Pathology & Oncology Research (2020) 26:433–442
https://doi.org/10.1007/s12253-018-0517-2

ORIGINAL ARTICLE

Radiation Therapy Reduced Blood Levels of LDH, HIF-1α, and miR-210 in OSCC

Marcela Gonçalves de Souza[1] · Sabrina Ferreira de Jesus[1] · Eloá Mangabeira Santos[1] · Emisael Stenio Batista Gomes[1] · Arlen de Paulo Santiago Filho[2] · Eliane Macedo Sobrinho Santos[3] · Luiz Henrique da Silveira[1] · Sérgio Henrique Sousa Santos[4] · Alfredo Maurício Batista de Paula[1] · Lucyana Conceição Farias[1] · André Luiz Sena Guimarães[1,2,5]

Received: 28 November 2017 / Accepted: 25 October 2018 / Published online: 8 November 2018

Abstract

Radiation Therapy (RT) is a treatment option for a large number of neoplasias. However, the effect of RT on the level of hypoxia markers is poorly understood. The present study aimed to investigate the effect of RT on the levels of hypoxic markers in Oral squamous cell carcinoma (OSCC). Evaluation of HIF-1α and miR-210 levels in OSCC was performed. Then a proteomic analysis was performed to identify candidate hypoxic targets of RT. To validate proteomic studies, the effect of RT on HIF-1α, miR-210, PDH-A and LDH-A levels under hypoxia was assessed by qRT-PCR. The impact of RT in hypoxia markers was evaluated in patients to confirm in vitro results. An increase in the HIF-1α levels was observed in OSCC. RT reduced OSCC cell proliferation and migration. Interestingly, hypoxia could revert the effect of radiation on OSCC phenotype. However, proteomics analyses suggested that LDH is one of the critical targets of RT even in hypoxia. Moreover, RT decreased HIF-1α, miR-210, and LDH even in hypoxia. The current study demonstrated that hypoxia could revert the effects of RT in the OSCC context. However, RT reduces the levels HIF-1α, miR-210 and LDH in vivo and in vitro. The consequences of RT in blood should be carefully investigated.

Keywords Radiotherapy · Warburg effect · OSCC

Introduction

Oral squamous cell carcinoma (OSCC) is a significant public health problem in many countries [1] and is the most common malignant neoplasia of the oral cavity [1]. Alcohol and tobacco consumption are the most critical risk factors currently described for OSCC [2]. However, individual intrinsic factors also play a significant role in the development and prognosis of cancer [3–5]. The commonly used treatments for OSCC are surgical resection and Radiation Therapy (RT) associated or not with chemotherapy [6]. OSCC treatment may offer morbid conditions, including functional deficits, as well as significant aesthetic damages, such as speech deficiency, swallowing and facial deformity [7].OSCC relapse is common despite commonly applied multimodal therapy [8, 9]. Hypoxia is a significant factor related to radioresistance [8]. Pyruvate is the final product of aerobic glycolysis [10].

In different types of neoplastic cells, pyruvate is highly converted to lactate, a phenomenon known as Warburg's

Marcela Gonçalves de Souza, Sabrina Ferreira de Jesus and Eloá Mangabeira Santos contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12253-018-0517-2) contains supplementary material, which is available to authorized users.

✉ André Luiz Sena Guimarães
andreluizguimaraes@gmail.com

[1] Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Minas Gerais, Brazil

[2] Hospital Dilson Godinho, Montes Claros, Minas Gerais, Brazil

[3] Instituto Federal de Educação, Ciência e Tecnologia do Norte de Minas Gerais (IFNMG), Araçuaí, Minas Gerais, Brazil

[4] Institute of Agricultural Sciences, Food Engineering College, Universidade Federal de Minas Gerais (UFMG), Montes Claros, Minas Gerais, Brazil

[5] Hospital Universitário Clemente Faria, Laboratório de Pesquisa em Saúde, Universidade Estadual de Montes Claros, 562 Av. Cula Mangabeira Santo Expedito, Montes Claros, MG 39401-001, Brazil

effect [11, 12]. The enzyme pyruvate dehydrogenase (PDH), when activated, can alter the metabolism of neoplastic glycolysis cells to glucose oxidation [13]. Lactate dehydrogenase (LDH) is responsible for the interconversion of reduced pyruvate and nicotinamide adenine dinucleotide (NADH) generated by glycolysis to lactate and NAD [14, 15]. The direction of the catalysis depends on the ratio of LDH-A and LDH-B. LDH-A promotes the conversion of pyruvate to lactate to regenerate NAD + from NADH, whereas LDH-B facilitates the reverse reaction [14, 15]. LDH-A is the key enzyme involved in the Warburg effect and in maintaining the glycolytic phenotype of cancer [12, 14, 16]. Hypoxia is the primary factor responsible for the abnormal glycolytic flow in cancer cells [10], with HIF-1α being the principal protein of the hypoxia pathway [17]. HIF-1α induces transcription several targets genes that promote adaptations to hypoxia [18–23]. Genetic products transcribed by HIF-1α influence cell metabolism, cell survival, cell migration, pH regulation and cytokine secretion to initiate the phenomenon called angiogenesis [24, 25]. High levels of HIF-1α expression are associated with a worse prognosis in breast cancer, upper aerodigestive tract carcinoma and colorectal cancer [18, 20, 21]. Recent studies have identified essential functions of microRNAs in several cellular processes, including carcinogenesis [26] and the literature has shown that high levels of miR-210 participate in the stabilization of HIF-1α during hypoxia [27]. Overexpression of miR-210 was detected in patients with breast cancer and head and neck cancer [28, 29]. Also, miR-210 on expression is a crucial element in endothelial cell response to hypoxia, affecting cell survival, migration, and differentiation [30]. Studies have shown that hypoxia in the neoplasia, promotes higher resistance to RT [31–36]. However, RT can diminish the activity of HIF-1α [37]. The present study aimed to investigate the role of radiation in the expression of HIF-1α, miR-210, PDH-A and LDH-A and phenotype of OSCC cells under hypoxic conditions.

Patients and Methods

Patients

Ethical approval for this study was obtained from the Institutional Review Board, and a signed informed consent form was obtained from all patients (process number CAAE 62425316.0.0000.5146). Sample calculation was based on the service data and literature [1, 38]. According to radiation therapy dentistry service OSCC is 90% of all oral malignant neoplasia, so the sample size calculation was performed as described before [39] to have alpha 0.05, beta 0.4 and study power 0.6.

Groups

The 26 OSCC patients were enrolled in the current study. OSCC patients were divided into two groups. Group 1 (n = 17) OSCC patients without any cancer treatment. On the other hand, Group 2 (N = 9) OSCC patients followed during radiation therapy. A third Group was comprised of 18 volunteers without OSCC.

The inclusion criteria for group 1 and 2 was the was the histopathological confirmation of the diagnosis according to the World Health Organization criteria [40, 41]. Specifically, Squamous cell carcinoma of the base of the tongue, Squamous cell carcinoma of other or unspecified parts of tongue, Squamous cell carcinoma of gum, Squamous cell carcinoma of floor of mouth, Squamous cell carcinoma of palate, Squamous cell carcinoma of other or unspecified parts of mouth and Squamous cell carcinoma of oropharynx were included in OSCC group. As exclusion criteria, all OSCC lesion with lip involvement were excluded.

Group 3 was comprised of individuals without oral cancer. The exclusion criteria for group 3 was individuals who presented past cancer medical history. All groups were collected between February 2016 to May 2017, met the requirements for participation in the study. Detailed clinical information about the subjects is disposed of in Table 1.

Tissue Specimens

The specimens from group 1 were10 primary lesions and 7 blood samples. From Group 2 only blood samples (N = 9) were ethically possible to be obtained. Two blood samples from all group 2 patients were taken after first and the last radiation therapy section. The oral mucosa samples (n = 10) from group 3 were collected during third molar surgery. Additionally, group 3 was comprised of blood samples from another 8 healthy donors. For all groups, qRT-PCR tissue specimens samples that presented the expression of lower endogenous expression or incompatible melting curve were also excluded from analyses qRT-PCR.

Cell Culture and Hypoxia

Cells were maintained as described before [10, 42]. Briefly, SCC9 cells were maintained in Dulbecco's modified Eagles medium (DMEM / F12, GIBCO, Billings, MT, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, Billings, MT, USA)*, 400 ng / ml hydrocortisone and an antibiotic / antimycotic solution (Invitrogen, Carlsbad, CA, USA) at 37 °C with 5% CO_2 in an atmosphere of humidified air. The cells were seeded into a 12-well plate and synchronized for 24 h by the absence of fetal bovine serum to obtain synchronized cultures of SCC9 cells (1×10^5). All treatments were performed in the lack of SBF. SCC9 cells were cultured in

Table 1 Detailed clinical information about the subjects

ID	Group	Tissue	Age	Sex	Alcohol use	Smoking	T	N	M	ICD	Daily/ Total (cG)	Follow up (days)
1	OSCC in RT	Blood	64	Male	Yes	Yes	T3-T4	N0	M0	2B61.0	200/7000	386
2	OSCC in RT	Blood	58	Male	Quit	Yes	T3-T4	N1-N2-N3	Mx	2B61.0	200/7800	677
3	OSCC in RT	Blood	51	Male	Quit	Quit	T3-T4	N1-N2-N3	M0	2B6A.0	200/6600	822
4	OSCC in RT	Blood	70	Male	Quit	Yes	T3-T4	N0	Mx	2B62.0	200/6600	691
5	OSCC in RT	Blood	88	Male	Quit	Yes	T3-T4	N0	M0	2B6A.0	200/7000	602
6	OSCC in RT	Blood	58	Male	Quit	Quit	T1-T2	N0	Mx	2B62.0	200/6600	533
7	OSCC in RT	Blood	55	Male	Yes	Yes	T3-T4	N1-N2-N3	Mx	2B6A.0	200/7000	348
8	OSCC in RT	Blood	59	Female	Quit	Quit	T3-T4	N1-N2-N3	Mx	2B62.0	200/7000	704
9	OSCC in RT	Blood	60	Male	Quit	Yes	T3-T4	N1-N2-N3	Mx	2B63.0	200/6600	783
10	OSCC	Blood	65	Male	Yes	Quit	T1-T2	Nx	Mx	2B62.0	N/A	N/A
11	OSCC	Blood	57	Male	Quit	Quit	T3-T4	N0	Mx	2B6A.0	N/A	N/A
12	OSCC	Blood	63	Male	Yes	Yes	T3-T4	N1-N2-N3	Mx	2B6A.0	N/A	N/A
13	OSCC	Blood	60	Female	No	Quit	T3-T4	N0	Mx	2B64.0	N/A	N/A
14	OSCC	Blood	75	Female	Yes	Yes	T3-T4	N1-N2-N3	Mx	2B62.0	N/A	N/A
15	OSCC	Blood	51	Male	Quit	Quit	T3-T4	N1-N2-N3	M0	2B6A.0	N/A	N/A
16	OSCC	Blood	56	Male	Yes	No	T1-T2	N0	Mx	2B62.0	N/A	N/A
17	OSCC	Lesion	57	Male	Yes	Yes	Tx	N1-N2-N3	M0	2B66.0	N/A	N/A
18	OSCC	Lesion	68	Male	Yes	No	T3-T4	N0	M0	2B62.0	N/A	N/A
19	OSCC	Lesion	41	Female	No	No	T3-T4	N0	M0	2B66.0	N/A	N/A
20	OSCC	Lesion	72	Male	Yes	No	T3-T4	N0	M0	2B66.0	N/A	N/A
21	OSCC	Lesion	54	Male	No	No	T3-T4	Nx	M0	2B66.0	N/A	N/A
22	OSCC	Lesion	56	Male	Yes	No	T3-T4	Nx	M0	2B66.0	N/A	N/A
23	OSCC	Lesion	75	Male	Yes	Yes	T3-T4	N1-N2-N3	M0	2B66.0	N/A	N/A
24	OSCC	Lesion	48	Male	Yes	Yes	T3-T4	N1-N2-N3	M0	2B62.0	N/A	N/A
25	OSCC	Lesion	67	Male	No	No	T3-T4	Nx	M0	2B62.0	N/A	N/A
26	OSCC	Lesion	47	Male	Yes	Yes	T1-T2	Nx	M0	2B63.0	N/A	N/A
27	Control	Mucosa	38	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
28	Control	Mucosa	19	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
29	Control	Mucosa	19	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
30	Control	Mucosa	22	Male	No	No	N/A	N/A	N/A	Control	N/A	N/A
31	Control	Mucosa	32	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
32	Control	Mucosa	24	Male	No	No	N/A	N/A	N/A	Control	N/A	N/A
33	Control	Mucosa	57	Male	No	No	N/A	N/A	N/A	Control	N/A	N/A
34	Control	Mucosa	29	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
35	Control	Mucosa	18	Male	No	No	N/A	N/A	N/A	Control	N/A	N/A
36	Control	Mucosa	56	Male	Yes	Yes	N/A	N/A	N/A	Control	N/A	N/A
37	Control	Blood	20	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
38	Control	Blood	28	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
39	Control	Blood	24	Male	Yes	No	N/A	N/A	N/A	Control	N/A	N/A
40	Control	Blood	19	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
41	Control	Blood	28	Male	No	Yes	N/A	N/A	N/A	Control	N/A	N/A
42	Control	Blood	30	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
43	Control	Blood	32	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A
44	Control	Blood	34	Female	No	No	N/A	N/A	N/A	Control	N/A	N/A

medium with the addition of 100 μM cobalt chloride ($CoCl_2$, Sigma, St. Louis, MO, USA) over a 24 h period to mimic hypoxic conditions. All culture experiments were performed in triplicate.

Radiation Assay and Groups

X- rays beam from GAMMATRON-S80, source CO-60 GK 60 T03 was used in this study. The cells were irradiate using the iso-centric technique with the linear accelerator positioned at the base of the adhered cell. Two vertical parallel opposed fields were used. The Source-axis distance (SAD) was 80 cm. The field size of 10×10 cm^2 at the isocenter plane was the same for both areas. The cells were irradiated with doses of 6 Gy. T25 tissue flasks were filled with DMEN/F12 to maintain the electronic equilibrium during X-rays irradiation. The effect of radiation on SCC9 cells was evaluated in all assays after 24 h of radiation. Comparisons were among the four groups, which included control, $CoCl_2$, Radiation, and Radiation+ $CoCl_2$.

RNA Isolation and qRT-PCR

RNA isolation and qRT-PCR were described before [10, 42]. RNA was isolated using the Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the

manufacturer Total RNA was treated with DNase I, Amplification Grade (Invitrogen, cat number 18068015, Carlsbad, CA, USA) and then 1.5 μg of RNA was reverse transcribed with the SuperScript® First-Strand Synthesis System for RT-PCR. (Invitrogen, cat number 11904018, Carlsbad, CA, USA). For qRT-PCR, 66 ng of the cDNA was added to SYBER GREEN reagent (Life Technologies, Carlsbad, CA, USA) with HIF-1α [43] LDHA and PDHA [10] primers whose primer sequences described in Supplementary Table 1. Amplification was performed on a StepOne QRT-PCR System (Life Technologies, Carlsbad, CA, USA). All reactions were done in triplicate, and Beta-Actin [44] was used as an endogenous control for gene expression analysis. For experiments with patient tissues, the normal mucosa was used as a calibrator group. For in vitro studies, untreated cells (control group) were used as a calibrator. The results were quantified as Ct values, where Ct was defined as the threshold cycle of PCR at which the amplified product is first detected and defined as relative gene expression (the ratio of target/endogenous). qRT-PCR was analyzed by the $2^{-\Delta\Delta Ct}$. method.

For miR-210 (ID: Hs04231470_s1, Life Technologies, Carlsbad, CA, USA), a TaqMan assay was performed according to the manufacturer's protocol. RNU44 was used as an endogenous control for miR-210 analysis (ID: 001094, Life Technologies, Carlsbad, CA, USA), All reactions were done in triplicate. For experiments with patient tissues, samples of healthy mucosa were used as calibrator. For in vitro studies, untreated cells (control group) were used as a calibrator. The following reaction steps were used for amplification: 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min. The Ct values of endogenous controls were subtracted from the Ct values of the respective targets to calculate the ΔCt. The ΔCt values from each experimental group were averaged and converted to log base 2 using the eq. $2^{-\Delta\Delta Ct}$ to compare expression among different samples.

Mass Spectrometry (MS): Preparation of Cell Samples

Cell lysates were treated with urea (Sigma, St. Louis, MO, USA) at final concentration of 1.6 M followed by reduction with dithiothreitol (Sigma, St. Louis, MO, USA) at 5 mM for 25 min at 56 °C, alkylation with iodoacetamide (Sigma, St. Louis, MO, USA) at 14 mM for 30 min at room temperature protected from light and digestion with trypsin (Promega, Madison, WI, USA) for 16 h at 37(ratio enzyme: substrate, 1:50). The reaction was stopped with formic acid (Merck; BDH Prolabo Chemicals Darmstadt Germany) to 0.4% and, after desalination using SepPack, the dried samples in a vacuum concentrator model SPD 1010 speedvac system (Thermo Fisher Scientific, Waltham, MA, USA). The samples were stored at −20 for subsequent analysis in a mass spectrometer.

For protein analysis was used previously described methods with appropriate modifications [45]. Briefly, an aliquot of 2.0 ul (4μg) of proteins resulting from peptide digestion was were separated by C18 (100 mm 6100 mm) RP-nanoUPLC nano Acquity (Waters, Borehamwood, Hertfordshire, UK) coupled with a Q-Tof Premier mass spectrometer (Waters, Borehamwood, Hertfordshire, UK) with nanoelectrospray source at a flow rate of 0.6 ul/min. The gradient was 2–90% acetonitrile in 0.1% formic acid over 10 min (spots) and 45 min (shotgun). The nanoelectrodes pray voltage was set to 3.5 kV, a cone voltage of 30 V and the source temperature was 100uC. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. After MS/MS fragmentation, the ion was placed on the exclusion list for 60 s and the analysis of endogenous cleavage peptides; a real-time exclusion was used.

For data analysis, the spectra were acquired using software Mass Lynx v.4.1 and the raw data files were converted to a peak list format (mgf) without summing the scans by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science, London, UK) and searched against the UniProt database, using Mascot engine v.2.3.01 (Matrix Science, London, UK), with carbamidomethylation as fixed modifications, oxidation of methionine as variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. After the data analyzed in Scaffold 4.8.4. The differentially expressed proteins between samples from each of the study groups were analyzed in MetaboAnalyst 3.0 with heatmap generation [46, 47].

Wound Scratch Assay

Cell migration was monitored in a wound risk assay as described previously [10, 42]. The images were obtained with an SC30 camera (Olympus, Center Valley, PA, USA) on an inverted IX81 microscope (Olympus, Center Valley, PA, USA). ImageJ software was used for analysis [48].

Acridine Orange/Ethidium Bromide Cell Death Assay

Simultaneous staining performed the detection of apoptotic cells simultaneous staining with both acridine orange (AO, Sigma, St. Louis, MO, USA) and ethidium bromide (EB, Sigma, St. Louis, MO, USA) as described before [10, 42]. Briefly, cells were incubated with 10 μg/ml of AO and 20 μg/ml of EB on the darkroom for 5 min and observed under a fluorescence microscope (FSX100, Olympus, Center Valley, PA, USA). The automatic count and threshold were performed in the merged image by ImageJ software [49].

Statistical Analysis

Kolmogorov Smirnov and the Shapiro-Wilk Tests were carried out to evaluate data distribution. After the definition of the data distribution, an appropriate test was conducted. Statistical significance was accepted at $p < 0.05$. Analyses were performed using SPSS (Version 18.0) and GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA).

Results

HIF-1α and miR-210 Present Higher Levels in Patients with OSCC

qRT-PCR was performed to compare HIF-1α and miR-210 levels in OSCC primary lesion and oral mucosa. Higher HIF-1α mRNA level was increased in patients with OSCC primary lesion compared to normal mucosa (Fig. 1a). On the other hand, no differences in miR-210 levels were observed between OSCC lesion and normal mucosa (Fig. 1b).

Effects of Radiation on OSCC Cell Phenotype under Hypoxic Conditions

The wound scratch assay was performed to clarify the impact of radiation on OSCC cells phenotype in a hypoxic environment. Radiation under normoxic conditions reduced OSCC migration. On the other hand, hypoxia could revert the effect of radiation after 24 h (Fig. 2a, b). In the same way, the cell death assay demonstrated that radiation under normoxic conditions increased OSCC cell death but, hypoxia could revert the effect of radiation after 24 h (Fig. 2c-d).

Proteomic Analyses Demonstrated that Radiation Therapy Could Change LDH Levels

LDH was the unique protein related to glycolytic metabolism which presented significant changes in expression for RT (Fig. 3a). In detail, LDH levels were drastically changed in hypoxic cells submitted to RT (Fig. 3b).

Radiation Reduced Hypoxic Factors and LDH Levels under Hypoxia In Vitro

Hypoxia is the primary stimulus that increases the expression of HIF-1α and mir-210. An in vitro assay was performed to assess if RT could interfere with HIF-1α, mir-210, LDH, and PDH level sin OSCC cells under hypoxia. RT did not promote changes in HIF-1α or mir-210 levels in normoxia. Moreover, RT did not improve LDH levels under normoxia (Fig. 4c). Hypoxia increased HIF-1α, mir-210 and LDH levels. On the other hand, RT reduced HIF-1α and mir-210 levels under hypoxia. (Fig. 4a, b respectively). Interesting enough RT also decreased LDH levels under hypoxia (Fig. 4c). Neither radiation nor hypoxia changed PDH level (Fig. 4d).

Radiation Therapy Reduced Hypoxic Factors and LDH Blood Levels in OSCC Patients

Blood levels of HIF-1α, miR-210, and LDH in patients under RT and its respective controls were evaluated to test the systemic effects of RT and confirm in vitro studies. Untreated OSCC patients presented higher blood levels of HIF-1α, miR-210, and LDH in comparison to the control group (Fig. 5a-c). RT reduced Blood levels HIF-1α, miR-210 and LDH (Fig. 5a-c). Additionally, no differences between control and RT patients were observed regarding HIF-1α, miR-210, and LDH blood levels. There were no differences between HIF-1α, miR-210, and LDH blood levels after RT treatment (Fig. 5a-c).

Discussion

Oral squamous cell carcinoma (OSCC) is characterized by its higher incidence worldwide [50]. Also, OSCC development and treatment produce distortions in the ability to interact socially [7, 51]. Thus, it is necessary to fully understand OSCC molecular signatures to improve the treatment [52]. The

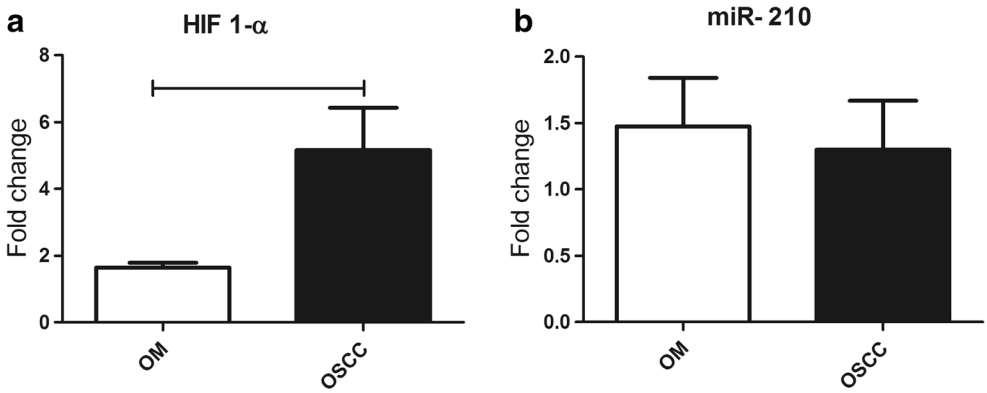

Fig. 1 HIF-1α and miR-210 expression in patients with OSCC primary lesion. In (**a**), the expression of HIF-1α was higher in OSCC patients with OSCC. HIF-1α mRNA levels were increased in comparison to control. In (**b**), no differences in miR-210 levels observes between OSCC and control

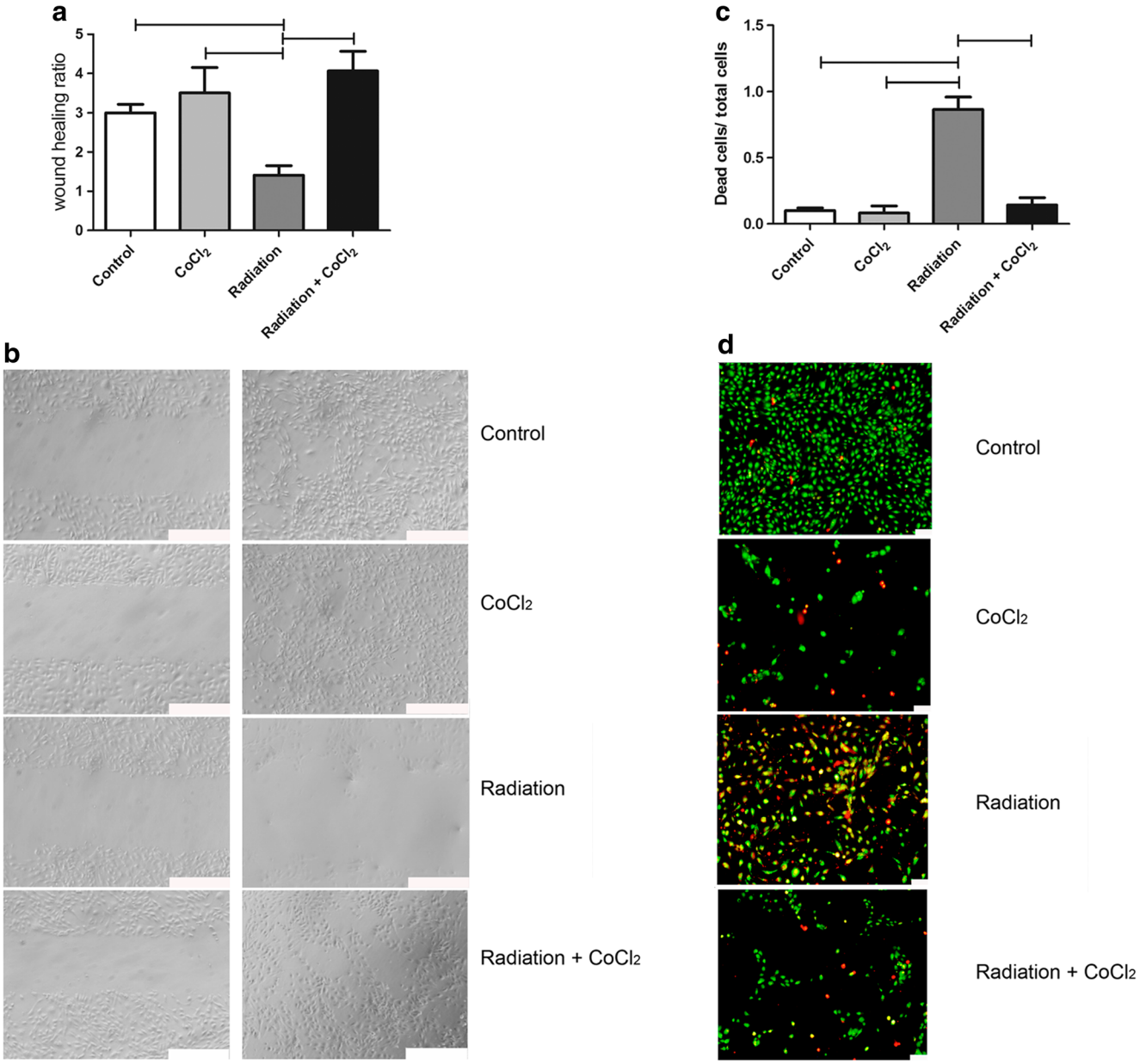

Fig. 2 Effect of Radiation and hypoxia in OSCC migration and death under hypoxic conditions. In (**a**, **b**) radiation decreases migration but hypoxia revert radiation effect. The scale represents 100 μm. AO/EB quantification (**c**) and representative figs. (d). Radiation increases cell death but hypoxia revert radiation effect. The scale represents 92 μm

current study is the first to investigate the impact of radiation therapy in miR-210, HIF-1α, and LDH in the context of OSCC under hypoxia.

In the current study, no differences in miR-210 levels were observed between OSCC and oral mucosa. Only one previous study [53] evaluated the levels of miR-210 in OSCC, but the comparison was performed with a lymph node. On the other hand, it was found that OSCC patients presented higher local and systemic levels of HIF-1α in comparison to control group. It was demonstrated that HIF-1α promotes OSCC development and metastasis [3]. Moreover, HIF-1α mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption in neoplastic cells [54, 55]. As the leading consequence, HIF-1α acts in favor of the Warburg's effect allowing higher pyruvate conversion to lactate [10]. Interestingly, lactate only activates HIF-1α in normoxic oxidative tumor cells [56].

The efficacy of radiotherapy depends on several factors, such as the mechanism of oxygenation of neoplastic cells [57]. There is a healthy relationship between poor prognosis and low oxygenation levels in neoplasias [3, 9]. Previous studies that suggest that RT of OSCC cells under hypoxia presented higher levels of HIF-1α when compared to OSCC cells only to radiated under normoxia [58]. Here, OSCC cells

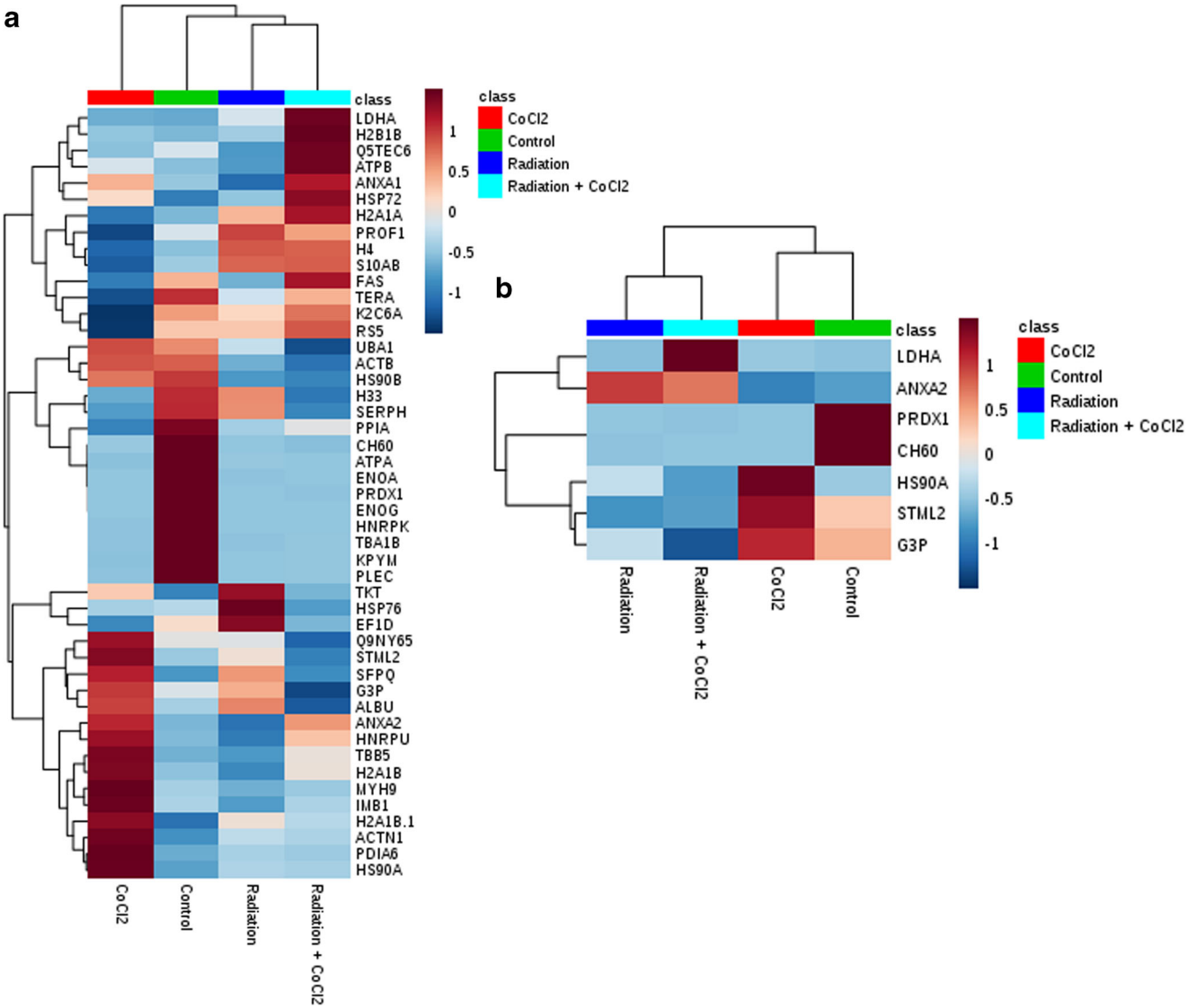

Fig. 3 Proteomic analyses. In (**a**) the essential proteins associated with hypoxia and Radiation Therapy. In (**b**) a selection of leading proteins related to OSCC. LDH was the unique protein associated with glycolytic metabolism which presented significant changes in expression for RT.

under hypoxia presented higher levels of HIF-1α, miR-210, and LDH. Additionally, In the current study, hypoxia reverts the phenotypic effect of RT in OSCC cells. The hypoxia in neoplasia microenvironment is an essential mechanism of

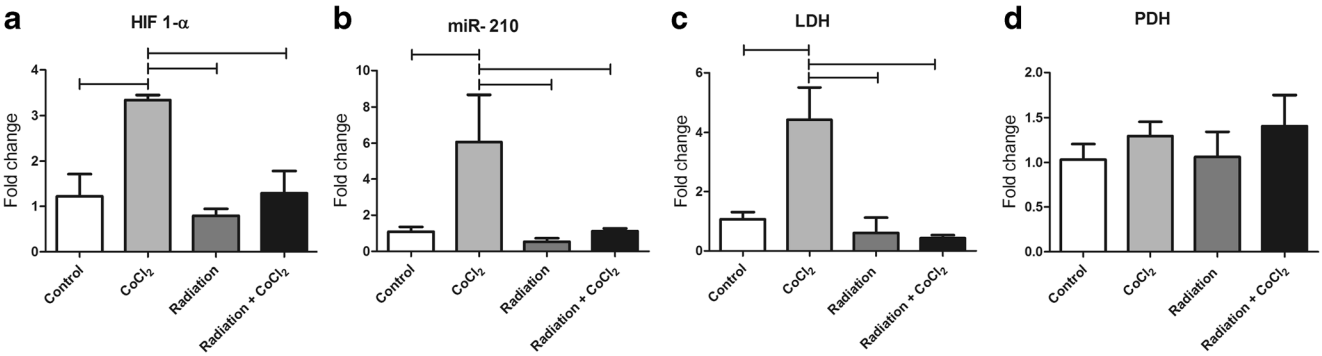

Fig. 4 Effect of radiation and hypoxia on HIF-1α, miR-210, LDH and PDH levels in OSCC cells. Radiation in hypoxia condition increases HIF-1α (4**a**) and miR-210 (4**b**) and LDH (4**c**) levels. Radiation reduced HIF-1α (4**a**) and miR-210 (4**b**) and LDH (4**c**) levels under hypoxia. Nighter radiation or hypoxia changed PDH levels. (4**d**)

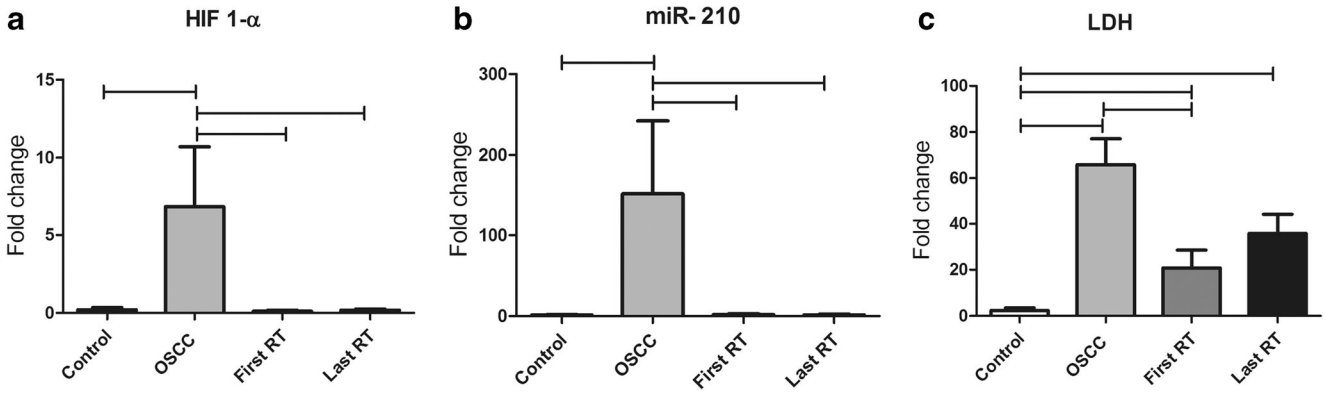

Fig. 5 HIF-1α, miR-210 and LDH expression systemic in patients with OSCC submitted to radiotherapy. Similar levels of HIF-1α (Fig. 5**a**), miR-210 (Fig. 5**b**) and LDH (Fig. 5**c**) were observed in patients with OSCC submitted to radiotherapy compared to control. Our results show that levels of HIF-1α, miR-210 and LDH had a significant decrease compared to OSCC patients without RT treatment (Fig. 5a-c respectively)

radioresistance and recurrence [9]. Activation and stabilization of the HIF-1α trigger angiogenesis, proliferation and glycolytic metabolism [36]. Also, HIF-1α can prevent pyruvate from entering the Krebs cycle [36].

RT, which is widely used as an OSCC treatment, promote the reduction of LDH activity [59, 60]. RT acts both, directly and indirectly, DNA strand break [61]. In the direct effect, the energy of the radiation works directly on atoms to promote breaking the DNA strand [61]. Divergently, RT indirect effect is a consequence of the increase in the production of Reactive Oxygen Species (ROS), which promotes DNA strand break [36]. RT dose reaches levels eliciting DNA damage, p53 is activated and diminishes the activity of HIF-1α and glycolysis [37]. In the current study, for the first time, a proteomic approach was performed to identify if there is any effect of RT in OSCC cells under hypoxia. The current study identified that LDH was the unique protein related to glycolytic metabolism which presented significant changes in expression for RT. It was demonstrated that RT might change levels of HIF-1α, miR-210, and LDH under hypoxia in vitro.

Additionally, in vivo results also demonstrated that RT promotes a reduction in blood levels of HIF-1α, miR-210, and LDH. The abscopal effect is defined as the capacity of ionizing radiation reduce tumor growth outside the radiation field, generating a systemic anti-tumor response [62]. LDH is responsible for malignant neoplasia maintenance and progression [16, 63]. Moreover, LDH-depletion promotes HIF-1α, reduction and consequently changes in the tumor microenvironment that modulates the immune [64]., In the current study, RT reduced systemic levels of LDH, HIF-1α, and miR-210. The systemic HIF-1α reduction might be associated with metastasis reduction [3] and might be a rationale for the abscopal effect. Moreover, fractionated RT might reduce HIF-1α and miR-210 because of the promotion of reoxygenation [65, 66]. Some preclinical studies had focused on the demonstration of an abscopal effect [67]. However, the exact mechanism of RT abscopal effects, specifically in OSCC, still needs to be clarified.

In conclusion, the current study demonstrated that hypoxia could revert the effects of RT in the OSCC context. However, RT reduces the levels HIF-1α, miR-210 and LDH in vivo and in vitro. The consequences of RT in blood should be carefully investigated.

Acknowledgements We thank the facility Laboratório Nacional de Biociências LNBio CNPEM-Brazil for the mass spectrometry analysis. We want to thank Bianca A. Pauletti in especial for technical assistance in mass spectrometry analysis.

Funding This study was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Instituto Federal de Educação, Ciência e Tecnologia do Norte de Minas Gerais (IFNMG) and the Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG). Dr. Guimarães, Dr. Gomez, Dr. Santos and Dr. de Paula are research fellows of the CNPq.

Compliance with Ethical Standards

Conflict of Interest The authors deny any conflicts of interest related to this study.

Ethical Approval Ethical approval for this study was obtained from the Institutional Review Board, and a signed informed consent form was obtained from all patients (process number 62425316.0.0000.5146).

References

1. Zhang S, Tian L, Ma P, Sun Q, Zhang K (2015) GuanchaoWang, et al. potential role of differentially expressed lncRNAs in the pathogenesis of oral squamous cell carcinoma. Arch Oral Biol 60(10): 1581–1587
2. Gupta A, Baxi S, Hoyne C (2017) Assessing feasibility, compliance and toxicity of concomitant chemo-radiotherapy in head and neck cancers in the Northern Territory: initial experience and challenges. J Med Radiat Sci 64(2):131–137
3. Fraga CA, de Oliveira MV, de Oliveira ES, Barros LO, Santos FB, Gomez RS et al (2012) A high HIF-1alpha expression genotype is

associated with poor prognosis of upper aerodigestive tract carcinoma patients. Oral Oncol 48(2):130–135
4. Gruber G, Greiner RH, Hlushchuk R, Aebersold DM, Altermatt HJ, Berclaz G, Djonov V (2004) Hypoxia-inducible factor 1 alpha in high-risk breast cancer: an independent prognostic parameter? Breast Cancer Res : BCR 6(3):R191–R198
5. Thongchot S, Yongvanit P, Loilome W, Seubwai W, Phunicom K, Tassaneeyakul W, Pairojkul C, Promkotra W, Techasen A, Namwat N (2014) High expression of HIF-1alpha, BNIP3 and PI3KC3: hypoxia-induced autophagy predicts cholangiocarcinoma survival and metastasis. Asian Pacific journal of Cancer Prevention : APJCP 15(14):5873–5878
6. Warnakulasuriya S (2009) Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 45(4–5):309–316
7. Stelzle F, Knipfer C, Schuster M, Bocklet T, Nöth E, Adler W, Schempf L, Vieler P, Riemann M, Neukam FW, Nkenke E (2013) Factors influencing relative speech intelligibility in patients with oral squamous cell carcinoma: a prospective study using automatic, computer-based speech analysis. Int J Oral Maxillofac Surg 42(11): 1377–1384
8. Nordsmark M, Bentzen SM, Rudat V, Brizel D, Lartigau E, Stadler P, Becker A, Adam M, Molls M, Dunst J, Terris DJ, Overgaard J (2005) Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 77(1):18–24
9. Barker HE, Paget JT, Khan AA, Harrington KJ (2015) The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. Nat Rev Cancer 15(7):409–425
10. Guimaraes TA, Farias LC, Santos ES, de Carvalho Fraga CA, Orsini LA, de Freitas TL et al (2016) Metformin increases PDH and suppresses HIF-1alpha under hypoxic conditions and induces cell death in oral squamous cell carcinoma. Oncotarget 7(34): 55057–55068
11. Otto AM (2016) Warburg effect(s)—a biographical sketch of Otto Warburg and his impacts on tumor metabolism. Cancer Metab 4(1): 5
12. Luc R, Tortorella SM, Ververis K, Karagiannis TC (2015) Lactate as an insidious metabolite due to the Warburg effect. Mol Biol Rep 42(4):835–840
13. Loeffelbein DJ, Eiber M, Mayr P, Souvatzoglou M, Mucke T, von Bomhard A et al (2015) Loco-regional recurrence after surgical treatment of oral squamous cell carcinoma: proposals for follow-up imaging based on literature, national guidelines and institutional experience. J Craniomaxillofac Surg 43(8):1546–1552
14. Allison SJ, Knight JR, Granchi C, Rani R, Minutolo F, Milner J et al (2014) Identification of LDH-A as a therapeutic target for cancer cell killing via (i) p53/NAD(H)-dependent and (ii) p53-independent pathways. Oncogene 3:e102
15. Read JA, Winter VJ, Eszes CM, Sessions RB, Brady RL (2001) Structural basis for altered activity of M- and H-isozyme forms of human lactate dehydrogenase. Proteins 43(2):175–185
16. Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9(6):425–434
17. Semenza GL (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–578
18. de Carvalho Fraga CA, Alves LR, Marques-Silva L, de Sousa AA, Jorge AS, de Jesus SF et al (2013) High HIF-1alpha expression genotypes in oral lichen planus. Clin Oral Investig 17(9):2011–2015
19. Hoffmann AC, Mori R, Vallbohmer D, Brabender J, Drebber U, Baldus SE, Klein E, Azuma M, Metzger R, Hoffmann C, Hoelscher AH, Danenberg KD, Prenzel KL, Danenberg PV (2008) High expression of heparanase is significantly associated with dedifferentiation and lymph node metastasis in patients with pancreatic ductal adenocarcinomas and correlated to PDGFA and via HIF1a to HB-EGF and bFGF. J Gastrointest Surg 12(10):1674–1681 discussion 81-2
20. Tuomisto A, Garcia-Solano J, Sirnio P, Vayrynen J, Perez-Guillermo M, Makinen MJ et al (2016) HIF-1alpha expression and high microvessel density are characteristic features in serrated colorectal cancer. Virchows Arch 469(4):395–404
21. van der Groep P, Bouter A, Menko FH, van der Wall E, van Diest PJ (2008) High frequency of HIF-1α overexpression in BRCA1 related breast cancer. Breast Cancer Res Treat 111(3):475–480
22. Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, Semenza GL (2001) Expression of hypoxia-inducible factor-1alpha: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res 61(7):2911–2916
23. Silva P, Slevin NJ, Sloan P, Valentine H, Cresswell J, Ryder D, Price P, Homer JJ, West CML (2008) Prognostic significance of tumor hypoxia inducible factor-1alpha expression for outcome after radiotherapy in oropharyngeal cancer. Int J Radiat Oncol Biol Phys 72(5):1551–1559
24. Langhammer S, Najjar M, Hess-Stumpp H, Thierauch KH (2011) LDH-A influences hypoxia-inducible factor 1alpha (HIF1 alpha) and is critical for growth of HT29 colon carcinoma cells in vivo. Target Oncol 6(3):155–162
25. Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441(7092):437–443
26. Karatas OF, Suer I, Yuceturk B, Yilmaz M, Oz B, Guven G et al (2016) Identification of microRNA profile specific to cancer stem-like cells directly isolated from human larynx cancer specimens. BMC Cancer 16(1):853
27. Puissegur MP, Mazure NM, Bertero T, Pradelli L, Grosso S, Robbe-Sermesant K et al (2011) miR-210 is overexpressed in late stages of lung cancer and mediates mitochondrial alterations associated with modulation of HIF-1 activity. Cell Death Differ 18(3):465–478
28. Camps C, Buffa FM, Colella S, Moore J, Sotiriou C, Sheldon H, Harris AL, Gleadle JM, Ragoussis J (2008) Hsa-miR-210 is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res 14(5):1340–1348
29. Gee HE, Camps C, Buffa FM, Patiar S, Winter SC, Betts G, Homer J, Corbridge R, Cox G, West CM, Ragoussis J, Harris AL (2010) Hsa-mir-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer 116(9):2148–2158
30. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G et al (2008) MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem 283(23):15878–15883
31. Hockel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93(4): 266–276
32. Okunieff P, Hoeckel M, Dunphy EP, Schlenger K, Knoop C, Vaupel P (1993) Oxygen tension distributions are sufficient to explain the local response of human breast tumors treated with radiation alone. Int J Radiat Oncol Biol Phys 26(4):631–636
33. Yang X, Zhu H, Ge Y, Liu J, Cai J, Qin Q, Zhan L, Zhang C, Xu L, Liu Z, Yang Y, Yang Y, Ma J, Cheng H, Sun X (2014) Melittin enhances radiosensitivity of hypoxic head and neck squamous cell carcinoma by suppressing HIF-1alpha. Tumor Biol 35(10):10443–10448
34. Moeller BJ, Dreher MR, Rabbani ZN, Schroeder T, Cao Y, Li CY, Dewhirst MW (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell 8(2):99–110
35. Liu J, Zhang J, Wang X, Li Y, Chen Y, Li K, Zhang J, Yao L, Guo G (2010) HIF-1 and NDRG2 contribute to hypoxia-induced radioresistance of cervical cancer Hela cells. Exp Cell Res 316(12):1985–1993
36. Dong G, Chen Q, Jiang F, Yu D, Mao Q, Xia W, Shi R, Wang J, Xu L (2016) Diisopropylamine dichloroacetate enhances

radiosensitization in esophageal squamous cell carcinoma by increasing mitochondria-derived reactive oxygen species levels. Oncotarget 7(42):68170–68178
37. Lall R, Ganapathy S, Yang M, Xiao S, Xu T, Su H, Shadfan M, Asara JM, Ha CS, Ben-Sahra I, Manning BD, Little JB, Yuan ZM (2014) Low-dose radiation exposure induces a HIF-1-mediated adaptive and protective metabolic response. Cell Death Differ 21(5):836–844
38. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136(5):E359–E386
39. Rosner B (2011) Fundamentals of biostatistics. Brooks/Cole, Cengage Learning, Boston
40. Katabi N, Lewis JS (2017) Update from the 4th edition of the World Health Organization classification of head and neck Tumours: what is new in the 2017 WHO blue book for tumors and tumor-like lesions of the neck and lymph nodes. Head Neck Pathol 11(1): 48–54
41. El-Naggar AK, Chan JKC, Grandis JR, Takata T (2017) Slootweg PJ. WHO Classification of Head and Neck Tumours, International Agency for Research on Cancer
42. Guimaraes TA, Farias LC, Fraga CA, Feltenberger JD, Melo GA, Coletta RD, Souza Santos SH, de Paula AMB, Guimaraes AL (2016) Evaluation of the antineoplastic activity of gallic acid in oral squamous cell carcinoma under hypoxic conditions. Anti-Cancer Drugs 27(5):407–416
43. Helman LJ, Gazdar AF, Park JG, Cohen PS, Cotelingam JD, Israel MA (1988) Chromogranin a expression in normal and malignant human tissues. J Clin Invest 82(2):686–690
44. Domingos PLB, Souza MG, Guimaraes TA, Santos ES, Farias LC, de Carvalho Fraga CA et al (2017) Hypoxia reduces the E-cadherin expression and increases OSCC cell migration regardless of the E-cadherin methylation profile. Pathol Res Pract 213(5):496–501
45. Aragao AZ, Belloni M, Simabuco FM, Zanetti MR, Yokoo S, Domingues RR et al (2012) Novel processed form of syndecan-1 shed from SCC-9 cells plays a role in cell migration. PLoS One 7(8):e43521
46. Xia J, Wishart DS (2010) MSEA: a web-based tool to identify biologically meaningful patterns in quantitative metabolomic data. Nucleic Acids Res 38(Web Server issue):W71–W77
47. Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0–making metabolomics more meaningful. Nucleic Acids Res 43(W1):W251–W257
48. Vaupel P, Harrison L (2004) Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. Oncologist 9(Suppl 5):4–9
49. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH image to ImageJ: 25 years of image analysis. Nat Meth 9(7):671–675
50. Mithani SK, Mydlarz WK, Grumbine FL, Smith IM, Califano JA (2007) Molecular genetics of premalignant oral lesions. Oral Dis 13(2):126–133
51. Speksnijder CM, van der Glas HW, van der Bilt A, van Es RJ, van der Rijt E, Koole R (2010) Oral function after oncological intervention in the oral cavity: a retrospective study. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons 68(6):1231–1237
52. Coutinho-Camillo CM, Lourenco SV, de Araujo LL, Kowalski LP, Soares FA (2015) Expression of apoptosis-regulating miRNAs and target mRNAs in oral squamous cell carcinoma. Cancer Genet 208(7–8):382–389
53. Scapoli L, Palmieri A, Lo Muzio L, Pezzetti F, Rubini C, Girardi A et al (2010) MicroRNA expression profiling of oral carcinoma identifies new markers of tumor progression. Int J Immunopathol Pharmacol 23(4):1229–1234
54. Golias T, Papandreou I, Sun R, Kumar B, Brown NV, Swanson BJ, Pai R, Jaitin D, le QT, Teknos TN, Denko NC (2016) Hypoxic repression of pyruvate dehydrogenase activity is necessary for metabolic reprogramming and growth of model tumours. Sci Rep 6:31146
55. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC (2006) HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 3(3):187–197
56. De Saedeleer CJ, Copetti T, Porporato PE, Verrax J, Feron O, Sonveaux P (2012) Lactate activates HIF-1 in oxidative but not in Warburg-phenotype human tumor cells. PLoS One 7(10):e46571
57. Saito K, Matsumoto S, Takakusagi Y, Matsuo M, Morris HD, Lizak MJ, Munasinghe JP, Devasahayam N, Subramanian S, Mitchell JB, Krishna MC (2015) 13C-MR spectroscopic imaging with hyperpolarized [1-13C]pyruvate detects early response to radiotherapy in SCC tumors and HT-29 tumors. Clinical cancer research : an official journal of the American Association for Cancer Research 21(22):5073–5081
58. Hosokawa Y, Okumura K, Terashima S, Sakakura Y (2012) Radiation protective effect of hypoxia-inducible factor-1alpha (HIF-1alpha) on human oral squamous cell carcinoma cell lines. Radiat Prot Dosimetry 152(1–3):159–163
59. Leung E, Cairns RA, Chaudary N, Vellanki RN, Kalliomaki T, Moriyama EH, Mujcic H, Wilson BC, Wouters BG, Hill R, Milosevic M (2017) Metabolic targeting of HIF-dependent glycolysis reduces lactate, increases oxygen consumption and enhances response to high-dose single-fraction radiotherapy in hypoxic solid tumors. BMC Cancer 17(1):418
60. Koukourakis MI, Giatromanolaki A (2018) Warburg effect, lactate dehydrogenase, and radio/chemo-therapy efficacy. Int J Radiat Biol:1–19. https://doi.org/10.1080/09553002.2018.1490041
61. Jackson SP, Bartek J (2009) The DNA-damage response in human biology and disease. Nature 461(7267):1071–1078
62. Demaria S, Ng B, Devitt ML, Babb JS, Kawashima N, Liebes L, Formenti SC (2004) Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys 58(3):862–870
63. Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM et al (2010) Inhibition of lactate dehydrogenase a induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci U S A 107(5):2037–2042
64. Serganova I, Cohen IJ, Vemuri K, Shindo M, Maeda M, Mane M, Moroz E, Khanin R, Satagopan J, Koutcher JA, Blasberg R (2018) LDH-A regulates the tumor microenvironment via HIF-signaling and modulates the immune response. PLoS One 13(9):e0203965
65. Herskind C, Ma L, Liu Q, Zhang B, Schneider F, Veldwijk MR, Wenz F (2017) Biology of high single doses of IORT: RBE, 5 R's, and other biological aspects. Radiat Oncol 12(1):24
66. Dewan MZ, Galloway AE, Kawashima N, Dewyngaert JK, Babb JS, Formenti SC, Demaria S (2009) Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. Clinical cancer research : an official journal of the American Association for Cancer Research. 15(17):5379–5388
67. Golden EB, Chhabra A, Chachoua A, Adams S, Donach M, Fenton-Kerimian M, Friedman K, Ponzo F, Babb JS, Goldberg J, Demaria S, Formenti SC (2015) Local radiotherapy and granulocyte-macrophage colony-stimulating factor to generate abscopal responses in patients with metastatic solid tumours: a proof-of-principle trial. Lancet Oncol 16(7):795–803