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MAIN ARTICLE

Oral squamous cell carcinoma and serum paraoxonase 1

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Abstract

Background: Serum paraoxonase 1 is involved in mechanisms that protect cells from oxidative stress damage. This study aimed to investigate the correlation between serum paraoxonase 1 activity and polymorphisms in patients with oral squamous cell carcinoma.

Methods and materials: Fifty-seven patients with oral squamous cell carcinoma and 59 matched healthy controls participated in the study. Serum paraoxonase 1 activity and polymorphisms in blood samples were compared with results for polymerase chain reaction and restriction fragment length polymorphism tests.

Results: Mean serum paraoxonase 1 activity levels were lower in patients than controls (mean ± standard deviation, 21.9 ± 5 units/l and 120.4 ± 2 units/l, respectively) ($p = 0.001$). The serum paraoxonase 1 192 glutamine polymorphism was more common in patients than controls.

Conclusion: Patients with oral squamous cell carcinoma had significantly lower serum paraoxonase 1 activity levels and a greater prevalence of the serum paraoxonase 1 192 glutamine allele, compared with controls. Serum paraoxonase 1 may play a role in the aetiology of oral squamous cell carcinoma.

Key words: Paraoxonase; Polymorphism, Genetic; Oral Cancer; Carcinoma, Squamous Cell; Genes

Introduction

Every year, more than 300 000 patients are diagnosed worldwide with oral carcinoma.[1] Oral cancers account for 14 per cent of all head and neck cancers excluding lip cancers.[2] Squamous cell carcinoma (SCC) is the most common type, accounting for 86.3 per cent of these types of cancers.[3]

Smoking and alcohol consumption are the most common aetiological factors, while dietary factors, occupational activities, socioeconomic status, human papilloma virus infection and genetic predisposition further contribute to the development of oral cancers.[4] The underlying mechanism may involve oxidative stress and free radical generation, which are known to be involved in the aetiology of many other different cancers.[5] Increased oxidative stress is well known to damage biological molecules such as DNA and to lead to carcinogenesis. The extent of oxidative stress is strongly correlated with the generation and detoxification of reactive oxygen molecules, which are believed to cause cancer in humans.[6] Lipid peroxidation, the cellular status of total antioxidants, and the oxidation of unsaturated fatty acids by free radicals are considered to be risk factors for oral cancers.[7–9]

The role of cellular antioxidants in cancer prevention is to eliminate liposoluble carcinogenic radicals produced by lipid peroxidation.[10] The serum paraoxonase 1 enzyme,[11] which destroys carcinogenic liposoluble radicals, belongs to a family of endogenous free radical antioxidant systems in the body. Serum paraoxonase 1 is synthesised in the liver and attaches to high-density lipoprotein.[8] In humans, the serum paraoxonase gene family is located in the q21.3 region of the long arm of chromosome 7. Serum paraoxonase 1 has two major well-known polymorphisms arising from amino acid substitution at position 55 (leucine *vs* methionine) and at position 192 (glutamine *vs* arginine).[11] Serum paraoxonase 1 is able to hydrolyse toxic organic molecules such as organophosphate neurotoxins, aromatic carboxylic acids esters, and fatal insecticides such as sarin and soman.[12]

Serum paraoxonase 1 concentration and activity level may vary between individuals due to consumption of dietary antioxidants, alcohol and/or medication (e.g. statins and fibrates).[12] Smoking is another factor that can irreversibly decrease the serum paraoxonase 1 concentration and activity level. Serum paraoxonase 1 concentration and polymorphisms have been associated with several forms of cancer, including

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non-Hodgkin's lymphoma,[13] multiple myeloma,[14] glioma and meningioma,[11] gastroesophageal malignancy,[15] ovarian epithelial carcinoma,[16] prostate cancer,[17] pancreatic cancer,[18] stomach cancer,[19] lung cancer,[20] and breast cancer.[21] As noted already, smoking and alcohol consumption are associated with a reduction in serum paraoxonase 1 activity. Thus, it is possible that serum paraoxonase 1 may play a role in SCC carcinogenesis via its antioxidant effects.

The aim of this study was to investigate the relationship between oral SCC, serum paraoxonase 1 activity levels, and the presence of serum paraoxonase 1 55 and 192 gene polymorphisms.

Materials and methods

Study design

The study was approved by the Research Ethics Committee of the Istanbul Medicine Faculty.

We enrolled in the study patients who were admitted to the otolaryngology department, Istanbul Faculty of Medicine, Istanbul University, or to the oral surgery and medicine department, Faculty of Dentistry, Istanbul University, and who volunteered to participate. Patients included in the study group had no other malignancies other than oral SCC. A total of 116 subjects were included: 57 in the patient group (31 men and 26 women) and 59 in the control group (25 men and 34 women, recruited from the polyclinic of the oral health department). Serum paraoxonase 1 activity and 55 and 192 gene polymorphism prevalence were compared between these two groups, using results from peripheral blood samples. Patients' smoking habits, alcohol consumption, histopathological findings and tumour–node–metastasis stage were evaluated and recorded in patients' follow-up documents.

Isolation of DNA

Blood specimens were collected into tubes containing ethylene diamine tetra-acetate. Leukocyte pellets underwent sodium dodecyl sulphate lysis, ammonium acetate extraction and ethanol precipitation, in order to isolate DNA.[22]

Paraoxonase genotyping

Paraoxonase 1 genotypes were determined using polymerase chain reaction conducted according to previously published protocols.[23,24]

The serum paraoxonase 1 192 polymorphism sense primer was 5′ TAT TGT TGC TGT GGG ACC TGA G 3′ (where T = thymine, A = adenine, G = guanine and C = cytosine), and the antisense primer was 5′ CAC GCT AAA CCC AAA TAC ATC TC 3′, which encompassed the 192 polymorphic region of the human serum paraoxonase 1 gene. The serum paraoxonase 1 55 polymorphism sense primer was 5′ GAA GAG TGA TGT ATA GCC CCA G 3′, and the antisense primer was 5′ TTT AAT CCA GAG CTA ATG AAA GCC 3′.

The polymerase chain reaction mixture contained 100 ng of DNA template, 0.5 μM of each primer, 1.5 mM $MgCl_2$, 200 μM 2′-deoxynucleotide triphosphates (also termed 4dNTP) and 1 U Taq DNA polymerase (MBI Fermentas, St Leon-Rot, Germany).

In order to identify the serum paraoxonase 1 192 polymorphism, after denaturing the DNA for 5 minutes at 94°C, the reaction mixture was subjected to 35 cycles of denaturing for 1 minute at 95°C, 1 minute annealing at 60°C and 1 minute extension at 72°C. The 99 base-pair polymerase chain reaction product was digested with 8 U AlwI (BspPI) restriction endonuclease enzyme (which recognises GGATC(4/5̂) sites) (MBI Fermentas), overnight at 55°C. The digested products were separated by electrophoresis on 4 per cent MetaPhor agarose gel (Lonza, Basel, Switzerland) and visualised using ethidium bromide. The arginine genotype contains a unique BspPI restriction site, which results in 66 and 33 base-pair products, whereas the glutamine genotype is not cleaved by this restriction enzyme, thereby allowing determination of the serum paraoxonase 1 192 genotype.[23]

To identify the serum paraoxonase 1 55 polymorphism, polymerase chain reaction and cycling were performed as described above. The polymerase chain reaction product (170 base-pairs) was digested with NlaIII (Hsp92II) (a restriction enzyme recognising CATG sites) (Promega, Madison, Wisconsin, USA) in the presence of bovine serum albumin (0.1 μg/μl final concentration) (37°C, overnight), and the digested products were separated and identified as above. The leucine allele did not contain the Hsp92II site, whereas the methionine allele did contain this site, giving rise to 126 and 44 base-pair products.[24]

Quantification of paraoxonase activity

Paraoxonase 1 activity was measured according to the method of Furlong *et al.*[25] The assay buffer contained 0.132 M trisaminomethane (Tris) HCl (pH 8.5), 1.32 mM $CaCl_2$ and 2.63 mM NaCl. The addition of 200 μl of 6 mM freshly prepared paraoxon (0,0-diethyl-0-*p*-nitrophenylphosphate; Sigma, Poole, UK) and 40 μl of serum was used to initiate the assay. The rate of generation of *p*-nitrophenol was determined at 37°C using a continuously recording spectrophotometer at 405 nm. A molar extinction coefficient of 18.05×10^3 was used for the calculation, using paraoxon as the substrate. The serum paraoxonase 1 activity was expressed as units/litre (where 1 unit = 1 μmol paraoxon hydrolysed/minute).

Statistical analysis

The Number Cruncher Statistical System 2007 (NCSS LLC, Kaysville, Utah, USA) and the Power Analysis and Sample Size 2008 (PASS, NCSS LLC) statistical software programs were used for the statistical analysis. For the data evaluation, in addition to the use of complementary statistical methods (calculating the median, standard deviation (SD), median, frequency and

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ratio), logarithmic conversion was applied when the serum paraoxonase 1 levels showed an abnormal distribution pattern. The one-way analysis of variance test was used when comparing the parameters in two or more groups, and the independent Student's *t*-test was used when comparing the parameters in three or more groups. Gender, smoking and alcohol parameters were evaluated with a chi-square test, comparing the patient and control groups. Results were evaluated utilising confidence intervals of 95 per cent and a level of significance of p less than 0.05.

Results

One hundred and sixteen individuals were included in this study, 57 (49.1 per cent) in the patient group and 59 (50.9 per cent) in the control group. The mean subject age was 57.4 years in the control group and 57.8 years in the patient group. There were no statistically significant differences (i.e. p values were 0.05 or greater) in gender, age or smoking between the two groups; however, the rate of alcohol consumption was significantly higher ($p < 0.01$) in the patient group compared with the control group.

Investigations aiming to localise patients' oral SCC revealed that 30 cases (52.6 per cent) had lesions of the tongue, 8 (14.0 per cent) had lesions on the gingival-alveolar crest, 7 (12.3 per cent) had lesions on the base of the mouth, 5 (8.8 per cent) had lesions in the retromolar trigon, 3 (5.3 per cent) had lip lesions, 2 (3.5 per cent) had hard palate lesions and 2 (3.5 per cent) had lesions in the buccal area. According to the classification system of the American Joint Cancer Committee, 14 per cent of these oral SCC cases were in stage 1, 10 per cent were in stage 2, 13 per cent were in stage 3 and 20 per cent were in stage 4. Twenty-four of the patients had early-stage disease (i.e. stage 1 or 2) and 33 had late-stage disease (i.e. stage 3 or 4). Of the 57 oral SCC patients, 19 (33.3 per cent) had histologically well-differentiated tumours, 31 (54.4 per cent) had moderately differentiated tumours and 7 (12.3 per cent) had poorly differentiated tumours.

In the total subject group, the mean serum paraoxonase 1 activity level ± SD was 96.7 ± 8 units/l (range, 0.7–331.3 units/l).

In the total subject group, the serum paraoxonase 1 position 55 leucine/leucine polymorphism was present in 60.6 per cent ($n = 66$), the methionine/methionine polymorphism in 1.8 per cent ($n = 2$) and the leucine/methionine polymorphism in 37.6 per cent ($n = 41$). The serum paraoxonase 1 position 55 leucine(+) polymorphism was found in 98.2 per cent ($n = 107$) of subjects, while the methionine(+) polymorphism was found in 39.4 per cent ($n = 43$). Regarding serum paraoxonase 1 position 192 polymorphism, 98.1 per cent ($n = 101$) of subjects had an arginine(+) polymorphism and 55.3 per cent ($n = 57$) had a glutamine(+) polymorphism. Table I shows the serum paraoxonase 1 activity level in the control and patient groups, together with the distribution of serum paraoxonase 1 55 and 192 genotypes and alleles. There was a statistically significant difference in serum paraoxonase 1 activity levels between the study and control groups ($p < 0.01$), with a significantly lower median serum paraoxonase 1 activity level in the patients (Figure 1). There was no significant difference between the frequency of the serum paraoxonase 1 position 55 and 192 genotypes and alleles,

TABLE I
SERUM PARAOXONASE 1 ACTIVITY AND GENOTYPE DISTRIBUTION

Parameter	Group			p
	Total	Control	Patient	
Serum PON1 activity (units/l)				
– Range	0.7–331.3	26.6–331.3	0.7–295.9	
– Mean ± SD	96.7 ± 8	120.4 ± 2	21.9 ± 5	0.001*§
– Median		132.9	21.9	0.001*§
PON1 55 genotype (*n* (%); pts)				
– Leu/Leu	66 (60.6)	33 (61.1)	33 (60.0)	0.331†
– Met/Met	2 (1.8)	2 (3.7)	0 (0)	
– Leu/Met	41 (37.6)	19 (35.2)	22 (40.0)	
– Leu(−)	2 (1.8)	2 (3.7)	0 (0)	0.243‡
– Leu(+)	107 (98.2)	52 (96.3)	55 (100)	
– Met(−)	66 (60.6)	33 (61.1)	33 (60.0)	0.906†
– Met(+)	43 (39.4)	21 (38.9)	22 (40.05)	
PON1 192 genotype (*n* (%); pts)				
– Arg/Arg	46 (44.7)	28 (54.9)	18 (34.6)	0.060†
– Gln/Gln	2 (1.9)	0 (0)	2 (3.8)	
– Arg/Gln	55 (53.4)	23 (45.1)	32 (61.2)	
– Arg(−)	2 (1.9)	0 (0)	2 (3.8)	0.495‡
– Arg(+)	101 (98.1)	51 (100)	50 (96.2)	
– Gln(−)	46 (44.7)	28 (54.9)	18 (34.6)	0.038†#
– Gln(+)	57 (55.3)	23 (45.1)	34 (65.7)	

*Student's *t*-test; †chi-square test; ‡Fisher's exact test. §$p < 0.01$; #$p < 0.05$. PON1 = paraoxonase 1; SD = standard deviation; pts = patients; Leu = leucine; Met = methionine; Arg = arginine; Gln = glutamine

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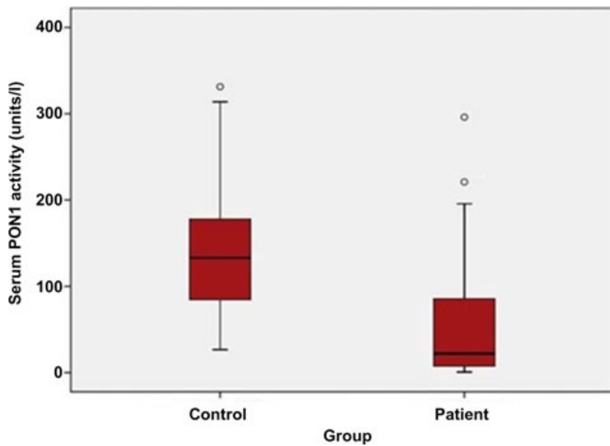

FIG. 1
Box and whisker plot showing distribution of serum paraoxonase 1 (PON1) activity in the control and patient groups. Central box rule = median; lower and upper box borders = first and third quartiles, respectively; whiskers = 95% confidence interval; circles = outliers

comparing the two groups ($p > 0.05$), except that the serum paraoxonase 1 192 glutamine(−) polymorphism was more frequent in the control group and the 192 glutamine(+) polymorphism was more frequent in the patient group ($p < 0.05$).

There was no significant association between serum paraoxonase 1 activity level and smoking status in either group ($p > 0.05$), as shown in Table II. As only two members of the control group consumed alcohol, its association with serum paraoxonase 1 activity could not be properly evaluated in this group. In the patient group, there was no significant association between serum paraoxonase 1 activity level and alcohol consumption ($p > 0.05$). No significant association was identified between serum paraoxonase 1 activity level and oral SCC tumour stage (comparing patients with early- versus late-stage carcinoma) ($p > 0.05$).

The mean serum paraoxonase 1 activity level was significantly lower both in smoking patients compared with smoking controls ($p < 0.01$), and in non-smoking patients compared with non-smoking controls ($p < 0.01$). The mean serum paraoxonase 1 activity level amongst patients who did not drink alcohol was significantly lower compared with non-drinking controls ($p < 0.01$). As only two control subjects consumed alcohol, we could make no meaningful comparison between serum paraoxonase 1 activity levels in alcohol-consuming patients versus alcohol-consuming controls.

A significant difference was observed between mean serum paraoxonase 1 activity levels in the patient versus control groups, for almost all the serum paraoxonase 1 position 55 and position 192 genotype and allele categories investigated (p 0.001) (Table III). None of the patients had the serum paraoxonase 1 55 methionine/methionine genotype, and none of the control subjects had the serum paraoxonase 1 192 glutamine/glutamine genotype, so comparisons of serum paraoxonase 1 activity could not be performed within these genotype groups.

TABLE II
SERUM PARAOXONASE 1 ACTIVITY BY GROUP, SMOKING, ALCOHOL, AND TUMOUR STAGE AND DIFFERENTIATION

Group	Exposure	Serum PON1 activity (units/l)		p*
		Mean ± SD	Median	
Patient	Smoking	24.1 ± 5	30.4	0.627†
	Non-smoking	19.7 ± 4	16.1	
Control	Smoking	114.6 ± 2	126.6	0.585†
	Non-smoking	125.1 ± 2	141.3	
Patient	Alcohol	20.8 ± 7	27.8	0.892†
	No alcohol	22.3 ± 4	19.7	
Control	Alcohol	190.2 ± 1	192.2	–
	No alcohol	118.4 ±2	126.7	
Patient	Stage 1 tumour	17.2 ± 7	21.6	0.860‡
	Stage 2 tumour	25.6 ± 6	22.7	
	Stage 3 tumour	27.9 ± 3	39.5	
	Stage 4 tumour	20.5 ± 5	19.7	
Patient	Early-stage tumour	20.3 ± 6	21.6	0.751†
	Late-stage tumour	23.2 ± 4	19.9	
Patient	Well differentiated tumour	15.1 ± 6	15.6	0.364‡
	Mod differentiated tumour	24.5 ± 4	27.9	
	Poorly differentiated tumour	37.3 ± 4	24.4	

Patient numbers: stage 1 = 14; stage 2 = 10; stage 3 = 13; stage 4 = 20; early stage (stage 1 or 2) = 24; late stage (stage 3 or 4) = 33; well differentiated tumour = 19; moderately (mod) differentiated tumour = 31; poorly differentiated tumour = 7. *Comparing mean values. †Student's t-test; ‡one-way analysis of variance. PON1 = paraoxonase 1; SD = standard deviation; – = too few data for meaningful calculation

Discussion

Serum paraoxonase 1 is an antioxidant enzyme which destroys endogenous free radicals.[10] Due to its antioxidant activity against free radicals and oxidative stress, a relationship exists between malignancies and serum

TABLE III
SERUM PARAOXONASE 1 ACTIVITY BY GENOTYPE AND ALLELE

Variant	Serum PON1 activity (mean ± SD; units/l)		p*
	Pt grp	Cntrl grp	
PON1 55 Leu(+)	21.2 ± 5	115.7 ± 2	0.001†
PON1 55 Met(+)	24.2 ± 5	123.3 ± 2	0.001†
PON1 192 Arg(+)	21.6 ± 5	119.5 ± 2	0.001†
PON1 192 Gln(+)	24.3 ± 5	136 ± 2	0.001†
PON1 55 Leu/Leu	19.3 ± 5	113.9 ± 2	0.001†
PON1 55 Met/Met	–	179.8 ± 2	–
PON1 55 Leu/Met	24.2 ± 5	118.5 ± 2	0.001†
PON1 192 Arg/Arg	20.4 ± 5	106.7 ± 2	0.001†
PON1 192 Gln/Gln	86.4 ± 1	–	–
PON1 192 Arg/Gln	22.4 ± 5	136 ± 2	0.001†

*Student's t-test. †$p < 0.01$. PON1 = paraoxonase 1; SD = standard deviation; Pt = patient; grp = group; Cntrl = control; Leu = leucine; Met = methionine; Arg = arginine; Gln = glutamine

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paraoxonase 1 genotypes.[26] Although many studies have investigated this relationship, to the best of our knowledge the present study is the first to investigate serum paraoxonase 1 activity levels and genotypes within oral cancer patients.

Lincz *et al.*[14] found a higher prevalence of the paraoxonase 1 position 192 glutamine/glutamine genotype in patients with multiple myeloma, compared with controls, whereas Van der Logt *et al.*[27] found no difference in genotype prevalence in colorectal cancer patients versus controls. Antognelli *et al.* reported that the risk of developing prostate cancer was greater in individuals with the serum paraoxonase 1 192 arginine/glutamine, 55 leucine/methionine and 55 methionine/methionine genotypes.[17] De Roos *et al.* found that the serum paraoxonase 1 methionine/methionine genotype was associated with an increased risk of non-Hodgkin's lymphoma.[13] Lurie *et al.* reported that the serum paraoxonase 1 192 arginine and 55 leucine alleles were associated with an increased risk of ovarian epithelial carcinoma.[28] In the present study, the prevalence of the serum paraoxonase 1 position 192 glutamine(+) genotype was high in the patient group and low in the control group, but there were no other statistically significant differences for other genotypes or alleles.

Several studies have reported significantly lower serum paraoxonase 1 activity levels in patients with malignancies of the stomach,[19] pancreas,[18] lung,[20] gastroesophagus[15] or ovaries,[16] compared with controls. In the present study, the mean serum paraoxonase 1 activity ± SD was 120.4 ± 2 units/l in the control group but only 21.9 ± 5 units/l in the patient group; this difference was statistically significant ($p = 0.001$).

- **Serum paraoxonase 1 protects cells from oxidative stress damage**
- **It may play a role in carcinogenesis**
- **In this study, serum paraoxonase 1 activity was lower in oral squamous cell carcinoma (SCC) patients than controls**
- **Serum paraoxonase 1 may play a role in the aetiology of oral SCC**

As noted above, smoking and alcohol consumption are well-known risk factors for the development of oral carcinoma, and both are known to decrease the serum paraoxonase 1 activity levels.[29] Elkiran *et al.* reported that smoking had no effect on serum paraoxonase 1 activity levels in patients with lung cancer.[30] In the current study, there was no significant difference in serum paraoxonase 1 activity levels between smokers and non-smokers, either in the patient group or the control group. Alcohol consumption was not associated with reduced serum paraoxonase 1 activity in the patient group, and no meaningful calculation was possible in the control group because of the small number of control subjects who consumed alcohol. Therefore, the decreased serum paraoxonase 1 activity level observed in patients with oral SCC was thought to be independent of smoking or alcohol consumption.

Krzystek-Korpacka *et al.* found no association between serum paraoxonase 1 activity level and tumour size or stage, in patients with gastroesophageal malignancy.[15] In our study, serum paraoxonase 1 activity level did not differ in patients with early-stage (stage 1 or 2) versus late-stage (stage 3 or 4) tumour, as determined by tumour–node–metastasis staging.

Conclusion

The present study identified significantly lower serum paraoxonase 1 activity levels in oral SCC patients compared with controls, independent of age and smoking habits. There was no significant difference in the prevalence of the various serum paraoxonase 1 genotypes and alleles in patients versus controls, except that the patient group had an increased prevalence of the serum paraoxonase 1 position 192 glutamine(+) polymorphism.

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