Cellular and Molecular Biology

Coskun *et al. Cell. Mol. Biol.*2016, 62 (5): 25-30
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.5.5

Original Research

Are there possible associations between MnSOD and GPx1 gene variants for laryngeal cancer risk or disease progression?

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Abstract: Laryngeal squamous cell carcinoma (LSCC) is a multifaceted and genomically complex disease and cellular and preclinical studies have demystified wide ranging molecular mechanisms which underpin its development and progression and resistance against wide ranging molecular therapeutics. Oxidative stress is a widely studied molecular mechanism and reportedly involved in carcinogenesis. Increasingly it is being realized that accumulation of Reactive Oxygen Species (ROS) activates defensive mechanism to counteract oxidative stress induced damage. Manganese superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) are important members of defensive machinery. We investigated whether the polymorphisms of MnSOD (Ala-9Val, rs4880) and GPx1 (Pro^{198}Leu, rs1050450) are associated with LSCC and also evaluated possible interactions between these polymorphisms and various lifestyle factors or pathological features of patients. For this purpose, 67 LSCC patients and 73 healty controls were enrolled. Molecular assessment of MnSOD and GPx1 variants were determined with polymerase chain reaction-restriction fragment length polymorphism techniques. We found that the frequency of both heterozygous PL genotype and P allele was considerably higher in patients with advanced tumor stage (T3/T4) than in those with early tumor stage (T1/T2) (OR= 5.106; 95% CI=1.372-19.004; p<0.001, OR=5.787; 95% CI =1.564-21.414; p<0.001 respectively). Although the frequency of ValVal/LL combine genotype was significantly decreased (OR=0.204, 95% CI=0.055-0.760; p=0.021), the frequency of ValAla/PL combine genotypes was higher in patients with stage T3/T4 than in those patients with stage T1/T2 (p=0.027). Consequently, we have concluded that variants of GPx1 and MnSOD should not be considered as a risk factor of LSCC, only may be accepted as a prognostic markers. Use of new technologies such as metabolomics and deep DNA sequencing will prove to be helpful in developing a deeper knowledge related to how cancer cell metabolism adapts and provides a buffer against increased oxidative stress.

Key words: Larynx cancer, genotype, MnSOD, GPx1, polymorphism.

Introduction

Laryngeal cancer is one of the most common malignancies among head and neck squamous cell carcinoma (1) and accounts for about 1% to 2.5% of all human cancers (2) and, approximately 85 to 90 % of malignant neoplasms of the larynx are squamous cell carcinomas (3). Data obtained through high-throughput technologies has considerably improved our understanding of the relationships between redox homeostasis, oxidative stress and activation of proliferation and survival pathways in laryngeal cancer. It is now clear that imbalance between ROS generation and elimination resulted in accumulation of ROS. Considerably higher levels of ROS also accumulate in cancer cells as a result of dysregulation of different intracellular signaling cascades that influence metabolism of the cells.

It is exciting to note that polymorphism data analysis has become an essential complementary tool to classical genetic analyses. Technological advancements in genomics and the clinical implementation of genome-wide assays have significantly enhanced our knowledge related to mechanisms underlying cancer development and progression and the contribution of structural variation to disease burden has evolved rapidly.

Therefore, genetic and environmental factors are contributory in larynx cancer development (4) and experimentally verified data has started to shed light on role of some single nucleotide polymorphisms (SNPs) as risk factors in cancer development (5, 6). Glutathione peroxidase (GPx) and Manganese Superoxide Dismutase (MnSOD) are important members of defensive machinery (4). There are mainly three forms of superoxide dismutase (SOD), i.e., cytosolic copper/zinc SOD (CuZnSOD, SOD1), mitochondrial MnSOD (SOD2) and extracelluler CuZnSOD (SOD3). MnSOD modulates conversion of superoxide anion into catalase (H_2O_2) because mitochondrion is major site for ROS production. After that, H_2O_2 is neutralized to water (H_2O) and oxygen (O_2) by GPx. (7, 8). GPx has four isozymes named as from GPx1 to GPx4. Even though all isotypes have similar function and substrat specifity, GPx1 is a selenium dependent enzyme that more efficiently converts H_2O_2 into H_2O and O_2 in particular (9, 10, 11, 12). It has previously been reported that polymorphisms of GPx1 and MnSOD were associated with

Received, March 23 2016; **Accepted**, April 12 2016; **Published** April 30 2016

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the susceptibility of cancer development in a number of molecular epidemiological studies but the results still remain controversial (13,14,15). In our study, we investigated whether the polymorphisms in MnSOD Ala-9Val (at codon -9 in exon 2, rs4880) and GPx1 C>T (Pro[198]Leu, rs1050450) present in 2[nd] exon of GPx1 gene were associated with larynx squamous cell carcinoma (LSCC) and also evaluated possible interactions between the polymorphisms and various lifestyle factors, or pathological features of patients with LSCC.

Materials and Methods

Study paricipants

Study groups consisted of 67 patients with LSCC (mean age 65.87 ± 8.64 years) and 73 control subjects (mean age 58.24 ±10.93 years) in current study. All participants provided written informed consent prior to study. Patients that were confirmed the diagnosis and cancer status by standardized questionnaire, pathological records and medical records were obtained from Otorhinolaryngology Clinic of Haydarpasa Numune Training and Research Hospital. Subjects in control group of which healty condition confirmed through face to face meeting was formed from hospital staff. They did not use smoking and not taking any regular medication and alcohol. The study was approved by the Medical Ethics Committee of Istanbul Medical Faculty.

Polymerase chain reaction (PCR)-based detection of MnSOD Ala-9Val (rs4880) and GPx1 Pro[198]Leu (rs 1050450) genotypes

Blood specimens were collected in tubes containing ethylenediaminetetraacetic acid (EDTA), and genomic DNA was isolated from leukocyte nuclei by the previously described method based on salting-out procedure (16). The genotyping of GPx1 Pro198Leu and MnSOD Ala-9Val polymorphisms were detected by polymerase chain reaction (PCR) with locus-specific primers and subsequent analysis of a restriction fragment length polymorphism (RFLP) as previously reported. All of the study protocols were performed under sterile conditions. The primers for PCR amplification of the GPx1 Pro198Leu region were forward 5'- AAG GTG TTC CTC CCT CGT AGG T-3' and reverse 5'-CTA CGC AGG TAC AGC CGC CGC T-3' and for MnSOD Ala-9Val region were forward 5'-ACC AGC AGG CAG CTG GCG CCG G-3' and reverse primer 5'-GCG TTG ATG TGA GGT TCC AC-3'. After the amplification the PCR products were digested with the proper restriction endonucleases, ApaI, PdiI (MBI Fermentas, Ontario, Canada) for detection of the genotypes of GPx1 Pro-198Leu and MnSOD Ala-9Val, respectively (17, 18). The digested DNAs were then separated on 2% agarose gel in 1XTris borate EDTA buffer followed by staining with ethidium bromide solution. The genotypes were typed by visualization under ultraviolet light.

Statistical methods

Clinical laboratory data are expressed as mean±SD. Mean values were compared between patients and controls by unpaired Student's t-test. Differences in the distribution of genotypes and alleles between cases and controls were tested using the Chi-square-statistic and Fisher's-exact tests. The Hardy-Weinberg equilibrium was tested for all polymorphisms. Allele frequencies were estimated by gene counting methods. The relative associations between laryngeal cancer patients and controls were assessed by calculating crude Gart's odds ratios (ORs) and 95% confidence intervals (95% CIs). The threshold for significance was $p<0.05$. The SPSS version 21.0 for Windows was used to perform statistical analysis (revision 21.0; SPSS Inc., Armonk, NY, USA).

Results

In the present study, MnSOD and GPX1 genotypes were determined by using PCR-RFLP analysis (Figures 1, 2). Patient and control groups had statistically similar distribution of age (65.87±8.64 years; 58.24 ±10.93 years, respectively, $p>0.05$). It was showed the demographic and clinicopathologic data of patients are shown in Table I. Clinicopathologic characteristics of patients were determined through tumor-node-metastais cancer staging system (19).

We did not found any significant association between LSCC patients and controls regarding the distribution of MnSOD and GPx1 genotypes and alleles ($p>0.05$, for each). (Table 2). The genotype distributions of MnSOD

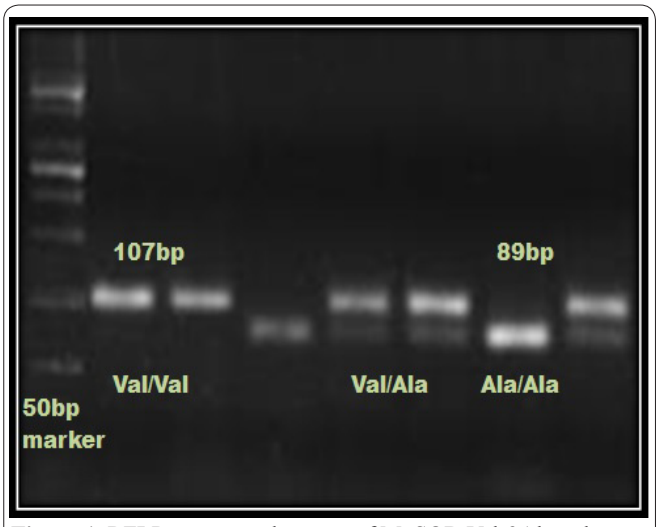

Figure 1. RFLP agarose gel pattern of MnSOD Val-9Ala polymorphism.

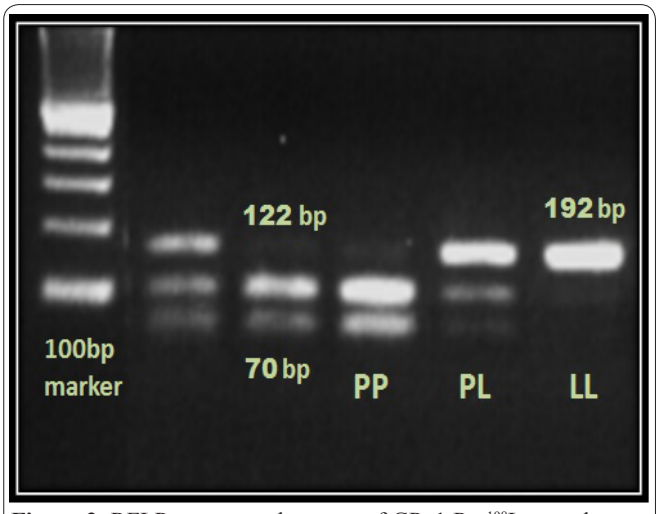

Figure 2. RFLP agarose gel pattern of GPx1 Pro[198]Leu polymorphism.

Table 1. Characteristics of patients with larynx squamous cell carcinoma.

Parameter		Patients with Larynx Cancer
Smoking status n (%)	Ever smokers	44 (65.7)
	Never smokers	23 (34.3)
Alcohol consumption n (%)	Yes	41 (61.2)
	No	26 (38.8)
Reflux n (%)	Yes	29 (43.3)
	No	38 (56.7)
Family history n (%)	Yes	22 (32.8)
	No	45 (67.2)
Tumor location n (%)	Glottic	50 (74.6)
	Supraglottic	17 (25.4)
Tumor stage n (%)	T1	6 (9.5)
	T2	10 (15.9)
	T3	28 (44.4)
	T4	19 (30.2)
Differentiation n (%)	Poor	10 (15.9)
	Medium	44 (69.8)
	Well	9 (14.3)
Perineural invasion n (%)	Yes	12 (19.0)
	No	51 (81.0)
Lymph node n (%)	N0	36 (57.1)
	N1	22 (34.9)
	N2	3 (4.8)
	N3	2 (3.2)
Metastasis n (%)	Yes	3 (4.8)
	No	60 (95.2)
Tumor recurrence n (%)	Yes	6 (9.5)
	No	57 (90.5)

Data presented as n (number of individuals) and, % (percentage of individuals). Some characteristic properties of patients were evaluated on 63 patients because of not achieving pathology reports of 4 patients.

Table 2. Genotype and allele frequencies of the MnSOD Ala-9Val and GPx1 Pro198Leu polymorphisms in study groups.

Genotypes and Alleles	Larynx Cases (n=67) Frequency (%)	Controls Cases (n=73) Frequency (%)	p-value
MnSOD			
Val/Val	20 (29.9)	27 (37.0)	
Val/Ala	29 (43.3)	31(42.5)	p=0.056
Ala/Ala	18 (26.9)	15 (20.5)	
Val Allele	69 (51.5)	85 (58.2)	p=0.258
Ala Allele	65 (48.5)	61 (41.8)	
GPx1			
PP	4 (6.0)	12 (16.4)	
PL	34 (50.7)	36 (49.3)	p=0.128
LL	29 (43.3)	25 (34.2)	
P Allele	42 (31.3)	60 (41.1)	p=0.09
L Allele	92 (68.7)	86 (58.9)	

Abbreviations: Val=Valine, Ala=Alanine, P=Proline, L=Leucine. Chi-square test was used to compare genotypes in the study group. It was accepted statistically meaningful p values as <0.05.

and GPx1 were in agreement with Hardy–Weinberg equilibrium in patient and control groups ($\chi2$=1.195, p=0.274; $\chi2$=1.179, p=0.278; $\chi2$=2.149, p=0.143; $\chi2$=0.025, p=0.870 respectively).

In patient group, when various genotypes of MnSOD and GPx1 were compared according to patient characteristics amongst themselves, we found that the frequency of heterozygous PL genotype in patient with T3/T4 (advanced tumor stage) severly increased compared to patients with T1/T2 (early tumor stage) [Odds ratio (OR)=5.106; 95% confidence interval (CI)=1.372-19.004; p<0.001). In addition, the frequency of P allele was also higher in patient with advanced tumor stage than in those with early tumor stage (OR=5.787; 95% CI=1.564-21.414; p<0.001) (Table 3).

Combine genotype analysis revealed that there were not significant differences between the patients and controls with regard to frequency of various combine genotypes (p>0.05). When combine genotype analyisis was carried out in LSCC patient groups considering clinicopathologic properties, the frequency of ValVal/LL combine genotype was significantly lower in patients with advanced tumor stage than in early tumor stage (OR=0.204, 95% CI=0.055-0.760; p=0.021). On the other hand, it was found that the frequency of ValAla/PL combine genotypes was higher in patients with advanced tumor stage than in those with early tumor stage (p=0.027) (Data not shown).

Table 3. The comparison of patient characteristics among the different genotypes of the MnSOD Ala-9Val and GPx1 Pro198Leu polymorphisms in the study groups.

Patient Characteristic		MnSOD Ala-9Val			GPx1 Pro198Leu		
		Val/Val	Val/Ala	Ala/Ala	PP	PL	LL
Smoking status n(%)	Yes	15 (36.6)	14(34.1)	12 (29.3)	3 (7.3)	21 (51.2)	17 (41.5)
	No	4 (18.2)	12(54.5)	6 (27.3)	1 (4.5)	11 (50.0)	10 (45,5)
Alcohol consumption n(%)	Yes	13 (33.3)	15 (38.5)	11 (28.2)	2 (5.1)	20 (51.3)	17 (43.6)
	No	6 (25.0)	11 (45.8)	7 (29.2)	2 (8.3)	12 (50.0)	10 (41.7)
Reflux n(%)	Yes	7 (25.0)	0 (35.7)	11 (39.3)	2 (7.1)	13 (46.4)	13 (46.4)
	No	12 (34.3)	16 (45.7)	7 (20.0)	2 (5.7)	19 (54.3)	14 (40.0)
Family history n(%)	Yes	5 (23.8)	10 (47.6)	6 (28.6)	1 (4.8)	12 (57.1)	8 (38.1)
	No	14 (33.3)	16 (38.1)	12 (28.6)	3 (7.1)	20 (47.6)	19 (45.2)
Tumor location n(%)	G.	13 (27.1)	20 (41.7)	15 (31.2)	2 (4.2)	25 (52.1)	21 (43.8)
	S.G.	5 (31.2)	9 (56.2)	2 (12.5)	2 (12.5)	7 (43.8)	7 (43.8)
Tumor stage n(%)	T1	2 (33.3)	0 (0.0)	4 (66.7)	0 (0.0)	1 (16.7)	5 (83.3)
	T2	3 (30.0)	5 (50.0)	2 (20.0)	0 (0.0)	1 (10.0)	9 (90.0)
	T3	8 (28.6)	12 (42.9)	8 (28.6)	3 (10.7)	19 (67.9)	6 (21.4)
	T4	6 (31.6)	9 (47.4)	4 (21.1)	1 (5.3)	11 (57.9)	7 (36.8)
Differantiatıon n(%)	Poor	4 (50.0)	3 (37.5)	1 (12.5)	1 (12.5)	4 (50.0)	3 (37.5)
	Med	7 (20.6)	16 (47.1)	11 (32.4)	2 (5.9)	15 (44.1)	17 (50.0)
	Well	4 (57.1)	2 (28.6)	1 (14.3)	0 (0.0)	5 (71.4)	2 (28.6)
Perineural invasion n(%)	Yes	3 (25.0)	5 (41.7)	4 (33.3)	1 (8.3)	7 (58.3)	4 (33.3)
	No	16 (31.4)	21 (41.2)	14 (27.5)	3 (5.9)	25 (49.0)	23 (45.1)
Lenf node involvement n(%)	N0	11 (30.6)	14 (38.9)	11 (30.6)	1 (2.8)	19 (52.8)	16 (44.4)
	N1	6 (27.3)	10 (45.5)	6 (27.3)	3 (13.6)	11 (50.0)	8 (36.4)
	N2	2 (66.7)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)	2 (66.7)
	N3	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)
Metastasis n(%)	Yes	2 (66.7)	0 (0.0)	1 (33.3)	0 (0.0)	2 (66.7)	1 (33.3)
	No	17 (28.3)	26 (43.3)	17 (28.3)	4 (6.7)	30 (50.0)	26 (43.3)
Tumor recurrence n(%)	Yes	4 (66.7)	1 (16.7)	1 (16.7)	0 (0.0)	2 (33.3)	4 (66.7)
	No	15 (26.3)	25 (43.9)	17 (29.8)	4 (7.0)	30 (52.6)	23 (40.4)

Combine genotype analysis revealed that there were not significant differences between the patients and controls with regard to frequency of various combine genotypes (p>0.05). When combine genotype analyisis was carried out in LSCC patient groups considering clinicopathologic properties, the frequency of ValVal/LL combine genotype was significantly lower in patients with advanced tumor stage than in early tumor stage (OR=0.204, 95% CI=0.055-0.760; p=0.021). On the other hand, it was found that the frequency of ValAla/PL combine genotypes was higher in patients with advanced tumor stage than in those with early tumor stage (p=0.027) (Data not shown).

Discussion

Substantial fraction of information has been added into the existing pool of knowledge related to laryngeal cancer. The genetic/epigenetic factors and environmental factors such as smoking and alcohol have a substantial roleplay in carcinogenesis (20, 21, 22). Previous studies supported that there was an association between genetic factors and the development of laryngeal cancer (4, 23, 24) and investigated various SNPs including the genes related to DNA repair mechanisms, cell cycle process and oxidative stress-related enzymes in these studies.

Some polymorphisms have earlier been identitifed in oxidative stress-related enzyme genes and are connected with enzyme activities (25-27) and, therefore modify the capacity to eliminate free radicals (28, 29). Moreover, oxidative stress induced damage resulted in cancer development (30-32). In this study, we also investigated whether the polymorphisms of MnSOD Ala-9Val and GPx1 Pro^{198}Leu increased the risk of cancer development (33).

It is now evident that genetic dimorphism encodes for either valine (Val) or alanine (Ala) in the mitochondrial targeting sequence (MTS) of MnSOD. Structural studies have shown that Ala-MnSOD/MTS allowed efficient import of MnSOD into the matrix of mitochondrion. Contrarily, Val-variant partially arrested precursor within inner mitochondrial membrane and formation of functionally active MnSOD tetramer was dramatically reduced in mitochondrial matrix (34). Information obtained from genetic studies conducted in different populations related to associatıon between MnSOD Ala-9Val and cancer susceptibility is inconsistent (25, 26, 35). In present study, we did not find any association between MnSOD genotype and LSCC cancer susceptibility. Similarly, Wang et al. reported that there was no correlation of MnSOD Ala-9Val polymorphism with cancer risk (15). Interestingly, Li et al. found that AA genotype for MnSOD was noted to be 5-fold higher risk of aggressive prostate cancer and associated with increased risk of breast cancer in premenopausal women who had lower consumption of antioxidants (35). Mitrunen et al. did not find any significant association between MnSOD genotype and breast cancer risk, except women with post-menopausal use of ostrogen (26). Cao et al. did not find any significant association of MnSOD Ala-9Val polymorphism with bladder cancer risk, Hung RJ et al. found that Val/Val genotype increased the risk of bladder cancer (14, 36).

GPx1 is part of the endogenous anti-oxidant defense system that prevents damage to DNA, proteins and lipids via detoxifying hydrogen and lipid peroxides (37, 38). Even though, it was reported that GPx1 was polymorphic at various positions (39) and, several specific GPx1 alleles may be associated with cancer risks (40).

But, it is still controversial whether GPx1 Pro[198]Leu polymorphism is associated with increased cancer risk (37, 41 - 44). In our study, both heterozygous PL genotype and P allele were seen more frequently in patients with advanced stage patients than in those with early stage disease even if there was not been the relationship between patients and control groups in terms of the distribution of GPx1 genotype and frequency of the alleles. Ichimura Y et al. reported that heterozygous PL genotype was associated with increased risk of bladder cancer (45). Lee CH et al. also found similar result in lung cancer patients (46). Contrarily, Vogel et al. and Ahn et al. did not find any association between GPX Pro[198]Leu genotype and risk of basal cell carcinoma and breast cancer respectively (47, 48). Sutton et al. reported that alcoholic liver cirrhosis patients expressing L allele had higher susceptibility than patients having homozygous PP genotype and this susceptibility was related to Ala/Val MnSOD polymorphism (49), however discordant findings have been reported in other studies that did not show any relationship between L allele carriers and increased cancer risk (43, 47, 48, 50, 51). We have observed that the frequency of ValVal/LL combine genotype is significantly lower in patients with advanced stage compared to early stage. Whereas, ValAla/PL combine genotypes were higher advanced stage patients than in early stage. As interesting finding, it was not encountered to ValAla/PL genotype among patients with early stage. This results were revealed that there may be combined effects of MnSOD/GPx1 regarding patients having advanced stage with LSCC. Kucukgergin et al. also demonstrated that MnSOD Ala allele and GPx1[198]Leu allele combinatorially enhanced bladder cancer susceptibility (52). Our study has several limitations, such as small sample size and ethnic differences. Keeping in view smaller number of subjects in this study, this is the first study that reveals information related to polymorhisms of MnSOD Ala-9Val and GPx1 Pro[198]Leu in Turkish patients with LSCC. Finally, we have concluded that variants of GPx1 and MnSOD should not be considered as a risk factor of LSCC, only may be accepted as a prognostic markers.

Acknowledgements

The present work was supported by a grant from the Scientific Research Projects Coordination Unit of Istanbul University (Project No: 40769).

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