Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Chlorine and Chromium Elements, Proteins of Oxidative Stress and Dna Repair Pathways Are Related to Tumor Aggressiveness and Prognosis of Patients With Oral Cancer

Arícia L. E. M. Assis
Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo
Anderson B. Archanjo
Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo
Raul C. Maranhão
Heart Institute (InCor), Medical School Hospital, University of São Paulo
Suzanny O. Mendes
Graduate Program in Biotechnology/RENORBIO
Rafael P. de Souza
Cancer Institute Arnaldo Vieira de Carvalho
Rafael de Cicco
Cancer Institute Arnaldo Vieira de Carvalho
Mayara M. de Oliveira
Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo
Aline R. Borçoi
Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo
Lucas de L. Maia
Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo
Fabio D. Nunes
Department of Stomatology, Faculty of Dentistry, University of São Paulo,
Marcelo dos Santos
Multicampi School of Medical Sciences of Rio Grando do Norte, Federal University of Rio Grande do Norte
Leonardo O. Trivilin
Department of Rural Engineering, Center for Agricultural Sciences and Engineering, Federal University of Espírito Santo
Christiano J.G. Pinheiro
Department of Rural Engineering, Center for Agricultural Sciences and Engineering, Federal University of Espírito Santo
Adriana M. Álvares-da-Silva

Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo

Breno Valentim Nogueira (brenovalentim@gmail.com)

Graduate Program in Biotechnology/RENORBIO, Federal Univerty of Espírito Santo

https://orcid.org/0000-0002-2199-0635

Research

Keywords: Metals, Synchrotron radiation, Oxidative stress, Repair enzymes, Oral cancer

Posted Date: March 8th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-281302/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

Background

Squamous cell carcinoma (OCSCC), the most frequent cancer of the oral cavity, is extremely aggressive, the response to treatment is poor and markers for prognosis of disease are scarce. The comparison of chemical and histopathological data obtained from the analysis of excised tumor fragments with the demographic and clinical evolution data is an effective strategy scarcely explored in OCSCC studies. The aim was to analyse OCSCC tissues for trace elements and protein expression of enzymes related with oxidative stress and DNA repair that can be candidates as markers of tumor aggressiveness and prognosis.

Methods

Seventy-eight tumor fragments from 78 OCSCC patients that had undergone ablative surgery were qualitatively analyzed by synchrotron micro-X-ray fluorescence (μ-XRF) for trace elements. Protein expression of superoxide dismutase (SOD-1) and thioredoxin (Trx) and DNA repair-related purinic/apyrimidinic endonuclease/redox factor-1 (Ref-1) and 8-oxoguanine glycosylase (OGG1/2) was performed by immunohistochemistry. Sociodemographic, clinical and hystopathological data were obtained from 4-year follow-up records.

Results

Disease relapse was higher in patients with chlorine and chromium presence and in those with tumors with strong OGG1/2 expression. Strong expression of SOD-1, Trx and Ref-1 was determinant of larger tumor. As expected, perineural, vascular invasions and alcohol consumption were markers of greater worse prognosis.

Conclusion

Presence of trace elements can be markers of disease prognosis. Strong expression of enzymes related with oxidative stress or DNA repair can be either harmful by stimulating tumor growth or beneficial by diminishing relapse rates. Interference on these players may bring novel strategies for the therapeutic management of OCSCC patients.

Background

Oral cancer squamous cell carcinoma (OCSCC) is the most common type of oral cavity cancers. These cancers predominanly occur between the fifth and the seventh decades of life and are the sixth most prevalent cancer type in the world population [1]. Despite the improvement in diagnostic and therapeutic procedures, tumor relapse after surgical removal is frequent and the overall 5-year survival rates are low [2, 3, 4]. Smoking is the chief risk factor for OCSCC, followed by alcoholic addiction. HPV infection and genetic susceptibility are other OCSCC risk factors [5].

It has now been well established that non-essential metals and metaloids can induce carcinogenicity by favoring the generation of reactive oxygen species (ROS). The formation of metal-mediated free radicals can cause several modifications in DNA bases, increases lipid peroxidation and elicits alterations in the body homeostasis of calcium and sulfhydryl [6].

Unbalance between ROS production and degradation leads to oxidative stress that may participate in the induction of inflammation and carcinogenesis [7]. Enzymes involved in the protection against damage from oxidative stress, such as superoxide dismutase (SOD-1), thioredoxin (Trx), and in DNA repair, such as purinic/apyrimidinic endonuclease/redox factor-1 (Ref-1) and 8-oxoguanine glycosylase (OGG) are important natural defenses against cancer development. Those enzymes may eventually be used as targets for acquisition of prognostic markers and therapeutic strategies [8].

SOD-1 catalyzes the dismutation of superoxide radical into oxygen and hydrogen peroxide (H_2O_2) [8]. In the intracellular medium, metals react with H_2O_2 and generate hydroxyl radical (OH^-) capable of acting against direct damages to DNA. Damaged DNA can be repaired by the action of OGG1 glycosylase, which excises the modified base [9]. Ref-1 protects the DNA structure against enzymatic degradation while the specific enzymes complete the repair [10]. The activity of Ref-1 is regulated by Trx. [10, 11].

Since OCSCC frequently relapses after surgery, it is of prime importance to determine the factors that contribute for the worse prognosis of those patients. In this study, we hypothesize whether differences in content in the tumoral tissue of metals and metaloids and in the protein expression of enzymes related with DNA repair and oxidative stress could be related to disease relapse. To this end, we analysed tumor fragments collected during the surgery of patients with OCSCC to match with the data of clinical evolution of those patients.

Materials And Methods

Study subjects

Tumor samples, demographic, hystopathological and clinical evolution data were obtained from 78 patients with hystopathological diagnosis of oral cavity squamous cell carcinoma (Table 1). They were selected at the Outpatient Clinic of the Arnaldo Vieira de Carvalho Cancer Institute (ICAVC) in São Paulo, Brazil, between January 2012 and May 2015. The follow-up period was 4 years. They were participants of an ongoing Genome Head and Neck Project (GENCAPO), involving a multi-institutional and multidisciplinary group that has been active since 2002. Tumor fragments inbedded in paraphin blocks were used for the analyses of protein and elementary characterization performed in this study.

Table 1
Epidemiological, clinicopathological and prognostic characteristics of patients with oral squamous cell carcinoma.

Characteristics	Total	
	N.	(%)
Gender		
Female	24	30.77
Male	54	69.23
Age, years		
Mean	63.69	
Standard Deviation	± 11.01	
Median	63	
Smoking		
Never	14	17.95
Yes, in the past	23	29.49
Yes, currently	41	52.56
Consumption of alcoholic beverages		
Never	17	21.79
Yes, in the past	27	34.62
Yes, currently	34	43.59
Tumor size (pT)[1]		
pT1	25	32.10
pT2	19	24.40
pT3	17	21.80
pT4	17	21.80
Lymph node (pN)[1]		
Negative	52	66.66

[1] TNM Classification (7th edition); [†]Did not enter in statistical calculations.

[2] pT3/pT4 (N0); [3] pT1/pT2 (N1).

Characteristics	Total	
	N.	(%)
Positive	26	33.34
Vascular Invasion		
Absent	55	70.50
Present	18	23.00
Not evaluable	5	6.50
Aggressiveness		
Less aggressive[2]	18	23.08
More aggressive[3]	14	17.95
Not evaluable[†]	46	58.97
Perineural Invasion		
Absent	43	55.13
Present	27	34.62
Not evaluable[†]	8	10.26
Recurrence		
No	63	80.77
Yes	15	19.23
Death due to Ilness		
No	50	64.11
Yes	28	35.99
Total	**78**	**100.00**
[1] TNM Classification (7th edition); [†]Did not enter in statistical calculations.		
[2] pT3/pT4 (N0); [3] pT1/pT2 (N1).		

Tissue microarrays

Tissue microarrays of the tumor fragments were made as previously described [12], with selection of two representative tumor areas, as evaluated by two experienced pathologists from tissue slides stained with hematoxylin/eosin, followed by extraction of two 1.5 mm diameter cylinders from each sample which

were then added to the microarray receptor block using a tissue microarrayer (BEECHER INSTRUMENTS, Silver Spring, MD, USA). Sections were then removed from the TMA and mounted on microscopy slides. A pathologist checked the content of each spot. Spots bent or missing more than 70% of the tissue were excluded.

Qualitative Elementary Characterization

For the qualitative elemental characterization, tumor tissue samples (mean thickness of 450 μm; mean density of 0.54 g/cm^3) were removed from the TMA and subjected to dewaxing and rehydration processes with xylene, alcohol (the quality and integrity of these reagents were checked or purity in each batch used) and ultrapure water. They were then deposited in plastic support with Ultralene® film (SPEX SAMPLEPREP, Metuchen, NJ, USA) and sent to the D09-XRF beamline equipment. For the elementary characterization, the synchrotron radiation-based μ-XRF technique was used to detect chemical elements from electron excitation energy absortion, which is specific for each element. To obtain the spectra, a white beam with a power range of 4 to 24 keV and dimensions of 2 mm^2 was applied to the samples for 20 seconds and excited the eletons. Nine measurements in a 3x3 matrix were performed and later an averaging was performed to obtain the final spectrum used in analyzes. For adjustment of characteristic X-ray spectra, determination of elements and their respective fluorescent intensities, an analysis of a certified reference sample was carried out, Standard Reference Material® 1577b “Bovine Liver”, produced by National Institute of Standards and Technology (Gaithersburg, MD, USA), under the same conditions as the test samples, PyMca 5.0.0 software program [13] was the basis for analyzes. Measurements of μXRF were performed in D09-X-Ray Fluorescence (D09-XRF) light line at the National Synchrotron Light Laboratory (Fig. 1), Campinas, São Paulo, Brazil [14]. Spectra were obtained by the average of nine measured points and analysis was peformed in program PyMca 5.0.0. Each spectrum was verified, the characteristic peaks of the elements identified, and then identification values were assigned as (0) for absence and (1) for the presence of the characteristic peak.

Immunohistochemistry

Antibodies anti-SOD- 11:400 (SC-11407, Santa Cruz Biotechnology), anti-Ref-1 1:400 (SC-17774, Santa Cruz), anti-OGG1/2 1:100 (SC- 376935, Santa Cruz Biotechnology) and anti-Trx 1:100 (SC-166393, Santa Cruz Biotechnology) were used in immunohistochemistry reaction with REVEAL Polymer-HRP (Spring Bioscience), according to the manufacturer's protocol. For each reaction negative controls (absence of primary and secondary antibody) were used. Protein expression was independently evaluated by two different observers, and conflicting results were submitted to re-analysis. Protein analysis was semiquantitative so that samples were classified according to % of cells stained at: 0 (0% of labeled cells), 1 (< 10%); 2 (10 ≤ 50%) and 3 (> 50% of labeled cells); and by staining intensity in: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). Scores estimated from the % and intensity of staining were multiplied and their means calculated for each sample. On the basis of the final score, each sample was cathegorized as negative (0), weak positive (1 ≤ 3) or strong positive (> 3), according to method used by studies that performed similar analyzes to the present study [15, 16].

Statistical Analysis

For association tests, Chi-square test was used in bivariate analysis and, when necessary, Fisher's exact test, with a 5% margin of error and with Bonferroni correction. Multivariate logistic regression by modeling was used to adjust odds ratio (OR) and confidence interval (CI ≥ 95%). The variables that obtained a p-value of less than 20% ($p < 0.20$) were inserted by the backward method in multivariate logistic regression model, with the significant variables remaining at the end of the model, at each stage ($p < 0.05$). For overall survival analysis, it was calculated the time interval (in months) between dates of surgery and death by disease of each patient or the last return in cases of survivors. The time interval for recurrence-free survival analysis was calculated using as end points the dates of global relapse, or the date of the last return in asymptomatic cases. The Kaplan–Meier model was used for survival analysis, using the Wilcoxon p-value and the Cox proportional hazards to adjust p-values, and to hazards ratio (HR) and CI (CI ≥ 95%). The values of OR and HR were adjusted for lymph node status (TNM). All analyses were performed using SPSS version 20 (IBM Corp., Armonk, NY, USA).

Results

Table 2 shows the results of the multivariate analysis between, on one hand, the data on demography, habits, histopathological features of the tumor and protein expression in the tumor cells and, on the other hand, of occurrence or not of relapse or death. Patients aged over 63 years were sevenfold less likely to relapse than those below this age cut-off ($p < 0.05$). In addition, we also observed that patients consuming alcoholic bevarages were eightfold more likely to relapse ($p < 0.05$) and sixfold more likely to die from the disease than those that did not consume alcohol ($p < 0.01$). Also, in Table 2, patients with tumors with vascular invasion were thirteenfold more likely to relapse ($p < 0.01$) and sixfold more likely to die ($p < 0.05$) as compared with those with tumors without vascular invasion. Perineural invasion was not related to disease relapse but death rates were increased fourfold in patients with tumors with this feature ($p < 0.05$).

Fifteen trace elements were identified in the different tumor fragments analysed by micro-XRF, namely iron and zinc (present in 100% of the tumor fragments), sulfur and calcium (99%), copper (95%), phosphorus (94%), potassium (83%), arsenic and bromine (68%), chromium (43%), chlorine (36%), manganese (33%), nickel (14%), magnesium and cobalt (12%). The finding of chlorine in the tumor fragments was determinant of reduction of frequency of disease relapse in the patients by thirteenfold, as compared to tumor fragments without detectable chlorine ($p < 0.05$). In contrast, the finding of chromium presence in the tumor fragment was determinant of an eightfold increase in the % of disease relapse ($p < 0.05$, Table 2).

Also shown in Table 2, all four proteins analysed here by immunohistochemistry were expressed in both nucleous and cytoplasm of the tumor cells (Fig. 2). In patients with tumors with strong expression of OGG1/2, the frequency of relapse was reduced by twenty-fourfold as compared to those with weak

expression of this protein ($p < 0.01$). Regarding the other three proteins, namely Trx, SOD-1 and Ref-1, no correlations were found with relapse or death rates.

Table 2
Logistic regression model between prognostic, clinicopathological characteristics, life habits and protein expression.

Features	Logistic regression model	
	Relapse	Death
Age		
> 63 / ≤ 63[1]		
OR	0.135	-
CI 95%	0.022–0.812	
P value	0.029	
Current alcohol consumption		
Yes / No [1]		
OR	8.437	6.391
CI 95%	1.098–64.806	1.672–24.422
P value	0.040	0.007
Vascular invasion		
Present / Absent[1]		
OR	13.516	6.072
CI 95%	1.900-96.129	1.276–28.902
P value	0.009	0.023
Perineural invasion		
Present / Absent[1]		
OR		4.054
CI 95%	-	1.00-16.443
P value		0.050
Chlorine		
Present / Absent[1]		
OR	0.076	-

[1] Reference variable.

Features	Logistic regression model	
	Relapse	Death
CI 95%	0.007–0.827	
P value	0.034	
Chrome		
Present / Absent[1]		
OR	8.003	-
CI 95%	1.315–48.709	
P value	0.024	
OGG1/2 Cytoplasmic		
Strong / Weak[1]		
OR	0.041	
CI 95%	0.004–0.414	-
P value	0.007	
SOD-1 Cytoplasmic		
Strong / Weak[1]		
OR	-	2.592
CI 95%		0.458–14.654
P value		0.281
Trx Cytoplasmic		
Present / Absent[1]		
OR	-	1.972
CI 95%		0.568–6.841
P value		0.285
[1] Reference variable.		

In Table 3 it is shown that strong protein cytoplasmatic expressions of Trx, and SOD-1 and strong nuclear expression of Ref-1 were associated with larger tumor sizes. Strong cytoplasmic expression of Trx ($p < 0.05$) and SOD-1 ($p < 0.05$) were related to three and to fivefold larger tumor sizes, respectively. The strong nuclear expression of Ref-1 was associated to sixfold larger tumor size ($p < 0.05$).

In Table 3, we also found by multivariate analysis that the lymph node vascular involvement was increased by fortysevenfold ($p < 0.001$) when tumors had vascular invasion. There was a trend for lymph node involvement in tumors of smokers that was not statistically significant ($p = 0.09$). The vascular invasion in tumors also increased the tumor aggressiveness by fifteenfold ($p < 0.05$), whereas the presence of perineural invasion increased the vascular invasion by tenfold ($p < 0.001$). No association was found between tumor size and tumor vascular invasion ($p = 0.211$).

Table 3
Logistic regression model concerning lymph node, tumor size, aggressiveness and vascular invasion with clinicopathological characteristics, life habits and protein expression.

Features	Logistic regression model			
	Lymph node	Tumor Size	Aggressiveness	Vascular Invasion
Smoking				
Yes / No[1]				
OR	3.38	-	-	-
CI 95%	0.81–14.09			
P value	0.093			
Tumor size				
≥ pT3 / ≤ pT2[1]				
OR	-	-	-	2.31
CI 95%				0.62–8.58
P value				0.211
Vascular invasion				
Present / Absent[1]				
OR	47.16	-	15.67	-
CI 95%	8.35-266.17		1.49–164.3	
P value	< 0.001		0.022	
Perineural invasion				
Present / Absent[1]				
OR	-	-	-	10.30
CI 95%				2.74–38.60
P value				0.001
Trx Nuclear				
Strong / Weak[1]				
OR	1.14	-	-	-

[1] Reference variable.

Features	Logistic regression model			
	Lymph node	Tumor Size	Aggressiveness	Vascular Invasion
CI 95%	0.256–5.11			
P value	0.861			
SOD-1 Cytoplasmic				
Strong / Weak[1]				
OR	-	4.63	-	2.81
CI 95%		1.12–19.08		0.28–28.34
P value		0.034		0.380
Trx Cytoplasmic				
Strong / Weak[1]				
OR	-	3.00	-	-
CI 95%		1.09–8.24		
P value		0.033		
Ref-1 Nuclear				
Strong / Weak[1]				
OR	-	6.12	-	-
CI 95%		1.14–32.83		
P value		0.034		
Potassium				
Present / Absent[1]				
OR	-	-	0.15	-
CI 95%			0.01–2.04	
P value			0.158	
[1] Reference variable.				

In Table 4 it is shown that current alcohol consumption is a risk factor for shorter relapse-free survival and increased threefold the probability of disease relapsing ($p < 0.05$; Table 4). Current alcohol consumption also decreased the overall survival ($p < 0.05$). The four-year survival after surgery was only 42% in alcohol consumption and 74% in non-alcohol addicted (Fig. 3). The multivariate analysis also

showed that alcohol consumption is a risk factor for shorter overall survival, with twofold increase in risk ($p < 0.05$; Table 4).

Patients with tumors with vascular invasion had lower probability of relapse-free survival ($p < 0.01$): within two years after surgery, 64% of patients with vascular invasion had disease relapse, as compared with 34% of those with tumors without vascular invasion. (Fig. 4). In the multivariate analysis, tumor vascular invasion appeared as fivefold increased risk for shorter disease-free survival (HR = 5.108, CI = 1.536–16.989; Table 4).

Two years after surgery, 93% of patients with tumor vascular invasion died, as compared with 26% of those whose tumors did not have vascular invasion (Fig. 5). Multivariate analysis showed that tumor vascular invasion was a risk factor for shorter overall survival, increasing threefold the probability of death (HR = 2.954, CI = 1.164–7.499; Table 4).

In Table 4 it is also shown that the presence in the tumor of chlorine was associated with fivefold decrease in the disease relapse rates (HR = 0.210, CI = 0.046–0.970). There was a trend not statiscally confirmed ($p = 0.069$) that presence of chromium would be related to shorter relapse-free survival.

Table 4
Cox model of prognostic factors and survival in patients which oral squamous cell carcinoma.

Features	Cox model	
	Recurrence-free survival	Overall survival
Current alcohol consumption		
Yes / No [1]		
OR	3.247	2.370
CI 95%	1.022–10.135	1.035–5.423
P value	0.046	0.041
Vascular invasion		
Present / Absent[1]		
OR	5.108	2.954
CI 95%	1.536–16.989	1.164–7.499
P value	0.008	0.023
Perineural invasion		
Present / Absent[1]		
OR		1.871
CI 95%	-	0.730–4.793
P value		0.192
Chlorine		
Present / Absent[1]		
OR	0.210	
CI 95%	0.046–0.970	-
P value	0.046	
Chrome		
Present / Absent[1]		
OR	2.980	-

[1] Reference variable.

Features	Cox model	
	Recurrence-free survival	Overall survival
CI 95%	0.917–9.688	
P value	0.069	
Manganese		
Present / Absent[1]		
OR	-	1.548
CI 95%		0.710–3.375
P value		0.272
Trx Cytoplasmic		
Strong / Weak[1]		
OR	2.019	-
CI 95%	0.632–6.450	
P value	0.236	
[1] Reference variable.		

Discussion

The results of this study respecting the relationships between the histopathological characteristics of OSCC tumors and the data of the clinical evolution of the patients were confirmatory of the reports from the literature [17–23]. In several previous studies, the presence of vascular and perineural invasion of the tumor has been related with worse prognosis, i.e., disease relapse, shorter survival and higher death rates [21, 23]. In fact, the vascular and perineural invasion of the tumor are fundamental mechanisms for metastatization and recurrence of tumors and constitute important prognostic factors [18–20]. Regarding the demographic relationships, our results are also in agreement with the previous reports of older age being a factor for better prognosis [24, 25]. The classical relation between alcohol consumption and worse prognosis [26–29] was also documented here. Unexpectedly, in contrast with previous reports [30–32] the smoking habit was not determinant of worse prognosis. It is possible that, in our study, the fact that among non-smokers few had never smoked, and many were ex-smokers, has concealed this rather classical relationship. Therefore, in general terms, the Brazilian population sample studied here had the typical prognostic features of those from other countries.

Our research group was the first to investigate the presence of metals in oral cancer by μ-XRF analysis [33]. Using this simple and straightforward approach, it is possible to perform multielemental analysis

without the disruptive preparation of the tissue samples. It is of note that among the fifteen different metals analysed in the tumor fragments only two had relationship with the disease evolution data of the patients. The presence of two of those two metals, chlorine and chromium was associated to disease relapse.

In the patients studied here, the concentration in the serum of the metals was not determined. Nonetheless, in none of the previous studies with elevated serum chlorine was found in oral cancer patients, in contrast, high concentrations of Cu and Zn were reported in patients with this cancer type but with no relationship with the clinical evolution or demographic data [34–36]. Our finding that the presence of chromium in the tumor was determinant of worse clinical evolution and that of chloride of better evolution suggests that the analysis of the tumor metal content may be important to unravel new mechanisms underlying the course of the disease.

Some hints of mechanisms whereby the presence of chromium may adversely affect the clinical evolution, with increased relapse rates, can be suggested by the studies of Shi et al. [37, 38]. Those authors postulate that reduction of hexavalent Cr to trivalent Cr generates oxygen radicals with activation of signaling pathways of apoptosis inhibition, via PI3K and AKT. The inhibition of apoptosis leads to the accumulation of mutations. This favors microenvironmental changes that stimulate tumor progression and relapse.

In respect to our finding of the relationship between presence of chlorine and decreased rate of tumor recurrence, it is possible that excess chlorine consequent to disturbances in the ionic channel function, specifically the chloride intracellular chanell 1 e 4 (CLIC1 CLIC4), lowers the cytoplasmic pH thereby inducing the tumor cell apoptosis. In fact, it has been recently shown that CLIC1 is involved in the regulation of the cell cycle and of cell volume, as well as in the regulation of apoptosis. It is postulated that CLIC1 has an important role in tumor development [33, 39, 40].

Regarding the protein expression of the four different enzymes studied here, only OGG1/2 expression showed relation with disease prognosis. Nonetheless, the expression of the three other enzymes, SOD1, Ref-1 and Trx were related to the tumor size. In patients with tumors exihibiting strong expression of OGG, there was less occurrence of disease relapse.

The protective action against disease relapse offered by strong OGG1/2 expression in the tumors can be ascribed to the reduction of ROS and of mitochondrial DNA damage resulting from the action of this enzyme. OGG1/2 inhibits the activation of p-AKT and of HIF1 which leads to decrease in progression and metastatization of tumors, as observed in mice models of breast tumor [41]. In OGG1/2 KO mice, ROS accumulation occurred, together with non-repair of oxidative damage generated in DNA by suppressing the Nrf2 pathway [42]. Progression of hepatocellular adenocarcinoma induced by phenobarbital to hepatocellular carcinoma was increased in the KO animals, which highlights the importance of OGG1/2 as a key enzyme acting in DNA repair [42]. Thus, our finding that disease relapse was less frequent in patients with strong OGG1/2 expression was in line with those anti-neoplastic actions of this enzyme.

Noteworthy was the fact that the expression of the three other enzymes that were unrelated to disease progression had otherwise strong relation with the size of the tumors. In this respect, strong nuclear expression of Ref-1 determined sixfold larger tumors, while cytoplasmic strong expression of SOD-1 and Trx were related to four and threefold larger tumors, respectively. The association between tumor size and the strong expression of these enzymes can be accounted for the activities of the enzymes that favor tumor growth. SOD-1 increases the oxidative burden, Ref-1 activates transcription factors such as early-response protein-1 (Egr-1), NF-κB, p53, HIF1α (AP-1), which are involved in various cellular processes, including cell survival and growth [43–46]. Trx-1 expression has been related to cancer development and spread [47]. Trx-1 has redox activity and is related to activation of different transcription factors of inflammation regulation, including NF-kB and activator protein-1 (AP-1) [11]. In addition, its action may increase expression of HIF1α, a hypoxia transcription factor [48], possibly by inhibiting the degradation of HIF1α [49]. Trx-1 binds and inhibits pro-apoptotic proteins, including apoptosis signal by regulating kinase-1 (Ask-1) [50]. Thus, increased protein expression of Trx-1 suggests that increase in the activity of this enzyme would favor tumor growth. This may occur by either altering inflammatory factors, or by activation of angiogenesis via HIF-VEGF.

Conclusion

The results show that detectable amounts chromium and chlorine in the tumor tissue, as well as the occurrence of strong protein expression of OGG1/2, SOD1, Ref-1 and Trx-1 may influence the tumor growth and predict the clinical evolution of OSCC. These findings may lead to the establishment of useful tools not only for disease prognosis but eventually for acquisition of new therapeutic targets.

Abbreviations

OCSCC = Squamous cell carcinoma; μ-XRF = micro-X-ray fluorescence; SOD-1= superoxide dismutase; Trx= thioredoxin; Ref-1= purinic/apyrimidinic endonuclease/redox factor-1; OGG1/2= 8-oxoguanine glycosylase; HPV= human papilloma virus; ROS= reactive oxygen species; ICAVC= Arnaldo Vieira de Carvalho Cancer Institute; GENCAPO= Genome Head and Neck Project; LNLS= The National Synchrotron Light Laboratory; Tissue microarrays= TMA; CNPEM= National Center for Energy and Materials Research; MCTIC= Social Organization overseen by the Ministry of Science, Technology, Innovations and Communications; OR= Odds Ratio; CI= confidence interval; Cl= chlorine; Cu= copper; Cr= chromium; Zinc= Zn; PI3K= Phosphoinositide 3-kinases; AKT= Protein kinase B; CLIC1= chloride intracellular chanell 1; CLIC4= chloride intracellular chanell 4; HIF1= Hypoxia-inducible factor 1; Nrf2= Nuclear factor erythroid 2-related factor 2; KO= Knockout; Egr-1= early-response protein-1; NF-κB= nuclear factor kappa B; AP-1= Activator protein 1; Ask-1= kinase-1; VEGF= vascular endothelial growth factor.

Declarations

Ethics approval and consent to participate

This study was approved by the Committees of Ethics Research of the Federal University of Espírito Santo [CAAE: 49091515.9.0000.5060] and the Arnaldo Vieira de Carvalho Cancer Institute [CAAE: 49091515.9.3002.5471]. The study was carried out in accordance with all relevant guidelines. As this was a retrospective study, the requirement for informed consent was waived. The GENCAPO project was approved by National Research Ethics Commission (CONEP) [Technical advice: 128/2012; CONEP: 16491].

Consent for publication

Not applicable.

Availability of data and materials

The data generated or analyzed during this study are included in this article and supplementary files. Extra information is available with the corresponding author upon request.

Competing interests

The authors declare that no competing interests exist.

Funding

This work was supported by Fundação de Amparo à Pesquisa e Inovação do Espírito Santo (FAPES), grant number 80706487/18. ALEMA was supported by FAPES fellowship (Process: 71896880). ABA was supported by FAPES/PROFIX (Process: 83757490) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001. AMAS is supported by FAPES (Process: 83965670). BVN is supported by CNPq fellowship (Process: 160990/2019-0). RCM holds a 1A Research Award from CNPq.

Authors' contributions

ALEMA and ABA carried out the experimental study and, analysis and interpretation of data and drafted the manuscript equally. RPS and RC collected clinical specimen and data. ALEMA, ABA, SOM and CJGP perfomed the elementary characterization. ALEMA, ABA, MMO, ARB, LLM, MS and LOT performed immunohistochemistry and data analysis. FDN and MS perfomed a critical review of the manuscript. RCM participated in the supervision and in the critical revision of the manuscript. BVN and AMAS contributed to the conception and supervision of the study, data interpretation and writing manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements

We thank Arnaldo Vieira de Carvalho Cancer Institute for making the OCSCC samples available through the Head and Neck Genome Project (http://www.gencapo.famerp.br/). We also thank Brazilian Synchrotron Light Laboratory for carrying out the analysis of micro-X-Ray Fluorescence [Proposal

20150102 - Elemental micro-imaging and quantifcation of squamous cell carcinoma head and neck, microenvironment tumor and its relationship with hypoxia gene expression and oxidative stress].

References

[1] Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians*, *68*(6), 394-424.

[2] Lindenblatt R de CR, Martinez GL, Silva LE, Faria PS, Camisasca DR, Lourenço S de QC. Oral squamous cell carcinoma grading systems – analysis of the best survival predictor. J Oral Pathol Med 2012;41:34–9. doi:10.1111/j.1600-0714.2011.01068.x.

[3] Liu S-A, Wang C-C, Jiang R-S, Lee F-Y, Lin W-J, Lin J-C. Pathological features and their prognostic impacts on oral cavity cancer patients among different subsites – A singe institute's experience in Taiwan. Sci Rep 2017;7. doi:10.1038/s41598-017-08022-w.

[4] Baxi SS, Pinheiro LC, Patil SM, Pfister DG, Oeffinger KC, Elkin EB. Causes of death in long-term survivors of head and neck cancer. Cancer 2014;120:1507–13. doi:10.1002/cncr.28588.

[5] Mendel JR, Baig SA, Hall MG, Jeong M, Byron MJ, Morgan JC, et al. Brand switching and toxic chemicals in cigarette smoke: A national study. PLoS One 2018;13:e0189928.

[6] Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006;160:1–40. doi:10.1016/j.cbi.2005.12.009.

[7] Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol Med 2010;49:1603–16. doi:10.1016/j.freeradbiomed.2010.09.006.

[8] Curtis CD, Thorngren DL, Nardulli AM. Immunohistochemical analysis of oxidative stress and DNA repair proteins in normal mammary and breast cancer tissues. BMC Cancer 2010;10:9. doi:10.1186/1471-2407-10-9.

[9] David SS, O'Shea VL, Kundu S. Base-excision repair of oxidative DNA damage. Nature 2007;447:941–50. doi:10.1038/nature05978.

[10] Shah F, Logsdon D, Messmann RA, Fehrenbacher JC, Fishel ML, Kelley MR. Exploiting the Ref-1-APE1 node in cancer signaling and other diseases: from bench to clinic. Npj Precis Oncol 2017;1:19. doi:10.1038/s41698-017-0023-0.

[11] Powis G, Kirkpatrick DL. Thioredoxin signaling as a target for cancer therapy. Curr Opin Pharmacol 2007;7:392–7. doi:10.1016/j.coph.2007.04.003.

[12] Cajaiba MM, Neves JI, Casarotti FF, de Camargo B, ChapChap P, Sredni ST, et al. Hepatoblastomas and Liver Development: A Study of Cytokeratin Immunoexpression in Twenty-Nine Hepatoblastomas. Pediatr Dev Pathol 2006;9:196–202. doi:10.2350/05-12-0002.1.

[13] Solé VA, Papillon E, Cotte M, Walter P, Susini J. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. Spectrochim Acta Part B At Spectrosc 2007;62:63–8. doi:https://doi.org/10.1016/j.sab.2006.12.002.

[14] Pérez CA, Radtke M, Sánchez HJ, Tolentino H, Neuenshwander RT, Barg W, et al. Synchrotron radiation X-Ray fluorescence at the LNLS: beamline instrumentation and experiments. X-Ray Spectrom 1999;28:320–6. doi:10.1002/(SICI)1097-4539(199909/10)28:5<320::AID XRS359>3.0.CO;2-1.

[15] Soini Y, Kahlos K, Puhakka A, Lakari E, Säily M, Pääkkö P, et al. Expression of inducible nitric oxide synthase in healthy pleura and in malignant mesothelioma. Br J Cancer 2000;83:880–6. doi:10.1054/bjoc.2000.1384.

[16] Santos M dos, Mercante AM da C, Louro ID, Gonçalves AJ, Carvalho MB de, da Silva EHT, et al. HIF1-Alpha Expression Predicts Survival of Patients with Squamous Cell Carcinoma of the Oral Cavity. PLoS One 2012;7.

[17] Yanamoto S, Yamada S, Takahashi H, Yoshitomi I, Kawasaki G, Ikeda H, et al. Clinicopathological risk factors for local recurrence in oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2012;41:1195–200. doi:10.1016/j.ijom.2012.07.011.

[18] Jardim JF, Francisco ALN, Gondak R, Damascena A, Kowalski LP. Prognostic impact of perineural invasion and lymphovascular invasion in advanced stage oral squamous cell carcinoma. Int J Oral Maxillofac Surg 2015;44:23–8. doi:10.1016/j.ijom.2014.10.006.

[19] Jones HB, Sykes A, Bayman N, Sloan P, Swindell R, Patel M, et al. The impact of lymphovascular invasion on survival in oral carcinoma. Oral Oncol 2009;45:10–5. doi:10.1016/j.oraloncology.2008.03.009.

[20] Kim JM, Kim TY, Kim WB, Gong G, Kim SC, Hong SJ, et al. Lymphovascular invasion is associated with lateral cervical lymph node metastasis in papillary thyroid carcinoma. Laryngoscope 2006;116:2081–5.

[21] Close LG, Burns DK, Reisch J, Schaefer SD. Microvascular Invasion in Cancer of the Oral Cavity and Oropharynx. Arch Otolaryngol Neck Surg 1987;113:1191–5. doi:10.1001/archotol.1987.01860110057008.

[22] Michikawa C, Uzawa N, Kayamori K, Sonoda I, Ohyama Y, Okada N, et al. Clinical significance of lymphatic and blood vessel invasion in oral tongue squamous cell carcinomas. Oral Oncol 2012;48:320–4. doi:10.1016/j.oraloncology.2011.11.014.

[23] Matsushita Y, Yanamoto S, Takahashi H, Yamada S, Naruse T, Sakamoto Y, et al. A clinicopathological study of perineural invasion and vascular invasion in oral tongue squamous cell carcinoma. Int J Oral Maxillofac Surg 2015;44:543–8. doi:10.1016/j.ijom.2015.01.018.

[24] Garavello W, Spreafico R, Gaini RM. Oral tongue cancer in young patients: A matched analysis. Oral Oncol 2007;43:894–7. doi:10.1016/j.oraloncology.2006.10.013.

[25] Bonifazi, Martina, et al. "Age–period–cohort analysis of oral cancer mortality in Europe: The end of an epidemic?." Oral Oncology 47.5 (2011): 400-407.

[26] Li Y, Mao Y, Zhang Y, Cai S, Chen G, Ding Y, et al. Alcohol drinking and upper aerodigestive tract cancer mortality: A systematic review and meta-analysis. Oral Oncol 2014;50:269–75. doi:10.1016/j.oraloncology.2013.12.015.

[27] International Agency for Reseach on Cancer (IARC). Section 2.2. Cancer of the oral cavity and pharynx. In: IARC Working Group on the Evalutionof Carcinogenic Risks to Humans.Alcohol Consumption and Ethyl- carbamate. Lyon, France: IARC Press; 2010:237-329.

[28] Nelson DE, Jarman DW, Rehm J, Greenfield TK, Rey G, Kerr WC, et al. Alcohol-Attributable Cancer Deaths and Years of Potential Life Lost in the United States. Am J Public Health 2013;103:641–8. doi:10.2105/AJPH.2012.301199.

[29] Praud D, Rota M, Rehm J, Shield K, Zatoński W, Hashibe M, et al. Cancer incidence and mortality attributable to alcohol consumption. Int J Cancer 2016;138:1380–7. doi:10.1002/ijc.29890.

[30] South, Andrew P. et al. Mutation signature analysis identifies increased mutation caused by tobacco smoke associated DNA adducts in larynx squamous cell carcinoma compared with oral cavity and oropharynx. Scientific reports, v. 9, n. 1, p. 1-9, 2019.

[31] Boyle, Jay O., et al. "Effects of cigarette smoke on the human oral mucosal transcriptome." Cancer prevention research 3.3 (2010): 266-278.

[32] Lin, Wen-Jiun et al. Smoking, alcohol, and betel quid and oral cancer: a prospective cohort study. Journal of oncology, v. 2011, 2011.

[33] Archanjo, Anderson Barros et al. elemental characterization of oral cavity squamous cell carcinoma and its relationship with smoking, prognosis and survival. Scientific Reports, v. 10, n. 1, p. 1-10, 2020.

[34] Chen, Fa et al. Serum copper and zinc levels and the risk of oral cancer: A new insight based on large-scale case–control study. Oral diseases, v. 25, n. 1, p. 80-86, 2019.

[35] Sachdev, Prageet K. et al. Zinc, copper, and iron in oral submucous fibrosis: A meta-analysis. International journal of dentistry, v. 2018, 2018.

[36] Khanna, Sunali et al. Trace elements (copper, zinc, selenium and molybdenum) as markers in oral sub mucous fibrosis and oral squamous cell carcinoma. Journal of Trace Elements in Medicine and Biology, v. 27, n. 4, p. 307-311, 2013.

[37] Shi X, Dalal NS. The role of superoxide radical in chromium(VI)-generated hydroxyl radical: The Cr(VI) haber-weiss cycle. Arch Biochem Biophys 1992;292:323–7. doi:10.1016/0003-9861(92)90085-B.

[38] Shi X, Dalal NS. Generation of hydroxyl radical by chromate in biologically relevant systems: role of Cr(V) complexes versus tetraperoxochromate(V). Environ Health Perspect 1994;102:231–6. doi:10.1289/ehp.94102s3231.

[39] Feng J, Xu J, Xu Y, Xiong J, Xiao T, Jiang C, et al. CLIC1 promotes the progression of oral squamous cell carcinoma via integrins/ERK pathways. Am J Transl Res 2019;11:557–71.

[40] Peretti M, Angelini M, Savalli N, Florio T, Yuspa SH, Mazzanti M. Chloride channels in cancer: Focus on chloride intracellular channel 1 and 4 (CLIC1 AND CLIC4) proteins in tumor development and as novel therapeutic targets. Biochim Biophys Acta - Biomembr 2015;1848:2523–31. doi:10.1016/j.bbamem.2014.12.012.

[41] Yuzefovych L V, Kahn AG, Schuler MA, Eide L, Arora R, Wilson GL, et al. Mitochondrial DNA Repair through OGG1 Activity Attenuates Breast Cancer Progression and Metastasis. Cancer Res 2016;76:30–34. doi:10.1158/0008-5472.CAN-15-0692.

[42] Kakehashi A, Ishii N, Okuno T, Fujioka M, Gi M, Fukushima S, et al. Progression of hepatic adenoma to carcinoma in Ogg1 mutant mice induced by phenobarbital. Oxid Med Cell Longev 2017;2017:16 pages. doi:10.1155/2017/8541064.

[43] Tell G, Damante G, Caldwell D, Kelley MR. The Intracellular Localization of APE1/Ref-1: More than a Passive Phenomenon? Antioxid Redox Signal 2005;7:367–84. doi:10.1089/ars.2005.7.367.

[44] Rani V, Deep G, Singh RK, Palle K, Yadav UCS. Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. Life Sci 2016;148:183–93. doi:10.1016/j.lfs.2016.02.002.

[45] Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. EMBO Rep 2002;3:420–5. doi:10.1093/embo-reports/kvf094.

[46] Kelley MR, Logsdon D, Fishel ML. Targeting DNA repair pathways for cancer treatment: what's new? Futur Oncol 2014;10:1215–37. doi:10.2217/fon.14.60.

[47] Hornsveld M, Dansen TB. The Hallmarks of Cancer from a Redox Perspective. Antioxid Redox Signal 2016;25:300–25. doi:10.1089/ars.2015.6580.

[48] Welsh SJ, Bellamy WT, Briehl MM, Powis G. The Redox Protein Thioredoxin-1 (Trx-1) Increases Hypoxia-inducible Factor 1α Protein Expression. Cancer Res 2002;62:5089–5095.

[49] Kim WJ, Cho H, Lee S-W, Kim Y-J, Kim K-W. Antisense-thioredoxin inhibits angiogenesis via pVHL-mediated hypoxia-inducible factor-1α degradation. Int J Oncol 2005;26:1049–52.

[50] Saitoh M, Nishitoh H, Fujii M, Takeda K, Tobiume K, Sawada Y, et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 1998;17:2596–2606. doi:10.1093/emboj/17.9.2596.

Figures

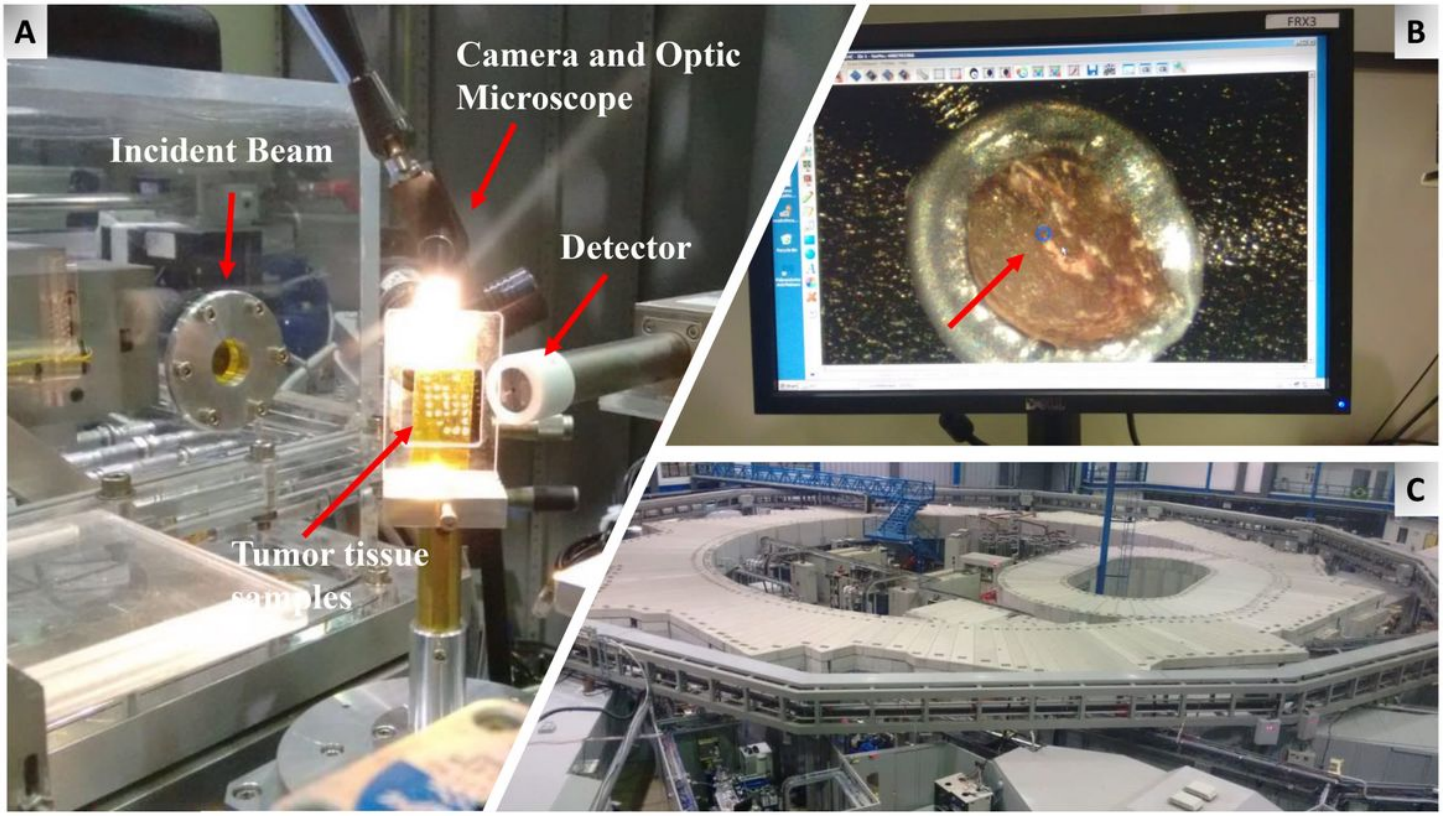

Figure 1

Cyclic accelerator of synchrotron light. A. The synchrotron micro-X-ray fluorescence (μ-XRF) radiation was used to detect chemical elements from electron excitation energy absorption performed on the D09-XRF beamline in oral cancer samples. The samples were deposited in plastic support with Ultralene® film and exposed to the synchrotron light to detect trace elements. B. Using a camera attached to an optic microscope it was possible to determine the spot of incidence of the beam on the sample (arrow). Nine measurements in a 3x3 matrix were performed. C. Overview of cyclic accelerator at the Brazilian Synchrotron Light Laboratory (LNLS), Campinas, SP, Brazil.

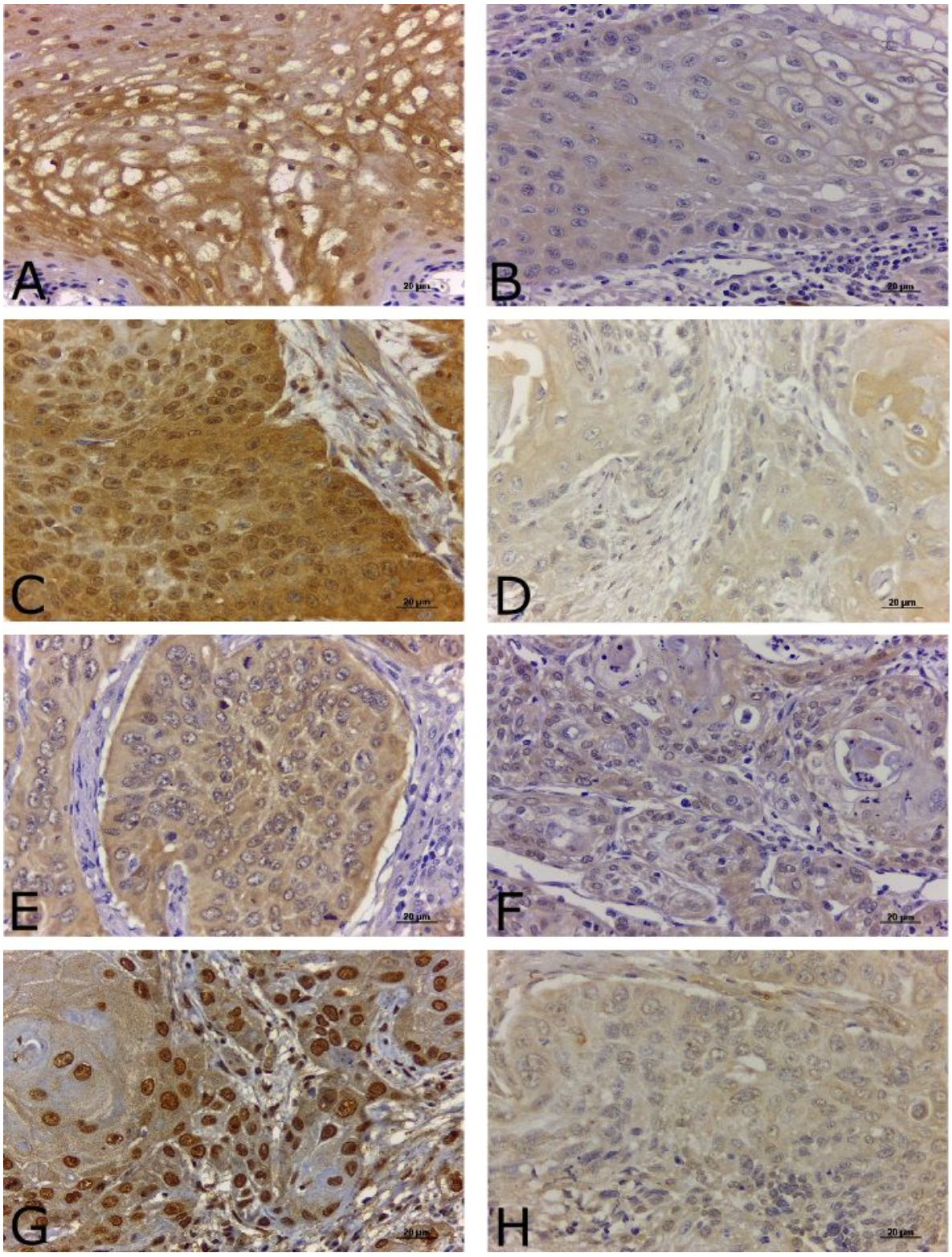

Figure 2

Imunnohistochemistry photomicrographs. (a) Low intensity cytoplasmic immunostaining of SOD-1. (b) Strong intensity cytoplasmic and nuclear immunostaining of SOD-1. (c) Strong intensity cytoplasmic and nuclear immunostaining of OGG1/2. (d) Low intensity cytoplasmic immunostaining of OGG1/2. (e) Strong intensity cytoplasmic immunostaining of Trx. (f) Low intensity cytoplasmic immunostaining of Trx. (g) Strong intensity cytoplasmic and nuclear immunostaining of Ref-1. (h) Low intensity cytoplasmic

immunostaining of Ref-1. SOD-1= superoxide dismutase; Ref-1= purinic/apyrimidinic endonuclease/redox factor-1; OGG1/2= 8-oxoguanine glycosylase. Original magnifications of 400x.

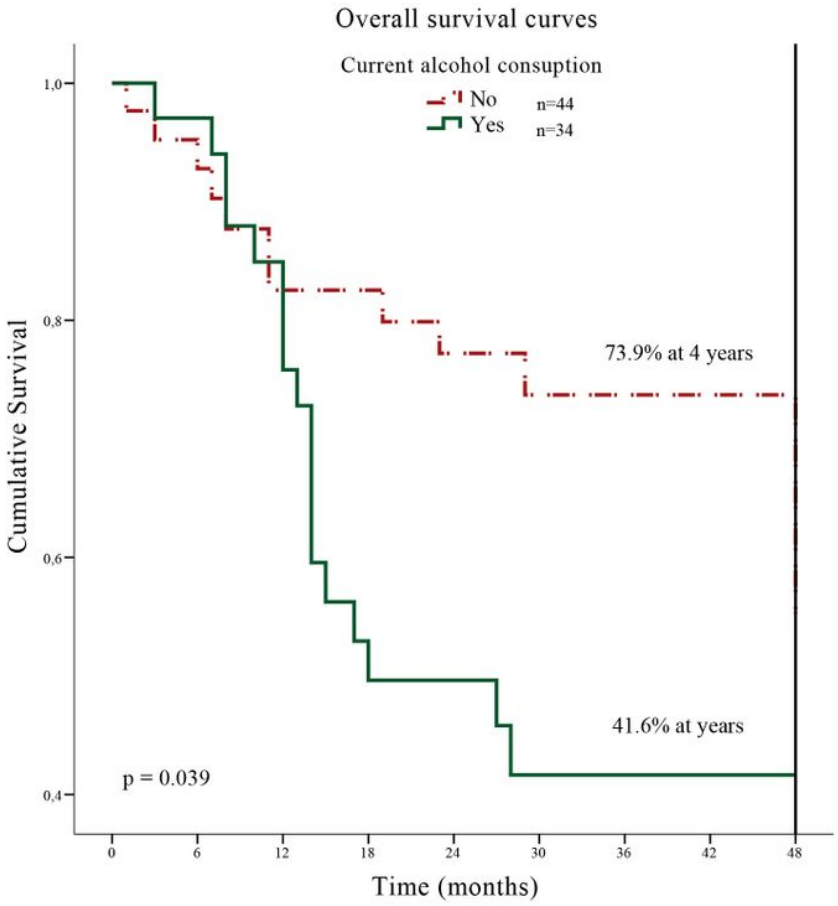

Figure 3

Overall survival plot. Kaplan-Meier curve is shown for overall survival in patients with squamous cell carcinoma of the oral cavity according to current alcohol consumption.

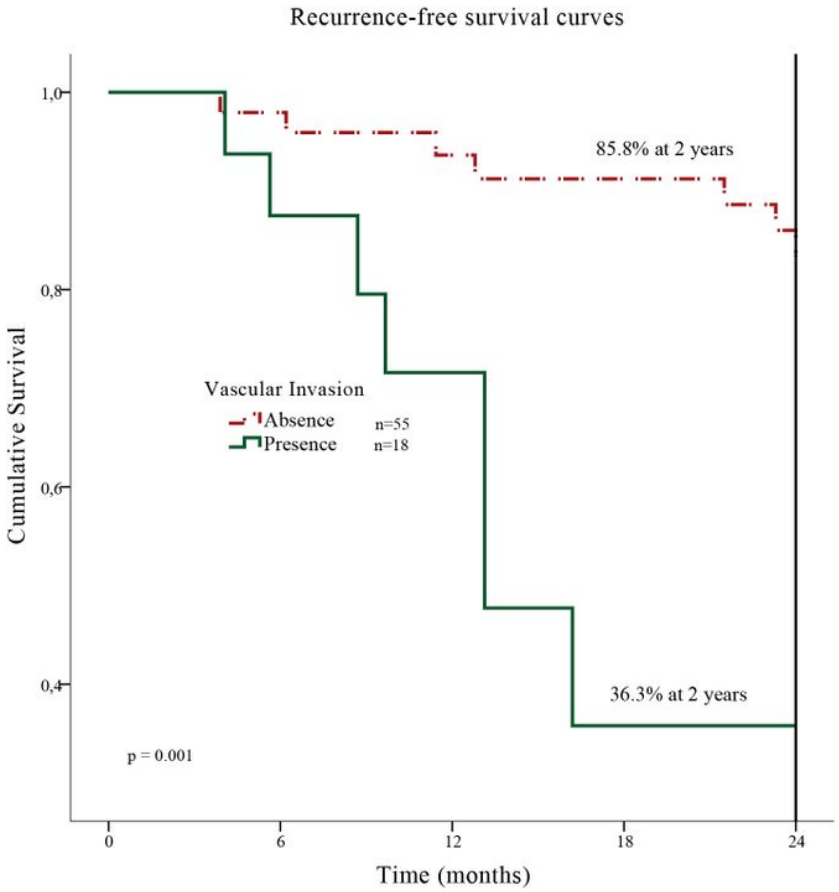

Figure 4

Recurrence-free survival plot. Kaplan-Meier curve is shown for recurrence-free survival in patients with squamous cell carcinoma of the oral cavity according to vascular invasion.

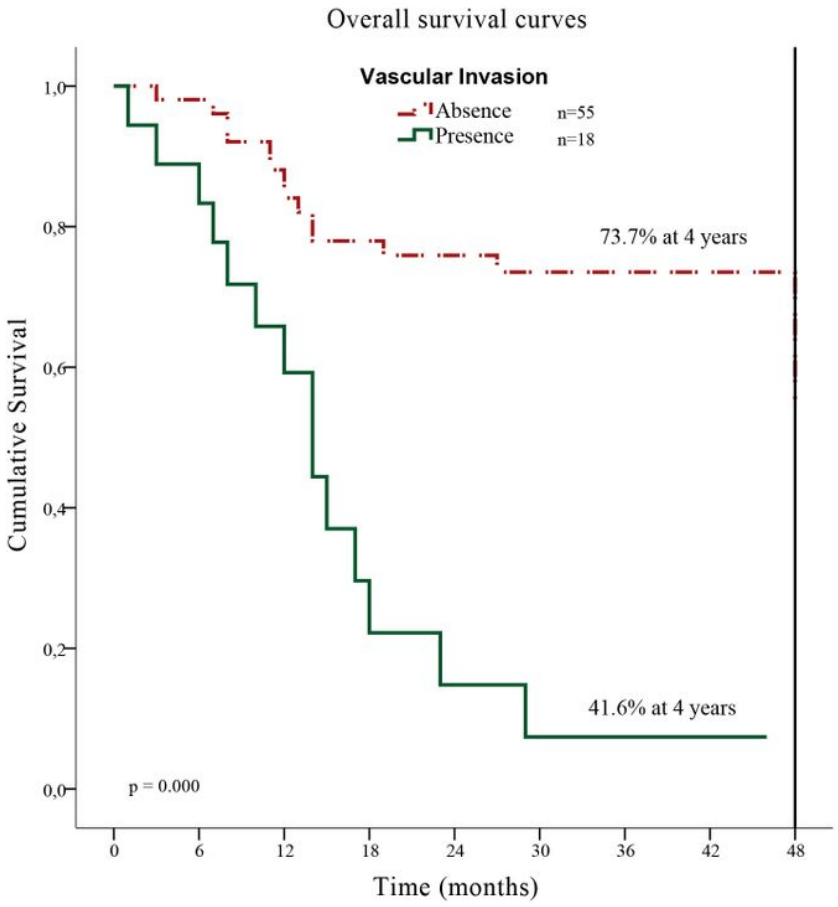

Figure 5

Overall survival plot. Kaplan-Meier curve is shown for disease-specific survival in patients with squamous cell carcinoma of the oral cavity according to vascular invasion.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.xlsx
- Supplementarytable2.xlsx