100 μl aliquots of washed RBC were added to 300 μl ice-cold 5% metaphosphoric acid (MPA). To precipitate proteins completely, the samples were vortexed and incubated on ice for 10 min. After centrifugation by 12,000 rpm for 10 min at 4 °C, the supernatants were then filtered through a 0.2-μm filter and diluted 5 times before being injected into the P/ACE-MDQ capillary electrophoresis system (Beckman Coulter, Brea, CA), equipped with a fixed wavelength UV detector. Beckman P/ACE-MDQ software was used for instrument control. Data were quantified on the basis of corrected peak areas with migration time.

2.6. Genotyping of the polymorphisms of MnSOD (1183 T>C), MPO (−463G>A), CAT (−15A>T) and GPx1 (Pro198Leu) genes

Blood samples from 244 subjects were drawn into sterile tubes containing sodium EDTA. Leukocyte DNA was extracted from the buffy coat by using a modified phenol-chloroform extraction method. The obtained DNA was resolved in a Tris-EDTA buffer and

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stored at 4 °C. The *MnSOD (*1183 T>C), *GPx1* (Pro198Leu), and *MPO* (−463G>A) SNPs were determined, respectively, by the real-time polymerase chain reaction (PCR) method (MJ Research PTC-200 Peltier Thermal Cycle, Waltham, MA), while the *CAT* (−15A>T) SNP was analyzed by the PCR-restriction fragment length polymorphism (RFLP) method (MJ Research). The real-time PCR assay consisted of 2 primers for PCR amplification of the sequence of the study and 2 allele specific probes. SNPs, primer and probe were: *MnSOD* (1183 T>C), primers: forward 5′-AGCCTGCGTAGACGGTCC-3′and reverse 5′-TCGGGGAGGCTGTGCTTC-3′, allele specific probes: forward 5′-6-FAM-AGCCCAGATACCCCAAAACCGGAGCC-TAMRA-3 and reverse 5′-HEX-AGCCCAGATACCCCAAAGCCGGAGCC-TAMRA-3; *GPx1* (Pro198Leu), primers: forward 5′-CCCCTACGCAGGTACAGC-3′ and reverse 5′-ACACCCTCATAGATGAAAAACCC-3′, allele specific probes: forward 5′-HEX-CGCGATCGTCTCAAGGGCCCAGCTGTGCCT-GATCGCG (BHQ1)-3′ and reverse 5′-6-FAM-CGCGATCGTCT-CAAGGGCTCAGCTGTGCCTGATCGCG (BHQ1)-3′; *MPO* (−463G>A), primers: forward 5′-GCTGGTAGTGCTAAATTCAAAGG-3′ and reverse 5′-TAGATACAGGGTTTCACCATGTTG-3′, allele specific probes: forward 5′-CGCGATCAGTGATCCACCCGCCTCAGCCTCGATCGCG-3′ and reverse 5′-CGCGATCAGTGATCCACCTGCCTCAGCCTCGATCGCG-3′. While the PCR-RFLP assay consisted of 2 primers for PCR amplification of the sequence of the study and one restriction enzyme for the assay. SNPs, primer and restriction enzyme were: *CAT* (−15A>T), primers: forward 5′-AATCAGAAGGCAGTCCTCCC-3′ and reverse 5′-TCGGGGAGCACAGAGTGTAC-3′, and using *Hinf* I as the restriction enzyme. The genotypes of the PCR products were confirmed by the DNA sequence analysis. In each experiment, DNA samples from the subjects, together with 2 or 3 previously sequenced DNA samples serving as quality controls (1 for each genotype) to validate genotyping procedures, were concomitantly amplified by the PCR-RFLP.

2.7. Statistical analysis

Data were expressed as mean ± SD. Statistical significance was assessed by using the Student's *t*-test to compare the means. Whereas, the odds ratio (OR) and its 95% confidence interval for each individual and interaction of two enzymatic polymorphisms were estimated by the unconditional logistic regression method. Values of 2-sided $p<0.05$ were considered significant. All these analyses were carried out by using the statistical package SPSS 10.0. In addition, the gene–gene interactions of 2-and-higher order for polymorphisms of different enzymes were also assessed by the multifactor dimensionality reduction method (MDR), proposed by Ritchie et al. [20] and Moore et al. [21], and followed by the odds ratio multifactor dimensionality reduction method (OR MDR method), a refined MDR, described by Chung et al. [22]. Briefly, the MDR method is designed to improve the identification of factors associated with disease risk by reducing the dimensionality of the multifactor information. The method involves several steps: First of all, the data were divided into a training set (consisting of 9/10 of the data) and an independent testing set (consisting of the remaining 1/10 of the data) as part of cross-validation. Secondly, a set of n factors (polymorphisms of enzymes) were selected, where $n=1$, 2, 3, and 4. Next, the "*n*" polymorphisms and their possible multifactor classes were represented in the n dimensional space. The ratio for the number of cases to the number of controls was calculated within each multifactor class. Each multifactor class in the *n* dimensional space was then labeled as "high risk" if the case to control ratio met or exceeded a threshold such as 0.5, or as "low risk" if that threshold was not exceeded, thus reducing the n dimensional space to 1 dimension with 2 levels (low risk and high risk). The model that gave the lowest misclassification error was selected for each set of the n polymorphisms. Finally, a prediction error was estimated for each model selected in the step mentioned above as a cross-validation procedure. The model with a lower prediction error and higher cross-validation is the best model. In the OR MAD procedure, the odds ratios for each combination of the genotypes were calculated. The 95% confidence intervals were constructed by using an empirical distribution of the odds ratios. The MDR and OR MDR methods were performed by free software named MDR 2.0 β6 (http://sourceforge.net/projects/mdr/).

3. Results

3.1. Risks of the polymorphisms of MnSOD (1183 T>C), MPO (−463G>A), CAT (−15A>T), or GPx1 (Pro198Leu) genes

In Table 2, *MnSOD*, *MPO*, *CAT*, and *GPx1* genotypic frequencies and the association between each genotype and the risk of oral cavity cancer were shown. In *MnSOD* (1183 T>C), the C/C genotype was found in 1.7%, the T/C genotype was found in 23.1%, and the T/T genotype was found in 75.2% in the patients. In the control group, frequencies of the genotypes were 1.6% for C/C, 26.2% for T/C and 72.1% for T/T. Their differences were found to be not significant (OR = 0.9 with 95% CI = 0.5–1.5, OR = 1.0 with 95% CI = 0.1–7.0 and OR = 0.9 with 95% CI = 0.5–1.5 for T/C vs. T/T, C/C vs. T/T and T/C + C/C vs. T/T, respectively). In *MPO* (−463G>A), the A/A genotype was found in 2.5%, the T/C genotype was found in 22.1%, and the T/T genotype was found in 75.4% in the patients. In the control group, frequencies of the genotypes were 1.6% for A/A, 22.1% for G/A, and 77.2% for G/G. Their differences were not significant (OR = 1.0 with 95% CI = 0.6–1.9, OR = 1.5 with 95% CI = 0.3–9.3 and OR = 1.0 with 95% CI = 0.6–1.9 for G/A vs. G/G, A/A vs. G/G and G/A + A/A vs. G/G,

Table 2
Frequencies and odds ratios of the genotypic polymorphisms for *MnSOD* (1183 T>C), *MPO* (−463G>A), *CAT* (−15A>T), and *GPx1* (Pro198Leu) genes in the patients with oral cavity cancer and the controls.

Genomic polymorphisms	Patients (n=122)	Controls (*n* = 122)	OR (95% CI)
MnSOD (1183 T>C)			
Genotype frequency, *n* (%)			
T/T	91 (75.2)	88 (72.1)	1
T/C	28 (23.1)	32 (26.2)	0.9 (0.5–1.5)
C/C	2 (1.7)	2 (1.6)	1.0 (0.1–7.0)
T/T vs.T/C + C/C, *n* (%)			
T/T	92 (75.4)	88 (72.1)	1
T/C + C/C	30 (24.6)	34 (27.9)	0.9 (0.5–1.5)
MPO (−463G>A)			
Genotype frequency, *n* (%)			
G/G	92 (75.4)	93 (77.2)	1
G/A	27 (22.1)	27 (22.1)	1.0 (0.6–1.9)
A/A	3 (2.5)	2 (1.6)	1.5 (0.3–9.3)
G/G vs. G/A + A/A, *n* (%)			
G/G	92 (75.4)	93 (76.2)	1
G/A + A/A	30 (24.6)	29 (23.8)	1.0 (0.6–1.9)
CAT (−15A>T)			
Genotype frequency, *n* (%)			
A/A	57 (46.7)	62 (50.8)	1
A/T	55 (45.1)	54 (44.3)	1.1 (0.7–1.9)
T/T	10 (8.2)	6 (4.9)	1.8 (06–5.2)
A/A vs.A/T + T/T, *n* (%)			
A/A	57 (46.7)	62 (50.8)	1
A/T + T/T	65 (53.3)	60 (49.2)	1.2 (0.7–2.0)
GPx1 (Pro198Leu)			
Genotype frequency, *n*(%)			
CC	108 (90.0)	112 (91.8)	1
CT	12 (10.0)	10 (8.2)	1.2 (0.5–3.0)
TT	0	0	

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respectively). In *CAT* (−15G>T), the T/T genotype was found in 8.2%, the A/T genotype was found in 45.1%, and the A/A genotype was found in 46.7% in the patients. In the control group, frequencies of the genotypes were 4.9% for T/T, 44.3% for A/T, and 50.8% for A/A. Their differences were not significant (OR = 1.1 with 95% CI = 0.7–1.9, OR = 1.8 with 95% CI = 0.6–5.2 and OR = 1.2 with 95% CI = 0.7–2.0 for A/T vs. A/A, T/T vs. A/A and A/T + T/T vs. A/A, respectively). In *GPx1* (Pro198Leu), the T/T genotype was not found in both of the patients and the controls of this study; however, frequencies of the C/T and C/C genotype were 10% and 90%, respectively, in the patients, and 8.2% and 91.8%, respectively, in the controls. The difference was not significant, either (OR = 1.2 with 95% CI = 0.5–3.0).

3.2. Gene–gene interactions among the polymorphisms of oxidative stress-related enzymes in the patients with oral cavity cancer

In Table 3, two-order of interactions between these polymorphisms were estimated by using binary logistic regression. All of the odds ratios for the interaction term were around 1 with 95% CI including 1, which indicated there were no significant interactions between the activities of any of these two enzymes in reducing risks regarding the development of oral cavity cancer. Nonetheless, in Table 4, the higher order (>2) of significant interactions could be detected by using the OR MDR method. The best model for the 3-order interaction included *CAT*, *MnSOD*, and *MPO* (OR = 1.72, 95% CI = 1.03–2.88). In addition, the 4-order interaction was also significant (OR = 1.83, 95% CI = 1.10–3.05).

Table 3
Epistasis of the genotypic polymorphisms for the enzymes related to oxidative stress by using binary logistic regression.

Polymorphisms of genes		Patients (n = 122)	Controls (n = 122)	Odds ratio (95% CI)
MnSOD	*MPO*			
TT	GG	68 (56.2)	70 (57.4)	1
C-carrier	GG	23 (19.0)	23 (18.9)	1.0 (0.5–2.0)
TT	A-carrier	23 (19.0)	18 (14.8)	1.3 (0.7–2.7)
C-carrier	A-carrier	7 (5.8)	11 (9.0)	0.7 (0.2–1.8)
MnSOD	*CAT*			
TT	AA	39 (32.2)	45 (37.8)	1
C-carrier	AA	17 (14.0)	16 (13.4)	1.2 (0.5–2.7)
TT	T-carrier	52 (43.0)	41 (34.5)	1.5 (0.8–2.6)
C-carrier	T-carrier	13 (10.7)	17 (14.3)	0.9 (0.4–2.0)
MnSOD	*GPx1*			
TT	CC	81 (65.6)	80 (68.1)	1
C-carrier	CC	27 (26.2)	32 (22.7)	0.8 (0.5–1.5)
TT	T-carrier	8 (6.6)	8 (6.7)	1.0 (0.4–2.8)
C-carrier	T-carrier	3 (2.5)	2 (1.6)	1.5 (0.2–9.1)
MPO	*CAT*			
GG	AA	45 (36.9)	49 (41.2)	1
A-carrier	AA	12 (9.8)	12 (10.1)	1.1 (0.4–2.7)
GG	T-carrier	47 (38.5)	42 (35.3)	1.2 (0.7–2.2)
A-carrier	T-carrier	18 (14.8)	16 (13.4)	1.2 (0.6–2.7)
MPO	*GPx1*			
GG	CC	82 (67.8)	83 (68.0)	1
A-carrier	CC	27 (22.3)	29 (23.8)	0.9 (0.5–1.7)
GG	T-carrier	10 (8.3)	10 (8.2)	1.0 (0.4–2.6)
A-carrier	T-carrier	2 (1.7)	0	–
CAT	*GPx1*			
AA	CC	50 (41.7)	55 (46.2)	1
T-carrier	CC	58 (48.3)	55 (46.2)	1.2 (0.7–2.0)
AA	T-carrier	7 (5.8)	6 (5.0)	1.3 (0.4–4.1)
T-carrier	T-carrier	5 (4.2)	3 (2.5)	1.8 (0.4–8.1)

3.3. Comparisons of the parameters related to the oxidative status in oral cavity cancer patients with different polymorphisms of oxidative stress-related enzymes

Since the frequencies of the mutation type were rare, we combined the mutation/mutation and mutation/wild to increase the sample size and compared the parameters between the patients with the combinations mentioned above and with the wild/wild genotypes. In *MnSOD* (1183 T>C), levels of O_2^- significantly decreased in the C-carrier when compared with those of T/T; on the contrary, the case of levels of GSSG was reverse. The other parameters including levels of GSH and total GSH, and the ratio of GSH to GSSG, were not significantly different. Regarding the *MPO* (−463G>A) polymorphism, only the level of O_2^- was significantly increased in those with the A-carrier when compared with those with G/G. The other parameters were not significantly different. In *CAT* (−15A>T), only the level of total GSH was significantly increased in those with the T-carrier when compared with those with A/A. The other parameters were not significantly different, neither. All of these parameters did not show significant differences between those with CT and with the CC genotype of *GPx1*. The detail results mentioned above were shown in Table 5.

4. Discussion

It is fully recognized that excessive generation of oxygen derived radicals with exhausted antioxidant defense systems can cause oxidative damages [23]. A growing body of evidence has indicated that these disproportionate oxidative damages were involved in carcinogenesis [11,24,25]. With the production of oxygen species and over-production of oxygen species, higher rates of lipid peroxidation have also been found in some neoplastic lesions or in cancer patients' blood [12–14,26]. However, it is a real challenge to provide "direct" evidence to confirm that continuing excess oxidative stress or damages involve carcinogenicity in the human body. In other words, it is hardly possible to follow a cohort who are suffering from oxidative stress and observe their eventual development of cancer or recruit a group of cancer patients and keep trace of the conditions of their previous oxidative stress. Therefore, we utilized the properties of the polymorphisms of oxidative stress-related enzymes genes such as *MnSOD*, *MPO*, *CAT*, and *GPx1*, as surrogates to provide evidence that excessive previous oxidative stress or damage could induce oral cavity cancer, given the facts that the polymorphisms of anti-oxidative enzymes could affect the capacity of eliminating excess free radicals generated by environmental factors such as cigarette smoking, alcohol consumption, or betel-quid chewing. In reality, it is meaningless to investigate the effect of genetic factors, which would interact with environmental factors on carcinogenicity, without any environmental exposures such as polymorphisms of metabolic enzymes or, in this study, polymorphisms of oxidative stress-related enzymes. Therefore, the oral cavity cancer cases with previous habits of cigarette smoking, alcohol

Table 4
Epistasis of the genotypic polymorphisms for the enzymes related to oxidative stress by using multifactor dimensionality reduction (MDR).

Variables	Balanced accuracy[a]	Odds Ratio (95% CI)	Kappa[b]	F-measure[c]
CAT	0.5246	1.22 (0.74–2.01)	0.05	0.53
CAT, MnSOD	0.5451	1.44 (0.87–2.38)	0.09	0.56
CAT, MnSOD, MPO	0.5656	1.72* (1.03–2.88)	0.13	0.60
CAT, MnSOD1, MPO, GPx1	0.5738	1.83* (1.10–3.05)	0.15	0.60

[a] (Sensitivity + Specificity)/2.
[b] A function of total accuracy and random accuracy.
[c] A function of sensitivity and precision.
* p<0.05.

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Table 5
Comparisons of oxidative parameters in oral cavity patients with different genotypes of *MnSOD* (1183 T>C), *MPO* (−463G>A), *CAT* (−15A>T), and *GPx1* (Pro198Leu).

Polymorphism	$O_2\cdot^-$ (counts/ 10 s×10^3WBC)	GSH (μmol/L)	GSSG (μmol/L)	GSH/GSSG	\Total GSH
MnSOD (T1183C)					
TT	351±332	866±232	136±60	7.68±4.23	1139±259
C-carrier	303±161*	886±278	170±93*	6.76±3.77	1126±256
MPO(G-463A)					
GG	298±170	886±229	144±74	7.59±4.26	1154±249
A-carrier	365±313*	884±283	147±61	7.08±3.77	1177±293
CAT (A-15 T)					
AA	320±228	842±224	151±79	7.01±3.73	1144±230
T-carrier	311±204	895±257	139±61	7.85±4.44	1173±284*
GPx1 (Pro198Leu)					
CC	319±221	872±245	146±71	7.25±3.63	1163±267
CT	303±170	810±208	144±68	7.89±6.41	1097±190

Values are expressed as mean±S.D.
*$p<0.05$, comparisons of different genotypes by using the *t*-test.

consumption, or betel-quid chewing were enlisted and we compared them with the controls with the same habits. The utilization of gene polymorphisms as risk markers on cancer or disease development with a case–control study design had been adopted by many previous studies [27,28]. Under this circumstance, the accuracy of gene polymorphism frequencies in a control group is very important before drawing any concrete conclusion. Because the publication in regard to the same population frequencies of SNPs which was adopted by us in this study is relatively scarce, it is hard to evaluate the deviation of gene polymorphism frequencies of our study away from those of the population we recruited. However, fortunately, the gene polymorphism frequencies in this study did not seriously violate the Hardy–Weinberg equilibrium.

Nevertheless, according to this study, the sole polymorphisms of oxidative related enzymes did not show significant protective effects on reducing the risk on the development of oral cavity cancer. There are several reasons to explain these situations. Firstly, too many carcinogens comprised in cigarettes or betel-quid which could trigger carcinogenesis other than excessive free radicals generated by cigarettes or betel-quid. Thus, the harm of the environmental factors could not be eliminated by the protection of the anti-oxidative enzymes. According to a previous study [4], the risk for combinations of cigarette smoking, alcohol consumption, and betel-quid chewing on oral cancer could be 123-fold higher in users than in non-users. It seemed that investigating genetic-effects with huge impact of environmental exposure could be inappropriate, especially, when the environmental effects, i.e. cigarette smoking, alcohol consumption, or betel-quid chewing in this study, outweigh much more than the genetic effect. Secondly, the environmental factors overtake genetic factors by generating too many free radicals and could not be compensated by any of these anti-oxidative enzymes solely. The balance of the free radical scavenging system is very complicated in the human body, in which antioxidant and oxidant enzymes can only play their designated roles.

Owing to the network of the free radical scavenging system, the concept of interaction between these antioxidant and oxidant enzymes had been proposed in investigating the prognosis of breast cancer patients [29]. Nevertheless, the rarities of mutant alleles make it nearly impossible to estimate higher order interaction by using traditional statistical methods, such as the logistical regression model. With the advancement in genetic techniques and knowledge, the importance of higher order interaction has been much more emphasized than before, which in turn pushes the development of new tools to meet this demand. The MDR method, first described by Ritchie et al. [20], was a nonparametric and genetic model-free data mining alternative to logistic regression for detecting and characterizing nonlinear interactions among discrete genetic attributes, which combines attribute selection, attribute construction, and classification with cross-validation and permutation testing to provide a comprehensive and powerful approach to detecting nonlinear interactions. It had been successfully applied to investigate a variety of diseases or clinical traits, such as asthma, cardiovascular disorder, even bladder cancer and their related genetic loci [20,30,31]. According to the analyses by using this new tool, we have revealed that certain combinations of the polymorphisms of these antioxidant and oxidant enzymes are related to the risks in the development of oral cavity cancer. This evidence could be strengthened by the analyses in Table 5, which showed that certain oxidative stress or status could be shaped by the polymorphisms of enzymes mentioned above. Therefore, even though the defense effect of any single antioxidant and oxidant enzyme activities is trivial, the joint effect could be significant on reducing risk of the development of oral cavity cancer.

Nevertheless, in Table 5, we just assessed the oxidative and GSH statuses in the cases, but not in the controls. The reason is that even though the cases used to be smokers, alcohol consumers, or betel-quid chewers, they had abstained from these habits according to their physicians' advice after diagnosis. The controls kept their habits that could affect the index we wanted to assess. Therefore, we just kept the blood of the cases for further assessment and presented these data in Table 5. It is worthwhile mentioning that the parameters related to oxidative stress in Table 5 were not highly correlated with each other. The reason could be the redox status is very complicated in the human body, affected by many factors which are often inextricably linked and, as a result, it is hard to judge the true meaning of the balance of the redox system by one of these parameters separately. Additionally, there remained a limitation in the assessments of the oxidative statuses and enzyme activities. Dietary differences have been reported to influence a person's oxidative status [32–34]. Even though we did not document any dietary information from all of the study subjects, the patients with oral cavity cancer lived in southern Taiwan, which is a relatively homogenous environment. Therefore, differences in dietary intake may be safely ignored.

In summary, in the present study, the changes of the levels of O_2^- and the activities of anti-oxidative enzymes according to certain genetic polymorphisms of oxidative stress-related enzymes in patients with oral cavity cancer were consistent with the decreasing/increasing risks by certain combinations of genetic polymorphisms regarding antioxidant and oxidant enzymes in the development of oral cavity cancer. We believe that genetic variations in the ROS defense genes and ROS producing genes mentioned above could modify the risks on oral cavity cancer brought out by cigarette smoking, alcohol consumption or betel-quid chewing, even though slightly, and infer that free radicals were involved in the development of this cancer to some extent.

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