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Research paper

Is HIF1-a deregulated in malignant salivary neoplasms?

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ABSTRACT

Background: There is significant controversy in the literature regarding the relationship between hypoxia and salivary gland neoplasms (SGNs).
Objective: The current study aims to investigate levels of hypoxia markers in both benign and malignant salivary neoplasms.
Patients and methods: The current study sample is comprised of a total of 62 samples. HIF-1α expression was evaluated by immunohistochemistry. Additionally, HIF-1α mRNA and miR-210 levels were assessed using qRT-PCR.
Results: No differences in HIF-1α expression were observed among the control group, benign and malignant SGNs. Similarly, HIF-1α mRNA levels were similar between benign and malignant SGNs. Also, there was no difference in miR-210 expression between case and control groups.
Conclusion: The angiogenic markers, miR-210 and HIF-1α, do not appear to distinguish malignancy in salivary glands.

1. Introduction

Head and neck cancers are associated with poor survival rate (Fonseca et al., 2012; de Oliveira et al., 2009; De Paula et al., 2009). In the case of salivary gland neoplasms (SGNs) a broad spectrum of phenotypic heterogeneity and are divided into five categories, according to the World Health Organization (WHO) (El-Naggar et al., 2017). Prognosis of SGNs is also related to the anatomical location of the neoplasia (Guzzo et al., 2010; Cerda et al., 2014). Recently, a large number of studies have attempted to evaluate the role of hypoxia in cancer development and prognosis (Guimaraes et al., 2016; Fraga et al., 2012). Hypoxia-inducible factor 1-alpha (HIF-1α) is a marker of hypoxia and is activated under hypoxic conditions (Wang and Semenza, 1993).

miRNAs are a class of single-stranded noncoding RNAs 21–22 nucleotides in length which regulate gene expression through the inhibition of RNA translation or degradation of target messenger RNA (mRNA) (Bartel, 2009). Recent evidence has suggested that miR-210 plays a crucial role in the cellular response to hypoxia. HIF-1α can promote isoform-specific stabilization of miR-210 by binding to the Hypoxia Responsive Element (HRE) present in the proximal promoter of miR-210 (Corn, 2008). Similar to HIF-1α, hypoxia induces miR-210 expression, which regulates cellular proliferation, DNA stability, mitochondrial metabolism, apoptosis and angiogenesis (Dang and Myers, 2015). Furthermore, the miR-210 expression is also significantly upregulated in other types of cancer, including non-small cell lung cancer (Zhu et al., 2016).

Although the first choice treatment for malignant SGNs is surgery (Green et al., 2016), adjuvant therapy has historically been determined based on data from studies focused on squamous cell carcinomas of the upper aerodigestive tract (Cerda et al., 2014). Importantly, there are

Abbreviations: SGNs, Salivary gland neoplasias; HRE, Hypoxia Responsive Element

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conflicting reports with regards to the relationship between hypoxia and clinical behavior or treatment responsiveness in patients diagnosed with SGNs (Wijffels et al., 2009). Recent studies have demonstrated that hypoxia might promote an increase in radioresistance (Harada, 2016), specifically via miRNA-mediated modulation of the hypoxic response (Gu et al., 2016). These data suggest that the use of chemoradiation might be a valuable alternative treatment option for patients presenting with radioresistant neoplasias (Cerda et al., 2014). Considering the controversial literature regarding the relationship between hypoxia and SGNs, the current study aimed to investigate levels of hypoxia markers in both benign and malignant salivary neoplasms.

2. Methods

2.1. Study design

A retrospective cross-sectional study was designed to evaluate the expression of the main hypoxia targets in SGNs.

3. Patients

Ethical approval for this study was obtained from the relevant Institutional Review Board. A total of 62 samples were included from the databases of the glandular tumor surgery services from 2010 to 2016. Samples were divided into three groups according to the world health organization classification of head and neck tumors (El-Naggar et al., 2017).

Group 1 ($n = 16$) was comprised of glandular tissue without neoplasia. Samples were obtained from mucocele excisions. The inclusion criteria for this group were histopathological diagnosis of mucocele.

Group 2 ($n = 22$) was comprised of patients with Pleomorphic Adenoma. The inclusion criteria for this group were histopathological diagnosis of pleomorphic adenoma.

The group 3 ($n = 24$) was comprised of one sample of Acinar Cell Carcinoma, two samples of Adenocarcinoma not otherwise specified, nine samples of Adenoid cystic carcinoma, ten Mucoepidermoid Carcinoma samples and two Myoepithelial Carcinoma samples.

4. Immunohistochemistry

Immunohistochemical reactions were performed from paraffin blocks of 49 patients. Including 13 controls (normal salivary gland samples), 16 benign tumors (Pleomorphic adenoma) and 20 malignant neoplasms (Acinar Cell Carcinoma $n = 1$, Adenocarcinoma not otherwise specified $n = 2$, Adenoid cystic carcinoma $n = 8$, Mucoepidermoid Carcinoma $n = 10$ and Myoepithelial Carcinoma $n = 2$). Protein expression of HIF-1α was evaluated through immunohistochemical reactions performed on 3.0 μm thick sections. The sections were deparaffinized with xylene and rehydrated with graded alcohol solutions. Antigen retrieval was conducted in an electric pressure cooker at 121 °C for 10 min in Trilogy buffer (Trilogy, Cell Marque, CA, USA). Endogenous peroxidase activity was blocked with 2 baths of 0.3% hydrogen peroxide for 15 min each. Samples were then incubated overnight with primary mouse monoclonal antibodies against HIF-1α (Clone H1a67; Sigma-Aldrich, MO, USA) at 4 °C at a dilution of 1:200. The samples were then incubated with a secondary biotinylated link and streptavidin-biotin-peroxidase complex using the LSAB kit (Dako Labs, Glostrup, Denmark), followed by incubation with the 3′3-diaminobenzidine-tetrahydrochloride (Cat.No.32750, Sigma-Aldrich, MO, USA). Counterstaining was performed using Mayer's hematoxylin (Cat. no. 109249, Merck, MA, USA). The samples were then dehydrated, and slides were mounted in ERV-mount (EasyPath, Brazil). Kidney samples were used as positive controls. Negative controls were performed by replacing the primary antibody with phosphate buffered saline (PBS). Ten microscopic fields of tumor parenchyma were photographed to quantify the staining at microscope FSX100 (Olympus, Center Valley, PA, USA). Cells were then counted using ImageJ software (Rueden et al., 2017). The ratio of positive cells to the total number of cells counted was used to quantify the staining.

5. RNA isolation and real-time PCR

Validation of the immunohistochemical results was performed with qRT-PCR. Samples of 24 patients, including 10 controls (normal salivary gland samples), 7 benign tumors (Pleomorphic adenoma) and 7 malignant neoplasms (Acinar cell carcinoma $n = 1$, Adenoid cystic carcinoma $n = 2$, Mucoepidermoid Carcinoma $n = 2$, Myoepithelial carcinoma $n = 2$) were used for qRT-PCR. RNA was isolated using Trizol (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol. Total RNA was treated with DNase I, Amplification Grade (Invitrogen, cat number 18068015, Carlsbad, CA, USA) and 1.5 μg of RNA were reversely transcribed with the SuperScript® First-Strand Synthesis System for qRT-PCR (Invitrogen, cat number 11904018, Carlsbad, CA, USA). Each reaction for SYBR green-based qRT-PCR (total volume 20 μl) contained 10 μl of SYBR Green master mix, 0.25 μM of both forward and reversed primers, 1 μl of cDNA (66 ng/reaction) and 8.5 μl H_2O. Non-Template Control (NTC) was included for each assay. The thermal cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of the following steps: 95 °C for 15 s and 60 °C for 1 min. The specific primers/probes (Life Technologies, Carlsbad, CA, USA) were described previously (Guimaraes et al., 2016). Specifically, the following primers were used for HIF1α: 5′-TCTGCAACATGGAAGGTATTGC-3′ and 5′-CTGAGGTTG GTTACTGTTGGTATCA-3. Beta-Actin was used to normalize HIF1α gene expression and was amplified using the following primers: 5′-TGCCGACAGGATGCAGAAG-3′ and 5′-CTCAGGAGGAGCAATGATCT TGA-3′. qPCR was performed on a StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).

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 mi-210 analysis (ID: 001094, Life Technologies, Carlsbad, CA, USA), All reactions were done in triplicate. Normal salivary glands were used as a control group. 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 Cq values of endogenous controls were subtracted from the Ct values of the respective targets to calculate the ΔCq. The ΔCq values from each experimental group were averaged and converted to log base 2 using equation 2^-ΔΔCq to compare expression among different samples.

5.1. Statistical analysis

Analyses were performed using SPSS (Version 18.0, IBM Software Inc., Armonk, New York, USA) and GraphPad Prism software (Version 5.0, GraphPad Software Inc., San Diego, CA, USA). The Kolmogorov-Smirnov and Shapiro-Wilk Tests were carried out to evaluate data distribution. These analyses revealed that the data were non-parametrically distributed; therefore, the Mann-Whitney and Kruskal-Wallis tests were performed. All data are given as means ± S.D. $p < .05$ was considered statistically significant.

6. Results

Considering all individuals, 35 (56%) were male, and 24 (44%) were female (Table 1). There is a significant association between hypoxic markers such as HIF-1α and miR-210 and angiogenesis (Dang and Myers, 2015; Zhang et al., 2017) (Fig. 1). However, the impact of hypoxic markers in SGNs still unknown. Qualitative analyses of HIF-1α immunoexpression is presented in Fig. 2(A–G). Most of the groups presented week staining for HIF-1α. The staining was not homogeneous considering the histological types. Quantitative analyses of HIF-1α immunoexpression and mRNA is shown in Fig. 3 (A and B respectively).

Table 1
Descriptive data of the patient population.

Group 1	Mucocele	Female	miRNA/Protein
Group 1	Mucocele	Female	miRNA
Group 1	Mucocele	Male	miRNA
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	miRNA/Protein
Group 1	Mucocele	Male	miRNA/Protein
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	RNA/miRNA/Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Male	Protein
Group 1	Mucocele	Female	RNA/miRNA
Group 1	Mucocele	Female	miRNA/Protein
Group 2	Pleomorphic Adenoma	Male	miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA/Protein
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Male	miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Male	RNA/miRNA
Group 2	Pleomorphic Adenoma	Female	RNA/miRNA
Group 2	Pleomorphic Adenoma	Female	miRNA/Protein
Group 2	Pleomorphic Adenoma	Female	RNA/Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Male	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 2	Pleomorphic Adenoma	Female	Protein
Group 3	Acinar Cell Carcinoma	Female	miRNA/Protein
Group 3	Adenocarcinoma (NOS)	Male	Protein
Group 3	Adenocarcinoma (NOS)	Male	Protein
Group 3	Adenoid Cyst Carcinoma	Male	miRNA/Protein
Group 3	Adenoid Cyst Carcinoma	Female	RNA/miRNA
Group 3	Adenoid Cyst Carcinoma	Female	Protein
Group 3	Adenoid Cyst Carcinoma	Female	Protein
Group 3	Adenoid cystic Carcinoma	Female	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Adenoid cystic Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Female	miRNA
Group 3	Mucoepidermoid Carcinoma	Male	miRNA
Group 3	Mucoepidermoid Carcinoma	Female	RNA/Protein
Group 3	Mucoepidermoid Carcinoma	Female	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Mucoepidermoid Carcinoma	Male	Protein
Group 3	Myoepithelial Carcinoma	Male	miRNA/Protein
Group 3	Myoepithelial Carcinoma	Male	RNA/miRNA/Protein

HIF-1α immunoexpression did not differ between the case and control groups or between benign and malignant SGNs (Fig. 3A). In the agreement, HIF-1α mRNA levels or immunoexpression did not differ between the case and control groups or between benign and malignant SGNs (Fig. 3B). miR-210 is an essential molecule associated with neoplastic hypoxia (Dang and Myers, 2015). Similarly to HIF-1α, miR-210 levels were similar among control, malignant and benign SGNs (Fig. 3C).

7. Discussion

Previous reports have suggested that hypoxia is an important mechanism related to radioresistance in specific cancers (Barker et al., 2015). Because the indirect actions of radiation depend on oxygen levels, hypoxia is believed to be the central mechanism leading to radioresistance (Cerda et al., 2014; Harada, 2016; Gu et al., 2016; Barker et al., 2015). Furthermore, it is important to highlight that vascular damage, which triggers an immune response, is a consequence of radiation-induced tumor hypoxia (Barker et al., 2015). However, fractionated radiation can induce subsequent tumor revascularization via recruitment of bone marrow-derived cells (BMDCs) in a HIF1α-dependent or independent manner (Kioi et al., 2010).

Traditionally, SGNs were considered radioresistant (Cerda et al., 2014). However, the primary biological mechanism that contributes to the radioresistance observed in SGNs is still not well established. As such, recommendations for the management and treatment of malignant SGNs have changed in recent years (Bell et al., 2005; Laramore et al., 1993). The shift in the treatment of SGNs is due to an improvement in imaging technology, as well as the development of various treatment options, including external beam radiation, neutron beam therapy, and chemotherapy (Bell et al., 2005; Laramore et al., 1993). It has been shown that miR-210 expression is induced by both HIF-1α (Wang et al., 2014; Kulshreshtha et al., 2007) as well as hypoxia (Kulshreshtha et al., 2007). The current study was the first to investigate miR-210 expression in SGNs. This report suggests that there are no significant alterations in miR-210 expression in this specific tumor subtype. Specifically, no differences in miR-210 levels were observed between SGNs and normal, healthy salivary glands. In support of the miR-210 findings reported here, our results regarding HIF-1α demonstrate that salivary gland neoplasms are not hypoxic. Instead, an elegant study has shown that SGNs are well oxygenated, and it is unlikely that hypoxia is a relevant factor in the clinical progression and treatment responsiveness of these tumors (Wijffels et al., 2009). Moreover, repair of sublethal damage during the interval between fractionated radiotherapy doses is minimal in neutron therapy but plays a significant role in external-beam radiotherapy (Hall et al., 1975), thus suggesting that radioresistance of SGNs is independent of HIF1α. This notion is supported by the central mechanism of chemotherapeutic agents that induce cell death in SGNs is DNA damage and not hypoxia (Cerda et al., 2014).

Hypoxia is associated with both radioresistance and tumor progression (Fraga et al., 2012; Dang and Myers, 2015; Wang et al., 2015). However, the importance of the hypoxic response appears to be specific to the tumor subtype. Correctly, a substantial positive correlation has been observed between adenoid cystic carcinomas and hypoxia (Wang et al., 2015). However, HIF-1α levels do not appear to be altered in pleomorphic adenomas, when compared with control samples (Wang et al., 2015).

It is important to note that there are limitations to the current study, including a small sample size, which precluded our ability to perform comprehensive analyses of different specimens and histological types. Irrespective of these limitations, the current study is the first to evaluate RNA levels of hypoxia markers in SGNs. Furthermore, the present study validates previously published data as we observed no differences in hypoxia markers between control, benign and malignant neoplasms.

In conclusion, our data suggest that salivary gland neoplasms do not display increased levels of hypoxia markers. Individually, the angiogenic markers, mir210 and HIF-α, do not seem to correlate with malignancy of salivary glands.

Conflict of interest statement

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

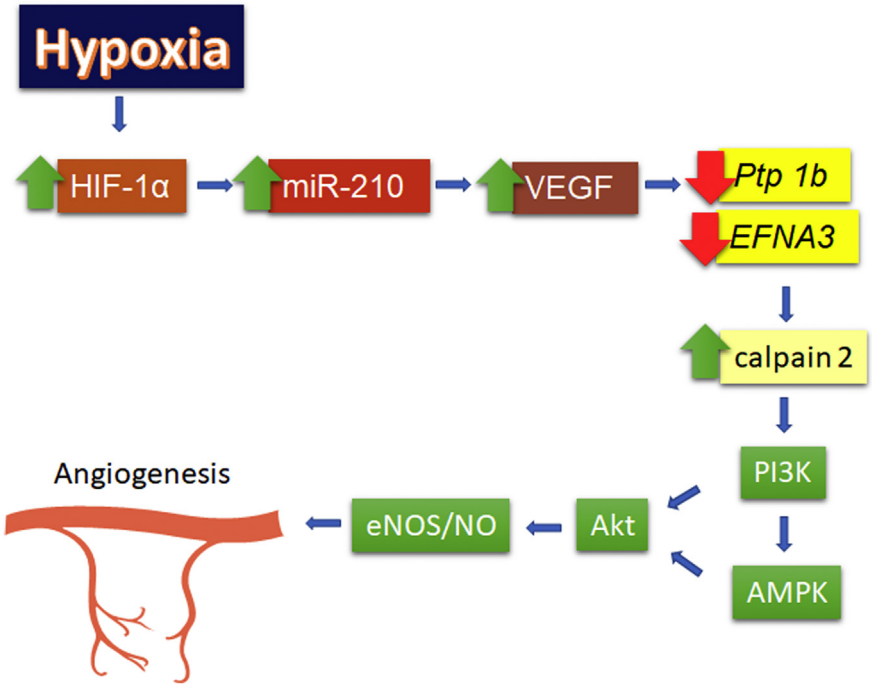

Fig. 1. Schematic representation of angiogenesis induced by Hypoxia.
Hypoxia induces the increase (green arrows) of Hypoxia-inducible factor 1-alpha (HIF-1α), miR-210 and Vascular endothelial growth factor (VEGF). Consequently, there is a transcription inhibition (red arrows) of both genes protein tyrosine phosphatase 1B (*Ptp1b*) and Ephrin A3 (*EPFNA3*). All these molecular events activate (green symbols) Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), 5′ adenosine monophosphate-activated protein kinase (AMPK), AKT serine/threonine kinase (AKT), Nitric oxide synthases (NOS) and Endothelial NOS (eNOS) which induces angiogenesis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

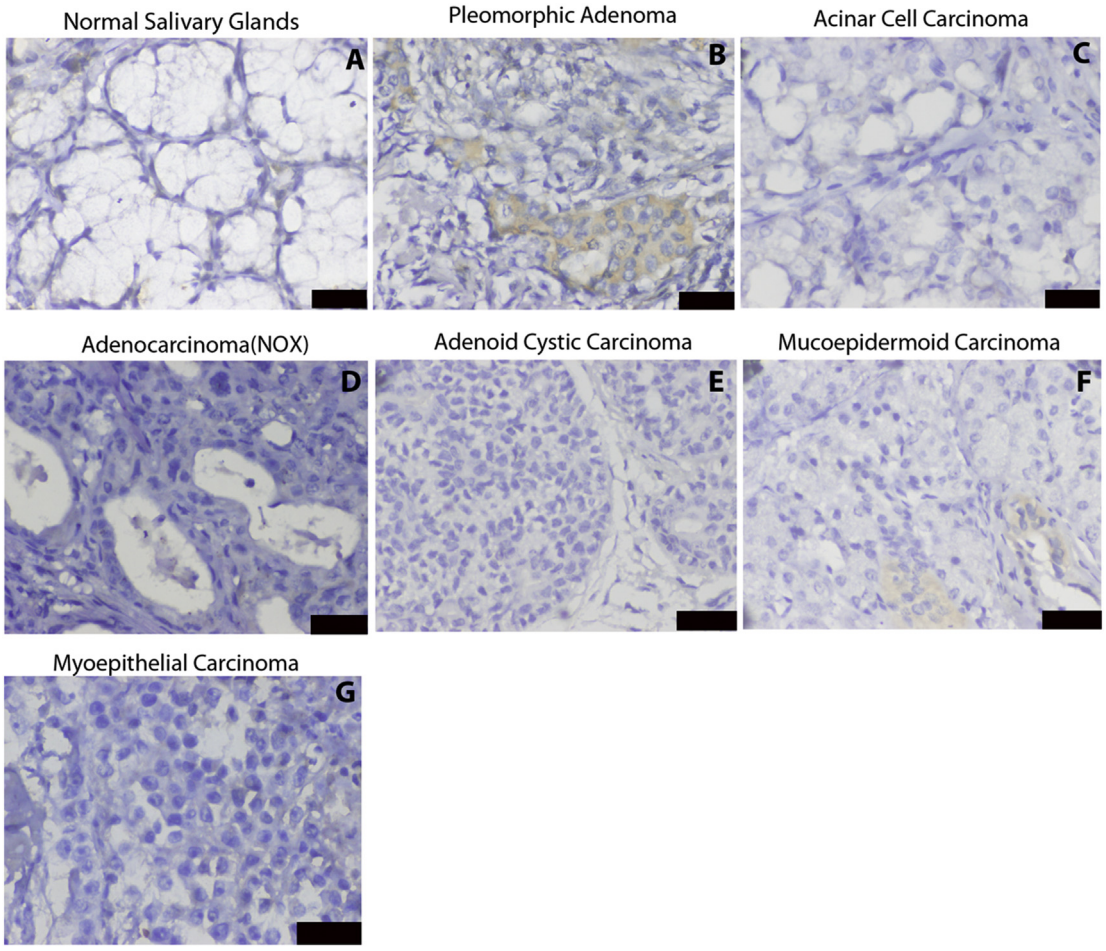

Fig. 2. Qualitative HIF-1α immunoexpression in samples.
HIF-1α expression qualitative results in Control (A), Pleomorphic Adenoma (B), Acinar Cell Carcinoma (C) Adenocarcinoma (NOX) (D), Adenoid cystic carcinoma (E), Mucoepidermoid Carcinoma (F) and Myoepithelial Carcinoma (G).

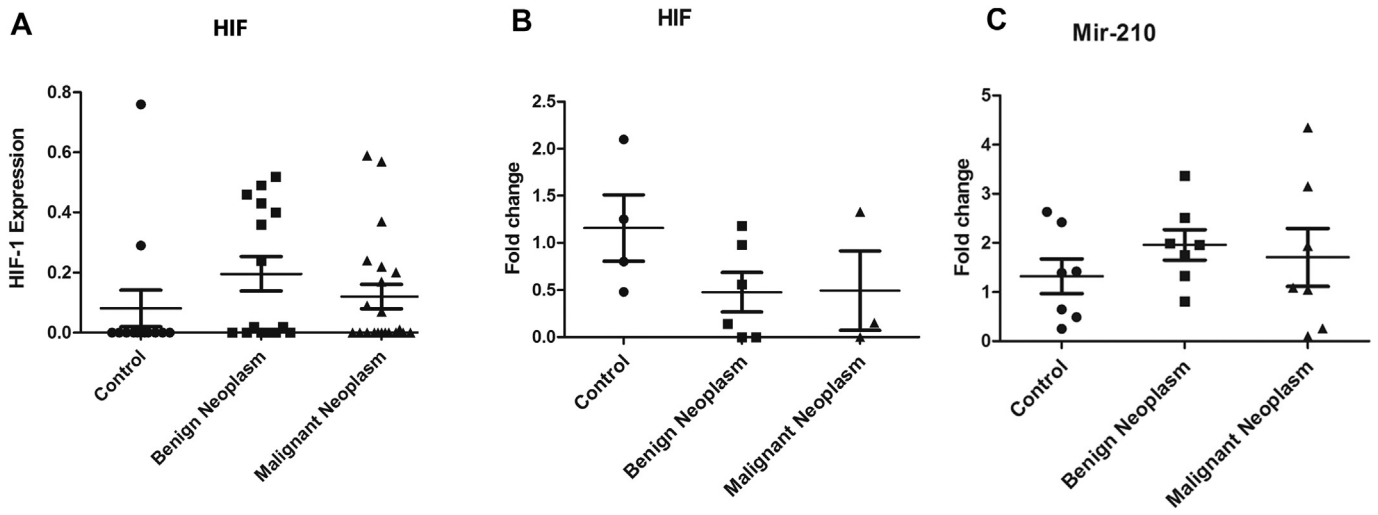

Fig. 3. Quantitative analyses of HIF-1α immunoexpression, HIF-1α mRNA and miR-210.
HIF-1α immunoexpression (A), HIF-1α mRNA (B) and miR-210 (C) in control, benign and malignant SGNs. (A). HIF-1α expression (A) or mRNA levels (B) is not altered in SGNs, compared with control samples. No difference in expression of miR-210 is observed between groups (C).

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Ethical approval

Ethical approval for this study was obtained from the relevant Institutional Review Board (52767316600005146). All patients signed informed consent.

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