Paraoxonase-2 expression in oral squamous cell carcinoma

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Corresponding Author:	Davide Sartini Universita Politecnica delle Marche Ancona, Ancona ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universita Politecnica delle Marche
Corresponding Author's Secondary Institution:	
First Author:	Roberto Campagna
First Author Secondary Information:	
Order of Authors:	Roberto Campagna
	Valentina Pozzi
	Alessia Salvucci
	Lucrezia Togni
	Marco Mascitti
	Davide Sartini
	Eleonora Salvolini
	Andrea Santarelli
	Lorenzo Lo Muzio
	Monica Emanuelli
Order of Authors Secondary Information:	
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Response to Reviewers:	REVIEWER'S CONCERN. Figure 1A: Please indicate the molecular weight of the target protein in the figure. AUTHOR'S REPLY. As requested by the reviewer, in Figure 1A, molecular weight of target protein (PON2) was reported. REVIEWER'S CONCERN. Figure 1E, legend: "Correlation between OSCC enzyme levels and tumor size" - this description may be misleading. Please reconsider. AUTHOR'S REPLY. We thank the reviewer for this observation. To address the raised issue, we have specified that, in Figure 1E, Spearman's rank correlation coefficient was calculated to explore the relationship between enzyme levels in OSCC samples and their size. REVIEWER'S CONCERN. Figure 1F and G: The authors should describe definitions of low and high expression in the figure legend. In addition, the number of patients in each group should also be described in the legend. AUTHOR'S REPLY. Concerning this request, we specified that RNA-sequencing data from TCGA database were obtained for HNSCC patients, with the aim to evaluate 10-

year overall survival (OS) and recurrence-free survival (RFS). Patients were divided in two groups, namely "low PON2" and "high PON2", based on the automatic function of KM plotter, that found and set the best cut-off value of PON2 expression at 833. According to these considerations, patients included in the "high expression category" displayed PON2 levels higher than 883, while those classified within the "low expression group" exhibited enzyme levels lower than 883. In the legend section related to Figure 1F and G, we also indicated the number of patients constituting each group, while in the main text of the letter, we added additional info regarding the tool used to perform the statistical analyses that generated data reported in those figure panels.

Title

Paraoxonase-2 expression in oral squamous cell carcinoma

Authors

Roberto Campagna[1†], Valentina Pozzi[1†], Alessia Salvucci[1], Lucrezia Togni[1], Marco Mascitti[1], Davide Sartini[1*], Eleonora Salvolini[1], Andrea Santarelli[1,2], Lorenzo Lo Muzio[3], Monica Emanuelli[1,4].

Authors' affiliations

[1] Department of Clinical Sciences, Polytechnic University of Marche, 60020 Ancona, Italy

[2] Dentistry Clinic, National Institute of Health and Science of Aging, IRCCS INRCA, 60124 Ancona, Italy

[3] Department of Clinical and Experimental Medicine, University of Foggia, 71122 Foggia, Italy

[4] New York-Marche Structural Biology Center (NY-MaSBiC), Polytechnic University of Marche, 60131 Ancona, Italy

[†] These authors contributed equally to this work.

[*]**Corresponding author**

d.sartini@univpm.it; Tel.: +39-0712204673

Oral squamous cell carcinoma (OSCC) is the most common head and neck squamous carcinoma (HNSCC) and is characterized by high aggressiveness and metastatic potential. Every year, more than 300,000 new cases of OSCC are diagnosed from any district of the oral cavity, including tongue, lip, gingiva, palate, floor of the mouth or buccal mucosa [1]. OSCC early detection greatly influences both treatment options and patient prognosis. Indeed, since OSCC is often diagnosed lately, about 30% of newly diagnosed patients is classified as stage I-II OSCC, while the majority of patients is diagnosed of locally advanced or metastatic disease. Advanced OSCC is treated by surgical excision of the primary tumor coupled with adjuvant chemotherapy and radiotherapy, whereas neoadjuvant chemotherapy is required in patients affected by unresectable tumors. Despite the recent significant improvements in pharmacological therapy, patients affected by advanced OSCC display a 5-year survival rate below 50%, due to the high recurrence rate of the neoplasm, drug resistance and frequent metastatic disease [2].

In the present study, we focused our attention on the enzyme paraoxonase-2 (PON2), whose involvement was recently reported in relation to many neoplasms, where it was shown to contribute to tumor progression and chemoradioresistance [3-5]. Western blot analysis and immunohistochemistry were carried out to evaluate PON2 expression levels in two different cohorts of matched cancer and normal tissue samples, obtained from patients affected from OSCC. Correlations between tumor enzyme levels and clinicopathological parameters were also explored. In order to evaluate PON2 expression level in OSCC, preliminary Western blot analyses were conducted on 11 tumor and corresponding normal mucosa specimens, collected after surgery for diagnostic purpose, and retrieved from the archives of Pathology of the Department of Biomedical Sciences and Public Health, Polytechnic University of Marche. Tissue samples, obtained from patients who underwent surgical treatment for OSCC, were immediately frozen in liquid nitrogen and stored at -80°C until use. The specimens included 6 males and 5 females (mean age 64, age range of 48-91 years; mean diameter 2.7 cm, range 1.2-5.0; 5 pT1, 4 pT2, 1 pT3, 1 pT4; Lymph node metastases: 7 N0, 4 N+; pathological stage I: 3, II: 3, III: 1, IV: 4; histological grading G1: 1, G2: 7, G3: 3). Subsequent immunohistochemistry was performed on a different cohort of 30 formalin-fixed and paraffin-embedded (FFPE) OSCC and adjacent normal tissue specimens, obtained from the archives of Pathology (Department of Biomedical Sciences and Public Health, Polytechnic University of Marche) and collected after surgery. Pathological staging was assigned according to the AJCC Cancer Staging Manual (8th edition) and histological grading was based on WHO classification of head and neck tumors (4th edition). The specimens included 12 males and 18 females (mean age 70, age range of 25-92 years; mean diameter 3.4 cm, range 0.7-5.5; 5 pT1, 7 pT2, 9 pT3, 9 pT4; Lymph node metastases: 13 N0, 12 N+, not assigned: 5; pathological stage I: 5, II: 6, III: 5, IV: 14; histological grading G1: 2, G2: 19, G3: 7, not assigned: 2). This retrospective study was conducted in accordance with the principles of the Declaration of Helsinki.

Western blot analysis demonstrated that PON2 expression was significantly (p=0.001) higher (3.15-fold increase) in tumor compared with those detected in adjacent normal oral mucosa. Indeed, lanes loaded with equal protein amounts showed markedly increased enzyme levels in OSCC samples compared to that in matched normal oral tissues, which often displayed a faintly detectable band (Figure 1A and B).

To confirm these data, subsequent immunohistochemistry was carried out in a different cohort of 30 FFPE samples. PON2 protein expression was markedly (p<0.0001) increased in OSCC cells (40.87±39.20) compared with that of healthy tissue margins (00.00±00.00) (Figure 1C and D), which displayed absent cytoplasmic immunoreactivity. Statistical analyses demonstrated that there was no significant association between intratumor PON2 expression and gender (p=0.5612), age (p=0.3656) pT parameter (p=0.3144), lymph node metastases (p=0.8395), pathological stage (p=0.1268) and histological grading (p=0.3490). Interestingly, a statistically significant (p=0.0384) inverse correlation (r=-0.3799) was observed between enzyme levels and tumor size (Figure 1E), thus suggesting a crucial role played by PON2 in early phase of oral tumorigenesis.

These results are in agreement with what reported in TNMplot public online database (http://tnmplot.com/analysis/), that illustrates differential gene expression analysis in tumor, normal and metastatic tissues. Regarding oral cavity neoplasms, PON2 was described to be markedly overexpressed in cancer compared with normal tissue, while appearing significantly downregulated in metastatic disease with respect to primary tumor (data not shown). Concerning PON2 prognostic potential, enzyme expression was

not found to be significantly related neither with overall survival (OS) or with recurrence-free survival (RFS) of OSCC patients enrolled in this study (data not shown). Interestingly, statistical analyses performed on transcriptome data available in The Cancer Genome Atlas (TCGA) public online database, using Kaplan-Meier (KM) plotter (https://kmplot.com/analysis/) website tool, revealed that high levels of PON2 were significantly associated with reduced OS (Hazard Ratio = 1.53, 95% CI 1.16–2.23, p = 0.0025) (Figure 1F), thus suggesting a promising prognostic value for the enzyme. On the contrary, Kaplan-Meier analyzes showed no significant differences for RFS (Hazard Ratio = 1.57, 95% CI 0.74-3.33, p = 0.23) (Figure 1G).

To the best of our knowledge, this is the first exploratory study to demonstrate PON2 overexpression in OSCC. Altogether, the results obtained confirm the significant potential of the enzyme as a diagnostic and prognostic biomarker in a variety of neoplasms [6, 7]. Despite the recent improvements in cancer treatment, OSCC lethality remains high, especially if diagnosed lately, due to its aggressiveness and high metastatic potential. In this light, the identification of lesions characterized by a high risk of progression is therefore critical for the therapeutic intervention, identifying patients that would benefit from larger demolition surgery and/or more aggressive chemoradiotherapy. Thus, although further studies are required in order to elucidate the molecular mechanisms underlying the PON2 contribution to oral tumorigenesis, the enzyme may be considered a promising biomarker for OSCC both for diagnosis and prognosis.
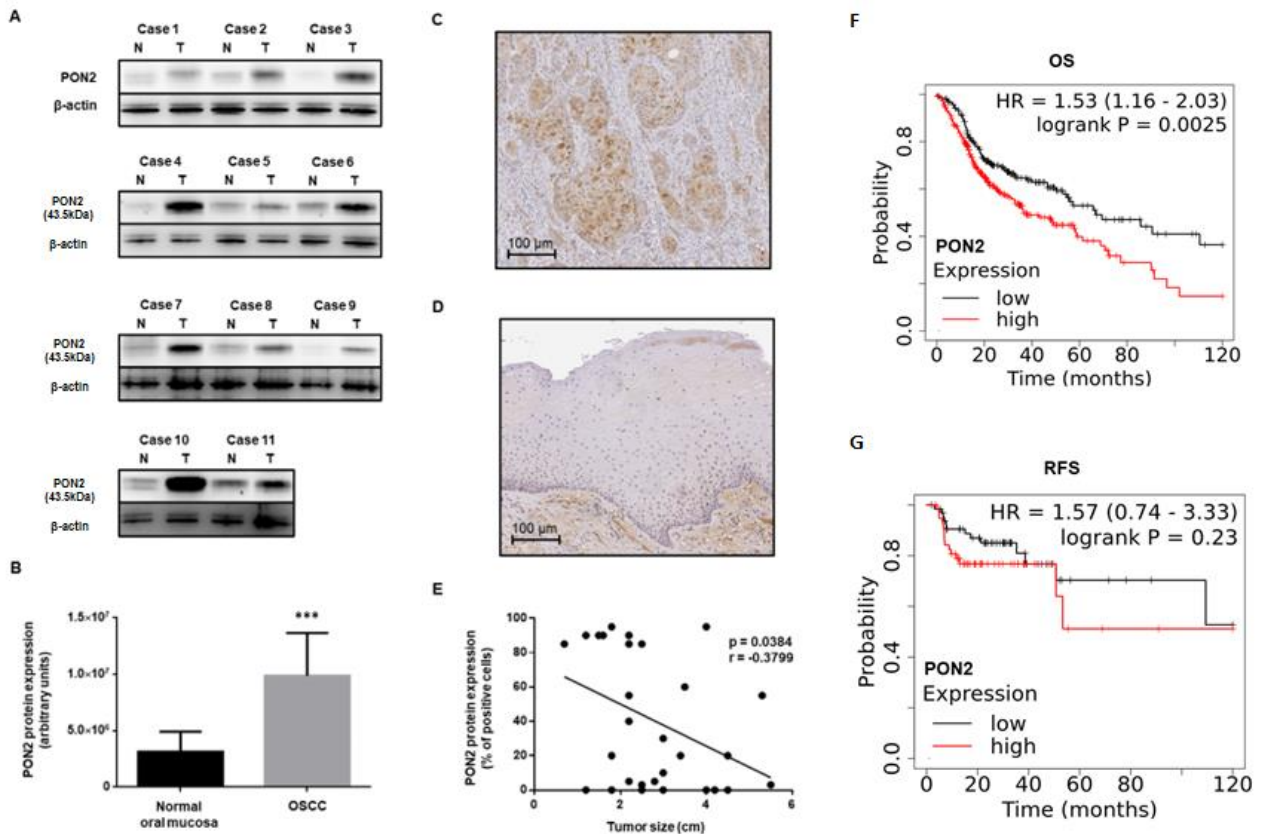

Figure 1. PON2 protein level was evaluated in 11 paired tumor (T) and normal (N) tissue specimens collected from patients affected with OSCC. Aliquots (20μg) of protein extract were subjected to 12.5% SDS-PAGE and transferred to polyvinylidene fluoride membrane. Blots were probed with rabbit anti-PON2 or anti-β-actin antibody, and analyzed with chemiluminescence (A). Densitometry was subsequently used to evaluate signal intensity of chemiluminescent bands and statistical analysis was performed to compare PON2 expression levels between tumor and normal tissue samples (B). Immunoistochemistry was carried out to evaluate PON2 expression in tumor (C) and surrounding healthy margin (D) related with FFPE tissue samples obtained from 30 OSCC patients (200x magnification). Spearman's rank correlation coefficient was calculated to explore the Correlation relationship between enzyme levels in OSCC samples and their size (E). Values are expressed as mean ± standard deviation (***p<0.005). Kaplan Meier curves were obtained by processing data available in The Cancer Genome Atlas (TCGA) public repository. In detail, RNA-sequencing data concerning enzyme levels were collected and analyzed with the aim to evaluate a significant relation with 10-year overall survival (OS) (panel F) and recurrence-free survival (RFS) (panel G) of HNSCC patients. The automatic function of KM plotter was used to find the best PON2 cut-off value, that was set at 833. In each case, patients were divided in two distinct groups, displaying low (<883) and high (>883) PON2 expression, respectively. In particular, patients with high PON2 expression (n=287) showed a reduced OS compared with patients with low PON2 expression (n=212). Regarding RFS, no difference was found between patients with low (n=66) and high (n=58) enzyme levels.

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