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ORIGINAL ARTICLE

Nrf2-peroxiredoxin I axis in polymorphous adenocarcinoma is associated with low matrix metalloproteinase 2 level

J. M. Brod[1] · Ana Paula Dias Demasi[1] · V. A. Montalli[1] · L. N. Teixeira[1] · C. Furuse[2] · M. C. Aguiar[3] · A. B. Soares[1] · M. Sperandio[1] · V. C. Araujo[1]

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Abstract Polymorphous adenocarcinoma (PAC) is a malignant epithelial neoplasm that affects almost exclusively the minor salivary glands, generally described as having a relatively good prognosis. Aberrant nuclear factor erythroid 2 (NF-E2)-related factor (Nrf2) activation in tumor cells has been associated with induction of antioxidant enzymes, such as peroxiredoxin I (Prx I) and increased matrix metalloproteinase (MMP) expression. In this context, the aim of the present study was to evaluate the expression of Nrf2 and correlate it with Prx I and MMP-2 secretion in PAC. Thirty-one cases of PAC from oral biopsies were selected and immunohistochemically analyzed for Nrf2 and Prx I. MMP-2 quantification was performed on primary cell cultures derived from PAC. Oral squamous cell carcinoma (OSCC) cell cultures were used as control. A high immunoexpression of Nrf2 was observed in both the cytoplasm and the nucleus of neoplastic cells from PAC. Nuclear staining for Nrf2 suggested its activation in the majority of the PAC cells, which was confirmed by the high expression of its target gene, Prx I. Quantification of MMP-2 secretion showed lower levels in PAC cell cultures when compared to OSCC cell cultures ($p < 0.05$). In conclusion, although Nrf2 overexpression has been frequently associated with high-grade malignancies, such relationship is not infallible and, in fact, the opposite may occur in low-grade tumors, such as PAC of minor salivary glands.

✉ Ana Paula Dias Demasi
demasiap@gmail.com

[1] Department of Oral Pathology, São Leopoldo Mandic Institute and Research Center, Rua José Rocha Junqueira 13, Swift, Campinas, SP 13045-755, Brazil

[2] State University of São Paulo, Araçatuba, SP, Brazil

[3] Federal University of Minas Gerais, Belo Horizonte, MG, Brazil

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Introduction

Polymorphous adenocarcinoma (PAC) is a malignant epithelial neoplasia that affects almost exclusively the minor salivary glands, being the second most common malignancy of such tissues [1]. It presents clinically as a painless slow-growing swelling, occasionally associated with bleeding and/or discomfort. PAC mainly affects the palate of females with a peak incidence between the fifth and eighth decade of life [2, 3].

PAC is characterized by cytological uniformity, morphological diversity, infiltrative growth, and low potential for metastasis [1]. Most tumor cells are uniform in size with ample cytoplasm and oval nuclei and inconspicuous nucleoli and also present indistinct outlines [4]. Tumor cells can be arranged in various histological patterns: solid, papillary, cribriform, and trabecular, and a single lesion may show more than one pattern. Mitotic figures are unusual and necrosis is not a typical finding. Immunohistochemically, PAC cells are positive for vimentin, cytokeratin 7 (CK 7), and S100 [1, 4].

It has been recognized that reactive oxygen species (ROS) may be involved in carcinogenesis through induction of DNA damage, especially under oxidative stress conditions, wherein their production overcomes the antioxidant defense system of the cell [5]. Nrf2 (nuclear factor erythroid 2 (NF-E2)-related factor) is a transcriptional factor first described in 1994 by Moi et al. [6] and found to play an important role in regulating the production of cytoprotective enzymes that safeguard cells from oxidative stress.

Under physiological conditions, Nrf2 is located in the cell cytoplasm forming an inactive complex with the cytoskeleton-

associated protein Keap1 (Kelch-like ECH-associated protein 1). An increased production of reactive oxygen species (ROS) and electrophiles promotes the release of Nrf2 from Keap1, allowing its translocation from the cytoplasm to the nucleus, where it binds to the antioxidant response elements (AREs), activating the transcription of genes that encodes antioxidant enzymes, such as peroxiredoxin I (Prx I) [7–9].

Prx I is the most abundant of the six isoforms of the mammalian peroxiredoxin family, a set of ubiquitous proteins able to catalyze the decomposition of peroxides with reducing equivalents provided through the thioredoxin system [10, 11]. Altered expression of Prx I has been described in diseases involving abnormal control of ROS, such as cardiovascular dysfunction, diabetes, neurodegenerative diseases, and different types of cancer [10–12].

Several studies have demonstrated the important and complex role that Nrf2 plays in the defense against carcinogens [13, 14]. Chemical carcinogenesis models using Nrf2-deficient mice revealed that they are more likely to undergo DNA damage than wild-type mice, which increased the susceptibility of the former to developing cancer [15]. Studies in vitro have shown that increased Nrf2 expression in cancer cells creates a favorable environment for proliferation; metastasis; evasion of apoptosis; and, ultimately, chemotherapy resistance [16–20]. In addition, the contribution of Nrf2 to tumor growth was also demonstrated by the association of its upregulation with increased matrix metalloproteinase (MMP) expression [21, 22]. MMPs are zinc-dependent endopeptidases with the ability to degrade extracellular matrix proteins and are involved in many phases of cancer progression including invasiveness and metastasis. On the other hand, recent report suggest that activation of the transcription factor Nrf2 has an antineoplastic effect in mucoepidermoid carcinoma (MEC) of the lung [23].

In this context, the aim of the present study was to evaluate the expression of Nrf2 and correlate it with Prx I labeling as well as MMP-2 secretion in PAC.

Materials and methods

This study was approved by the Research Ethics Committee of the São Leopoldo Mandic Dental School, Campinas, São Paulo, Brazil. Thirty-one cases of PAC biopsies were retrieved from the Oral Pathology archives of São Leopoldo Mandic Institute and Research Center, State University of São Paulo and Federal University of Minas Gerais. Tumor morphology was carefully evaluated using Hematoxylin and Eosin (H&E)-stained sections and the diagnosis confirmed by concomitant immunopositivity for CK-7, Vimentin, and S-100. For this study, only cases formerly classified as polymorphous low-grade adenocarcinoma were used. The clinical data of cases are depicted in the Table 1.

Table 1 Clinicopathological data of polymorphous adenocarcinoma used in the study

Case	Age (year)	Gender	Site
1	72	M	Palate
2	50	F	Palate
3	62	F	Buccal mucosa
4	48	F	Palate
5	51	F	Palate
6	35	F	Palate
7	83	F	Palate
8	48	F	Buccal mucosa
9	57	M	Palate
10	68	F	Upper Buccal Sulcus
11	66	M	Buccal mucosa
12	73	F	Upper lip
13	51	F	Lower Buccal Sulcus
14	67	F	Upper lip
15	70	M	Palate
16	52	F	Palate
17	69	F	Palate
18	58	M	Palate
19	77	M	Alveolar ridge and palate
20	41	M	Maxilla
21	36	F	NA
22	65	F	Palate
23	55	F	Palate
24	70	F	Palate
25	68	M	Alveolar ridge
26	45	F	Alveolar ridge
27	64	F	Palate
28	41	F	Upper lip
29	51	F	Palate
30	33	F	Upper Buccal Sulcus
31	67	M	Palate

F female, *M* male, *NA* not available

Immunohistochemistry

Thirty-one paraffin blocks were selected, from which only 29 cases had sufficient tissue available for both Nrf2 and Prx I immunostaining. Therefore, Nrf2 was tested in 31 cases and Prx I in 29 cases. Oral squamous cell carcinoma (OSCC) was used as positive control for Nrf2 and Prx I immunostaining.

Five-micrometer sections were deparaffinized and hydrated, and endogenous peroxidase activity was quenched by immersing the slides in 3% hydrogen peroxide for 30 min (Dinâmica, Diadema, SP, Brazil). Antigen retrieval (AR) was achieved by boiling the slides in a steamer immersed in a citrate buffer (pH 6.0) for 30 min. The sections were subsequently incubated with the primary antibodies for Nrf2 (1:50, overnight, Abcam, La Jolla, CA) and Prx I (1:500, 2 h,

Abcam). Peroxidase-linked secondary antibody and diaminobenzidine tetrahydrochloride (DAB) (Peroxidase Envision Kit, Dako Corp., Carpinteria, CA) were used to detect specific binding. The sections were counterstained with hematoxylin, dehydrated, and mounted. Appropriate positive and negative controls were used throughout.

Staining evaluation

Using a double-headed microscope, two examiners interpreted the immunohistochemical reactions for Nrf2. The labeled sections were evaluated semi-quantitatively. Positive nuclear and/or cytoplasmic staining were evaluated. Expression scores were assigned according to the percentage of nuclear positivity for Nrf2 and cytoplasmic positivity for Prx I into 0, 1, or 2 (0, negative or less than 5%; 1, between 5 and 50%; 2, more than 50% of nuclear/cytoplasm positive cells).

Cell culture experiments

To verify the possible involvement of the Nrf2-Prx I axis in the synthesis of MMPs in PAC, MMP-2 secretion by PAC-derived cells was also evaluated. OSCC cells were selected as positive controls, since this aggressive tumor is known to exhibit an increased nuclear expression for Nrf2 [24] and high levels of both Prx I [25–27] and MMP-2 [28–31].

Primary cell culture derived from polymorphous adenocarcinoma

The primary cell culture of PAC was established using the explant method followed by density gradient centrifugation in Percoll in order to separate epithelial cells from mesenchymal cells [32]. The neoplastic epithelial cells were then seeded onto 25 cm^2 culture flasks (Corning Incorporated, Costar, Corning, New York, NY) containing DMEM (Nutricell, Campinas, SP, Brazil) supplemented with 10% (*v*/*v*) fetal bovine serum (Cultilab, Campinas, SP, Brazil), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich, St. Louis, MO). Once the culture reached confluence, the cells were detached using a solution containing 1 mM EDTA (Gibco/Life Technologies, Grand Island, NY) and 2.5 mg/mL trypsin (Gibco/Life Technologies). The cells were cultured in 24-well polystyrene plates (Corning Incorporated) at 2×10^4 cells/well. During the culture period, cells were incubated at 37 °C in humidified atmosphere of 5% CO_2 and 95% air and the medium was changed every 3 days.

Oral squamous cell carcinoma cell culture

The CAL 27 (CRL-2095™) cell line of malignant keratinocytes derived from tongue squamous cell carcinoma were acquired from *the American Type Culture Collection* (ATCC, Manassas, VA) and cultured in 25 cm^2 flasks (Corning Incorporated) with DMEM/F-12 medium (LGC Biotechnology, São Paulo, SP, Brazil) supplemented with 10% (*v*/*v*) bovine fetal serum (Cultilab), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma Aldrich). At confluence, the cells were detached and cultured in 24-well plates in the same conditions described above.

MMP-2 quantification in polymorphous adenocarcinoma by ELISA

The quantification of MMP-2 in PAC and OSCC cells was assayed at day 7. Briefly, the culture medium was collected and centrifuged at 336×*g* for 10 min. The resulting supernatant was collected, aliquoted, and stored at − 80 °C. The cells were then detached using a solution containing 1 mM EDTA (Gibco/Life Technologies) and 2.5 mg/mL trypsin (Gibco/Life Technologies) and counted using a hemocytometer. MMP-2 quantification was performed using the Human MMP-2 DuoSet ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The experiment was carried out in quintuplicate, and MMP-2 concentration was established by spectrophotometry (ELX, Epoch Bioteck Instruments, Inc) at 590 nm. The absorbance values were normalized by the total number of cells and the data expressed as ng/10^4 cells.

Statistical analyses

Nrf2 and Prx I immunoexpression scores were analyzed using the Pearson's correlation test. Nrf2, Prx I, and MMP-2 quantification was analyzed using the Mann-Whitney test. The level of significance was established at 5%. All statistical calculations were performed on GraphPad Prism 6.

Results

Immunohistochemical detection of Nrf2 and Prx I in polymorphous adenocarcinoma

The immunohistochemical analysis showed homogeneous and strong positivity to Nrf2 in both the nuclei and the cytoplasm of the neoplastic cells (Fig. 1a). The nuclear expression scores for PAC were 0 in a single case (3%), 1 in 7 cases (23%), and 2 in 23 cases (74%). Regarding cytoplasmic expression, no case scored 0; score 1 was recorded in 4 cases (13%) and score 2 in 27 cases (87%) (Fig. 1b). All normal mucous cells from salivary gland tissue used as internal control were negative to Nrf2 (Fig. 1c), while OSCC, used as control, was strongly positive for Nrf2 (Fig. 1d).

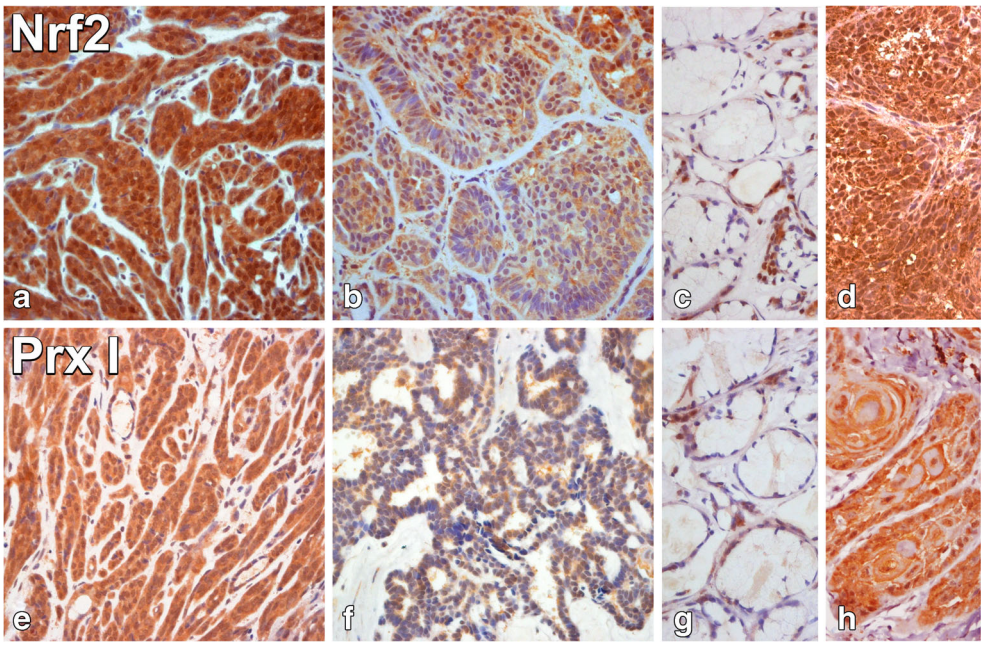

Fig. 1 Immunohistochemical detection of Nrf2 and Prx I in polymorphous adenocarcinoma (PAC). Tumor cells exhibited a strong positivity for Nrf2 in both the nuclei and the cytoplasm (score 2) (**a**). Seven cases were classified as score 1 (**b**). Prx I expression was predominantly observed in the cytoplasm of the tumors cells (**e**). Ten cases were classified as score 1 (**f**). All normal mucous cells, used as internal control, were negative for both Nrf2 (**c**) and Prx I (**g**), while oral squamous cell carcinoma (OSCC) was strongly positive for both proteins (**d**, **h**). Magnification ×400

Since Prx I is under Nrf2 regulation, its expression was evaluated in PAC cells to confirm Nrf2 activation. Prx I staining was observed in the cytoplasm of PAC cells, presenting score 0 in 3 cases (10.4%), score 1 in 10 cases (34.5%), and score 2 in 16 cases (55.1%) (Fig. 1e, f). All normal mucous cells from salivary gland tissue used as internal control were negative to Prx I (Fig. 1g), while OSCC, used as control, was strongly positive for Prx I (Fig. 1h), as it has been described in the literature [25–27].

Expression scores for Nrf2 and Prx I presented a significantly positive correlation ($r = 0.591$, $p = 0.0007$).

MMP-2 quantification in polymorphous adenocarcinoma by ELISA

Higher secretion of MMP-2 was detected in OSCC compared with PAC ($p < 0.05$) (Fig. 2).

Discussion

Nrf-2 expression has been associated with highly malignant tumors [16–20]. However, high levels of Nfr-2 expression was also reported in a mucoepidermoid carcinoma, which exhibits a favorable prognosis [23]. Tempted by these controversial results, we decide to elucidate whether this latter is a common event in salivary gland tumors. For this, we have studied the Nfr2-Prx I axis in PAC, which is a slow-growing tumor that rarely recurs or metastasizes [2–4].

In this study, we demonstrated a high Nfr2 expression in both the cytoplasm and the nucleus of neoplastic cells from PAC. Nuclear staining of Nrf2 demonstrated its activation in the majority of the PAC cells, which was confirmed by the correlated high expression of one of its target genes, Prx I. Although we have not compared PAC to different salivary gland tumors, the literature showed that in adenoid cystic carcinoma (ACC), a tumor more aggressive than PAC, Nfr-2, was highly expressed; meanwhile, it was lower in pleomorphic adenoma, a benign tumor [33, 34]. The intense expression of Nfr2 in both PAC and ACC suggests that Nfr2 is not related with the malignancy grade in salivary gland tumors.

Kim et al. [9] demonstrated the activation of Nrf2 and upregulation of *PRDX1* gene by changes in oxygenation status, which is a common microenvironmental finding in the natural selection process seen in tumourigenesis, suggesting that the Nrf2-Prx1 axis may be a promising prognostic marker

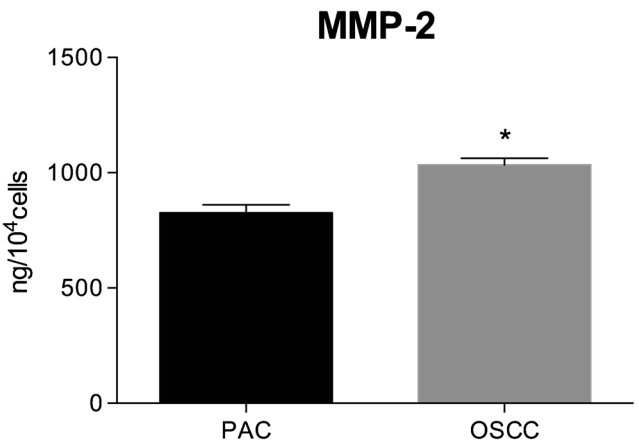

Fig. 2 Metalloproteinase-2 (MMP-2) quantification in the supernatant obtained from polymorphous adenocarcinoma (PAC) and oral squamous cell carcinoma (OSCC) cell cultures by ELISA. The data were normalized by the number of cells and are reported as the mean ± SD. Asterisk indicates statistical significance ($p < 0.05$)

as well as a potential target for therapeutic strategies [9]. Cycling hypoxia predisposes cells to damage associated with cellular exposure to ROS, which are generated at high levels following reperfusion, leading to an abrupt oxygen tension increase in the previously hypoxic cells [35, 36]. Thus, ROS-mediated Nrf2 activation and Prx I upregulation in PAC tumor cells observed herein could provide antioxidant protection to PAC cells in the unstable oxygenation tumor environment without interfering with the malignancy status.

Although Nrf2 mediates cancer chemoprevention in normal cells, recent studies have also demonstrated that aberrant Nrf2 activation confers an advantageous environment in which cancer cells can proliferate and metastasize [16–20]. The otherwise cytoprotective Nrf2-mediated signaling cascade is probably hijacked by cancer cells for their own survival, thus suggesting a possible role for Nrf2 in cancer biology. The evidence for such assumption comes from studies showing elevated levels of Nrf2 and its target protein heme oxygenase-1 (HO-1) in various tumors with high metastatic potential [18, 37–39].

A recent study has identified, on the contrary, that activation of the transcription factor Nrf2 has an antineoplastic effect in mucoepidermoid carcinoma (MEC) of the lung [23]. MEC of the lung is a rare subtype of NSCLC that originates from the submucosal bronchial glands and is associated with more favorable prognoses than other forms of intrathoracic malignancies. Such study was based on a previous report showing that low-grade pulmonary MECs are characterized by significantly attenuated expression of MMP-2 and MMP-9 when compared to typical lung cancer [40]. Contrary to what has been demonstrated for the commonest types of non-small cell lung cancer (NSCLC) [37], activation of the transcription factor Nrf2 in mucoepidermoid carcinoma diminished MMP levels attenuating tumor cell migration. The roles played by activated Nrf2 are largely attributable to the ARE-regulated gene HO-1, implying that the Nrf2/HO-1 axis could be one of the major molecular pathways contributing to the relatively mild clinical presentations of lung MECs [40]. The effects of Nrf2 and HO-1 expression on tumor metastasis and clinical prognosis are largely tumor-specific, and the exact mechanisms that determine how upregulation of the Nrf2/HO-1 axis will influence MMP expression and secretion remain to be clarified.

Based on the findings by Tertil et al. 2015 [23], the present study has also demonstrated that MMP-2 secretion was indeed much attenuated in PAC cell cultures when compared with the aggressive OSCC cells, despite the similar expression of Nrf2 and Prx I in both tumors, thus corroborating the parallel pattern observed between minor salivary gland PAC and lung MEC.

The findings from the present study demonstrated that Nrf2 is highly expressed in PAC, while MMP-2 secretion is lower in PAC than in OSCC, suggesting that despite significant associations between metastasis and Nrf2 overexpression in most high-grade malignancies, such relationship is not infallible and, in fact, the opposite may occur in low-grade neoplasms, such as PAC of the minor salivary glands.

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Authors' contributions The design of the present study was conceived by APD and VCA. Data acquisition was carried out by JMB and VAM. Data interpretation was made by LNT, CF, MCA, ABS, and MS. Work drafting was made by JMB, APD, LNT, MS, and VCA. Critical review was carried out by VAM, CF, MCA, and ABS. The final approval of the version to be published was done by all authors.

Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethics committee approval This study was approved by the Research Ethics Committee of the São Leopoldo Mandic Dental School (no. 42315715.1.0000.5374), Campinas, São Paulo, Brazil.

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