Abstract
Objective
The aim of this study was to assess whether C1772T and G1790A HIF-1α polymorphisms are associated with odds ratio of oral squamous cell carcinoma (OSCC) development.

Materials and Methods
Restriction fragment length polymorphism analysis was used to investigate hypoxia-inducible factor (HIF)-1α C1772T and G1790A polymorphisms in 48 patients with epithelial dysplasia (ED) and 40 patients with OSCC. Additionally, 88 elderly individuals without head and neck squamous cell carcinoma were enrolled as a control group.

Results
The frequency of the TT, GA and AA genotypes was higher in patients with ED and OSCC when compared with controls. However, CT genotype was associated with moderate epithelial dysplasia in ED patients, while TT genotype was more frequent in OSCC patients.

Conclusions
In conclusion, our study demonstrated that the T and A alleles of C1772T and G1790A polymorphisms of the HIF-1α gene increased the risk of ED and OSCC. C1772T and G1790A polymorphisms of the HIF-1α gene had differing patterns of allelic imbalance in the precancerous lesions and subsequent carcinoma, suggesting a complex genetic pattern of progression from dysplasia to carcinoma. These findings suggest an additional role for HIF-1α in OSCC development. Further studies are necessary to elucidate the HIF-1α pathway in carcinogenesis, which would facilitate the development of novel therapeutic strategies for the prevention and treatment of OSCC and other solid tumours.

Introduction
Oral squamous cell carcinoma (OSCC) is a major health problem in many parts of the world and is associated with significant morbidity and mortality. While OSCC may often be preceded by a period during which the affected epithelium shows histological evidence of epithelial dysplasia, this may not always be clinically apparent. The annual percentage of malignant transformation varies in different parts of the world, owing to variations in the use of tobacco, alcohol consumption and dietary habits. The rate of malignant transformation varies from nearly 0% to almost 20% in 1 to 30 years.

During the progression from normal oral mucosa to OSCC, hypoxia-like proteins can be associated to the epithelial malignization. Early stages of oral cancer development present increased expression of matrix metalloproteinase (MMP) proteins, which favour the development of premalignant lesions. Higher expression of MMPs is associated with an increase in vascular endothelial growth factor (VEGF). A protein expression, and both proteins lead to malignization. VEGF is a major modulator of angiogenesis as it promotes endothelial cell migration through a hypoxic area and its regulation is promoted by hypoxia-like proteins.

An imbalance between oxygen delivery and consumption results in hypoxia, a hallmark of human cancers that contributes to resistance to therapy. The condition of hypoxia inducible factors is controlled by hypoxia-inducible factor-1 (HIF-1), which is a basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β, the former being the major gene involved in the development of hypoxia. The contribution of HIF-1α to tumour progression is largely attributed to its ability to induce the expression of genes whose products contribute to metabolic reprogramming, angiogenesis and metastasis. Several studies have demonstrated that both C1772T and G1790A polymorphisms in exon 12 of the HIF-1α gene can increase the transcriptional activity of this gene compared with the wild-type isoform. Moreover, both polymorphisms may regulate some factors secreted by primary tumours that alter the microenvironment and support the establishment of metastasis in lymph nodes, having a negative impact on survival in head and neck squamous cell carcinoma (HNSCC). However, to our knowledge, no study has associated C1772T and G1790A HIF-1α polymorphism to ED. Based on these data, the aim of this study was to assess whether C1772T and G1790A HIF-1α polymorphisms are associated with odds ratio of OSCC development.

Materials and methods
Patients and samples
Ethical approval for this study was obtained from the relevant Institutional Review Boards. All patients gave their informed consent prior to their inclusion in the study. The study was conducted according to the principles of the Helsinki Declaration. All authors abide by the Association for Medical Ethics (AME) ethical rules of disclosure.

DNA isolation and HIF-1α genotyping

DNA was isolated from ten 10-µm tissue sections from each tissue block of HNSCC specimens using the DNeasy Tissue kit (Qiagen, Chatsworth, CA, USA), as previously described. Oral mucosal cell samples from healthy patients were collected during oral clinical examinations. DNA extraction was carried out as described by Boom et al.14 and modified as described by Farias et al.15. HIF-1α (C1772T and G1790A) polymorphisms were assessed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). PCRs for HIF-1α were performed in a total volume of 25 µL containing approximately 100 ng genomic DNA as a template, 0.5 µL of each primer (20 pmol/µL), 2.5 µL dNTP mix (25 mM of each; Amresco, Ohio, CA, USA), 2.5 µL 10× PCR buffer, 1.25 µL magnesium chloride (50 mM) and 2.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). DNA sequencing of each product was performed to confirm the PCR-RFLP genotyping. The conditions for the PCR assay were denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 57.4°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The forward primer sequence was 5′- AAG GTG TGG CCA TTG TAA AAA CTC 3′ and the reverse primer sequence was 5′- CAG TGG TAG TGG TGG C 3′. This primer pair produced a fragment of 240 bp. The 240-bp PCR product of the HIF-1α gene was digested using the SsiI (Acil) restriction endonuclease (Fermentas Life Sciences, Hanover, MD, USA) for the G1790A polymorphism (rs11549467). The substitution from a G to an A allele produces a single cut site to produce two bands of 131 and 109 bp.15

Electrophoresis

Digested fragments of the PCR products were analysed on a 6.5% polyacrylamide gel that was electrophoresed at 120 V at constant voltage for 1.5 h and stained with Safe DNA gel stain (Invitrogen). Electrophoresis results were estimated by comparing fragment sizes to a 100-bp ladder.

Statistical analysis

OL samples were grouped into two categories: normal epithelium/mild dysplasia and moderate/severe dysplasia. Chi-square and Fisher’s exact frequency tests were applied for the statistical analysis of results. All statistical analyses were performed with SPSS® v13.0 for Windows®. P values <0.05 were considered significant.

Results

Sociodemographical and clinicopathological characteristics

Clinicopathological characteristics of the study group are presented in Table 1. The mean age among the controls, leukoplakia and cancer cases was 71.74 ± 6.77, 45.0 ± 6.52 and 62.10 ± 13.99 years, respectively.

Molecular data in controls, OL and OSCC samples

To determine whether the presence of either of the two single-nucleotide polymorphisms of the HIF-1α gene is associated with the development of ED or OSCC in the study population, 88 individuals without HNSCC were enrolled as a control group. The frequency of the T and A alleles was higher in patients with ED and OSCC when compared with the controls (Table 2).

TT and AA genotypes were increased in patients with OSCC when compared with ED. Moreover, CT genotype was associated with moderate epithelial dysplasia, while TT
Table 1 Sociodemographical and clinicopathologic characteristics of control, ED and OSCC patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Controls n (%)</th>
<th>Epithelial dysplasia n (%)</th>
<th>OSCC n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>61 (69.3)</td>
<td>14 (22.9)</td>
<td>18 (45.0)</td>
</tr>
<tr>
<td>Yes</td>
<td>27 (30.7)</td>
<td>34 (77.1)</td>
<td>22 (55.0)</td>
</tr>
<tr>
<td>Tobacco use</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>62 (70.5)</td>
<td>11 (22.9)</td>
<td>13 (32.5)</td>
</tr>
<tr>
<td>Yes</td>
<td>26 (29.5)</td>
<td>37 (77.1)</td>
<td>27 (67.5)</td>
</tr>
<tr>
<td>TNM clinical staging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>16 (40.0)</td>
<td>24 (60.0)</td>
<td></td>
</tr>
<tr>
<td>III/IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumour size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1/T2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3/T4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locoregional metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>23 (57.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>17 (42.5)</td>
<td></td>
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</tbody>
</table>

All values were calculated using the χ² test.
Comparisons: *controls × ED, **ED × OSCC patients.

In bold, statistically significant results.

demonstrated that genetic and epigenetic alterations could be associated with an increased risk for oral cancer development\(^6,25\). OL is a clinical diagnosis that describes white patches or plaques that cannot be attributed to any other disease. Approximately 15% of OL is diagnosed as mild or moderate dysplasia and about 5% may be diagnosed as severe dysplasia or carcinoma in situ\(^1\). The investigation of genetic polymorphisms associated with factors that play a role in the mechanisms that lead to tumour formation is of significant interest\(^22,23,26\).

Functional genetic polymorphisms have been demonstrated in vast majority of oral diseases\(^27-30\). Genetic polymorphisms C1772T and G1790A in exon 12 of HIF-1α have been associated with increased transcriptional activity and increased levels of HIF-1α, influencing the progression of the disease\(^5,12,13,31,32\). It has been demonstrated that high HIF-1α protein levels in lymph nodes may regulate some factors secreted by primary tumours that alter the microenvironment and support the establishment of metastasis in lymph nodes. Moreover, HIF-1α G1790A polymorphism together with HIF-1α protein expression may have a negative impact on the prognosis of patients with HNSCC\(^13\).

To our knowledge, the present study is the first one that investigates the association between C1772 and G1790A polymorphisms of HIF-1α gene in premalignant lesions and OSCC in a Brazilian population. We observed that TT, GA and AA genotypes were frequently higher in ED samples when compared with the controls. However, when we compared OSCC and ED patients, the former presented increased TT and AA genotype frequencies. This demonstrates that the presence of TT and AA genotypes may increase susceptibility to the development of malignant diseases.

In the present study, we focused on HIF-1α polymorphisms in ED and OSCC patients. The genotype was more frequent in OSCC (Table 2). No significant association was observed between HIF-1α polymorphisms and dysplasia grade (data not shown).

Table 2 Genotype and allele frequencies of the C1772T and G1790A polymorphisms of the HIF-1α gene in control, ED and OSCC patients

<table>
<thead>
<tr>
<th>Gene variant/genotype</th>
<th>Controls n (%)</th>
<th>Grading of oral dysplasia</th>
<th>OSCC n (%)</th>
<th>P value*</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mild dysplasia n (%)</td>
<td>Moderate/severe dysplasia n (%)</td>
<td></td>
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</tr>
<tr>
<td>C1772T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>85 (96.6)</td>
<td>13 (17.1)</td>
<td>1 (7.7)</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CT</td>
<td>3 (3.4)</td>
<td>14 (40.0)</td>
<td>8 (61.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td></td>
<td>15 (42.9)</td>
<td>4 (30.8)</td>
<td></td>
<td></td>
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<tr>
<td>G1790A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>81 (92.0)</td>
<td>5 (14.3)</td>
<td>2 (15.4)</td>
<td>&lt;0.001</td>
<td>2 (5.0)</td>
</tr>
<tr>
<td>GA</td>
<td>7 (8.0)</td>
<td>23 (65.7)</td>
<td>9 (69.2)</td>
<td></td>
<td>1 (2.5)</td>
</tr>
<tr>
<td>AA</td>
<td>0 (0)</td>
<td>7 (20.0)</td>
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Discussion

Oral carcinogenesis is a multistep process whose underlying mechanisms are still unclear\(^22-24\). Several studies regarding cancerization have demonstrated that genetic and epigenetic alterations could be associated with an increased risk for oral cancer development\(^6,25\). OL is a clinical diagnosis that describes white patches or plaques that cannot be attributed to any other disease. Approximately 15% of OL is diagnosed as mild or moderate dysplasia and about 5% may be diagnosed as severe dysplasia or carcinoma in situ\(^1\). The investigation of genetic polymorphisms associated with factors that play a role in the mechanisms that lead to tumour formation is of significant interest\(^22,23,26\).

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Discussion

Oral carcinogenesis is a multistep process whose underlying mechanisms are still unclear\(^22-24\). Several studies regarding cancerization have demonstrated that genetic and epigenetic alterations could be associated with an increased risk for oral cancer development\(^6,25\). OL is a clinical diagnosis that describes white patches or plaques that cannot be attributed to any other disease. Approximately 15% of OL is diagnosed as mild or moderate dysplasia and about 5% may be diagnosed as severe dysplasia or carcinoma in situ\(^1\). The investigation of genetic polymorphisms associated with factors that play a role in the mechanisms that lead to tumour formation is of significant interest\(^22,23,26\).

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Discussion

Oral carcinogenesis is a multistep process whose underlying mechanisms are still unclear\(^22-24\). Several studies regarding cancerization have
OSCC patients. Although there are no reports of HIF-1α polymorphisms in oral carcinogenesis, some studies have investigated HIF-1α protein levels in carcinogenesis\textsuperscript{33–36}. There is increasing evidence suggesting that HIF-1 activation occurs in the early stages of carcinogenesis. HIF-1α and HIF-1 target genes were shown to be overexpressed in hyperplastic and dysplastic skin lesions during multistage epidermal carcinogenesis\textsuperscript{37}. Moreover, overexpression of HIF-1α and activation of its target genes from normal tissue through premalignant lesions to carcinomas have already been observed in colorectal\textsuperscript{39}, hepatocellular\textsuperscript{33,38}, prostate\textsuperscript{39}, gastric\textsuperscript{35}, breast\textsuperscript{40}, cervical\textsuperscript{34} and endometrial\textsuperscript{41} carcinogenesis.

Several studies highlight hypoxia-like proteins, particularly epidermal growth factor (EGF), MMPs and VEGF upregulation, as frequent and important components of multistage oral carcinogenesis\textsuperscript{4}. It has been proposed that MMP increases VEGF activity of pathological angiogenesis during tumourigenesis. Deletion of MMP-9 in a mouse model suppressed tumour progression due to the inhibition of VEGF mobilization from extracellular matrix\textsuperscript{43–44}. In early stages of oral cancer development, the expression of matrix MMPs favours the development of premalignant lesions, and the expression of MMPs, EGF and VEGF\textsuperscript{45} is associated to the progression to malignization\textsuperscript{46,47}.

Recent studies have further shown that hypoxia-independent HIF-1α activation is induced by various mechanisms such as oncogene activation\textsuperscript{48}, inactivation of tumour suppressor genes\textsuperscript{49} and activation of growth factor signalling\textsuperscript{50}. In particular, phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase signalling activate the mammalian target of rapamycin and protein-synthesizing machinery, which in turn upregulates HIF-1α expression\textsuperscript{51}. Several alterations in the activity and oligomerization of the m2 isoform of pyruvate kinase by the HPV-16 E7 oncoprotein is a potential link between enhanced aerobic glycolysis (HIF-1 α function) and HPV in cancer development\textsuperscript{37}. These findings suggest that HIF-1α and HPV-16 may also cooperate at the level of cellular metabolism. High-risk HPV infection has emerged as a possible aetiologic factor for HNSCC and potential malignant lesions in the oral cavity. In a previous study, we observed that HPV-16 is present in 64.6% and 26.7% of mild/moderate ED and HNSCC samples, respectively\textsuperscript{53}. Coordinate functions of both HPV E7 and HIF-1/HIF-1α could enhance the metabolic adaptation of HPV-expressing cells to focal regions of oral cancer development. However, this hypothesis needs to be confirmed in further studies.

These results, in conjunction with previous studies\textsuperscript{5,33,34}, suggest that HIF-1α has an important role in the initial stages of oral cancer. Furthermore, we suggest that HIF-1α polymorphisms can alter HIF-1α protein expression and simultaneously enhance its target genes, thereby altering the microenvironment and supporting sequential epithelial dysplasia and carcinomas from the oral cavity.

**Conclusion**

In conclusion, our study demonstrated that the T and A alleles of C1772T and G1790A polymorphisms of the HIF-1α gene increased the risk of ED and OSCC. C1772T and G1790A polymorphisms of the HIF-1α gene had differing patterns of allelic imbalance in the precancerous lesions and subsequent carcinoma, suggesting a complex genetic pattern of progression from dysplasia to carcinoma. These findings suggest an additional role for HIF-1α in OSCC development. Further studies are necessary to elucidate the HIF-1α pathway in carcinogenesis, which would facilitate the development of novel therapeutic strategies for the prevention and treatment of OSCC and other solid tumours.

**Abbreviations list**

ED, epithelial dysplasia; EGF, epidermal growth factor; HIF, hypoxia-inducible factor; HNSCC, head and neck squamous cell carcinoma; MMP, matrix metalloproteinase; OL, oral leukoplakia; OSCC, Oral squamous cell carcinoma; VEGF, vascular endothelial growth factor

**Acknowledgements**

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**References**

8. Salceda S, Caro J. Hypoxia-inducible factor 1 alpha (HIF-1alpha) protein is rapidly