UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

GLAUCE GUIMARÃES PEREIRA

AVALIAÇÃO DE MUTAÇÕES NOS GENES TP53, PTEN E AKT EM CARCINOMA DE CÉLULAS ESCAMOSAS ORAL E LEUCOPLASIA ORAL DE FUMANTES E NÃO FUMANTES

ASSESSMENT OF TP53, PTEN AND AKT GENE MUTATION IN ORAL SQUAMOUS CELL CARCINOMA AND ORAL LEUKOPLAKIA OF SMOKERS AND NON-SMOKERS

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Estomatopatologia, na Área de Estomatologia.

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ORIENTADOR: PROF. DR. HELDER ANTONIO REBÊLO PONTES

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- ORCID do autor: https://orcid.org/0000-0002-6115-3743
- Currículo Lattes do autor: http://lattes.cnpq.br/2738227347840803

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PROF. DR. NICOLAU CONTE NETO

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RESUMO

O Carcinoma Espinocelular Oral (CEC) é o tumor maligno mais comum da cavidade oral, que pode ser precedido por lesões potencialmente malignas, das quais a leucoplasia oral (LO) é a mais prevalente. A LO apresenta uma taxa de transformação maligna de 3,5%, e ocorre mais comumente em lesões de língua. Neste estudo, nosso objetivo foi investigar a perda de heterozigosidade (LOH), bem como a frequência e os padrões das mutações nos genes *PTEN, AKT* e *TP53* em amostras de LO e carcinoma espinocelular em língua de pacientes fumantes e não fumantes. Amostras de tecido foram obtidas de 73 pacientes: 35 casos de CEC e 38 casos de LO de alto grau, ocorrendo na borda lateral da língua. Análise de sequenciamento de DNA foi realizada para identificar mutações nos genes *PTEN, AKT* e *TP53* e hibridização in situ por fluorescência foi utilizada para examinar a presença dos locus *PTEN, AKT* e *TP53*. Foram detectadas pequenas diferenças entre as alterações genéticas nos genes estudados tanto em pacientes fumantes quanto em não fumantes. As alterações nos genes *TP53* não ocorreram precocemente, no estágio LO, enquanto as alterações no gene *PTEN* ocorreram tanto nas LO quanto nos carcinomas. Além disso, alterações no gene *AKT* não ocorreram com alta frequência nem em LO nem em carcinomas, demonstrando que alterações na importante via PI3K são devidas a alterações genéticas no *PTEN* e não no gene *AKT*.

Palavras-chave: *TP53, PTEN, AKT,* Perda de Heterozigosidade e Hábito de Fumar.

ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity. It can be preceded by Oral leukoplakia (OL), the most prevalent potentially malignant disorder, which presents a malignant transformation rate of 3,5%, found most commonly in tongue lesions. In this study we aimed to investigate loss of heterozygosity (LOH) as well as the frequency and patterns of the mutations in the *PTEN, AKT* and *TP53* genes in OL and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients. Tissue specimens were obtained from 73 patients: 35 cases of TSCC and 38 cases of high grade OL occurring in the lateral border of the tongue. Sequencing analysis was performed to identify mutations in the *PTEN, AKT* and *TP53* genes. Fluorescence in situ hybridization was used to examine the presence of the *PTEN, AKT* and *TP53* locus. Slight differences between the genetic changes studied in the *TP53, PTEN* and *AKT* genes in smoking and non-smoking patients were detected. Changes in the *TP53* genes did not occur early, in the OL stage, while changes in the *PTEN* gene occurred in both OL and carcinomas. In addition, changes in the *AKT* gene did not occur with high frequency neither in OL nor in carcinomas, demonstrating that changes in the important PI3K pathway are due to genetic changes in *PTEN* rather than in the *AKT* gene.

Keywords: *TP53, PTEN, AKT,* Loss of Heterozygosity and Smoking behavior.

SUMÁRIO

1 INTRODUÇÃO

O Carcinoma Espinocelular Oral (CECO) é o tumor maligno mais comum em região de cabeça e pescoço [1] e pode ser precedido por lesões assintomáticas, coletivamente referidas como lesões potencialmente malignas. A leucoplasia oral (LO) é a lesão potencialmente maligna mais comum, com uma prevalência global de 2–3%. A taxa relatada de transformação maligna da LO é de 3,5%, sendo a língua o local mais comumente afetado.[2] Os mecanismos moleculares subjacentes responsáveis pela progressão de LO para carcinoma espinocelular ainda permanecem pouco conhecidos [3].

PTEN é um dos genes supressores de tumor mais frequentemente mutados no câncer humano. Desempenha um papel importante na via de sinalização PI3K/AKT da sobrevivência celular, regulando negativamente a atividade de AKT, a qual controla uma variedade de vias celulares críticas durante o processo carcinogênico, incluindo aquelas que levam à inibição da apoptose e aumento da proliferação celular, bem como aumento da invasão de células tumorais, angiogênese e metabolismo celular. Portanto, a perda da função do PTEN leva ao aumento da atividade de AKT, o que tem sido associada à metástase em vários tipos de malignidades [4,5,6].

Mutações somáticas, supressão gênica ou silenciamento epigenético que levam à perda de *PTEN* têm sido relatadas em uma variedade de lesões potencialmente malignas e neoplasias [7]. No entanto, em relação aos carcinomas espinocelulares em região de cabeça e pescoço, mutações e deleções pontuais do gene *PTEN* parecem ser eventos raros, demonstrando que podem não constituir um fator direto responsável pela regulação negativa da proteína PTEN [8].

A proteína quinase B (AKT) é um importante alvo do receptor do fator de crescimento tirosina quinase que sinaliza via PI3K. Evidências crescentes sugerem que perturbações em AKT desempenham um papel importante em malignidades humanas. Estudos anteriores em

CECO demonstraram expressão de níveis significativamente mais elevados de p-Akt, comparado à displasia epitelial e ao epitélio normal, sugerindo que a expressão de p-Akt pode ser um evento precoce e potencialmente crítico em casos de lesões displásicas que progridem para câncer oral. Além disso, a superexpressão de p-Akt demonstrou ser um indicador de mau prognóstico para CECO. No entanto, apesar de mutações em *AKT* ocorrerem em 3-5% dos cânceres humanos, em carcinomas espinocelulares orais, mutações do gene *AKT* foram raramente documentadas [9, 10].

O gene supressor de tumor humano p53 (*TP53*) detecta aberrações de DNA e está envolvido em um grupo de eventos biológicos, como controle do ciclo celular, apoptose e preservação da estabilidade genômica. Algumas variações genéticas do gene *TP53* foram relatadas como associadas à carcinogênese humana e, por ter sido um gene extensivamente estudado, tem sido apontada como um biomarcador para predizer tanto o grau de displasia quanto o potencial de transformação maligna da LO [6]. Portanto, juntamente ao *KMT2C*, *TP53* tem sido descrito como o gene mais frequentemente mutado em LO com ou sem displasia [11].

A carcinogênese oral é um processo complexo e muitos estudos têm focado em alterações moleculares presentes em leucoplasias orais na tentativa de identificar biomarcadores preditivos para sua transformação maligna. Desse modo, vários marcadores moleculares, usados sozinhos ou em combinação, têm sido reconhecidos como importantes no estudo da displasia epitelial oral e da carcinogênese [12, 13]. No entanto, a frequência de mutação nos genes *PTEN, AKT* e *TP53* em LO e seu impacto na transformação maligna dessa lesão permanece por ser investigado. Assim, neste estudo, pretendemos investigar a perda de heterozigosidade (LOH), bem como a frequência e padrões das mutações nos genes *PTEN*, *AKT* e *TP53* em LO e amostras de carcinoma espinocelular em língua de pacientes fumantes e não fumantes.

2 ARTIGO

Artigo submetido ao periódico _____ Oral Oncology

ASSESSMENT OF *TP53, PTEN* AND *AKT* GENE MUTATIONS IN TONGUE SQUAMOUS CELL CARCINOMA AND ORAL LEUKOPLAKIA OF SMOKERS AND NON-SMOKERS

Glauce Guimarães Pereira [a]; Flávia Sirotheau Corrêa Pontes [b]; Carolina Cavalieri Gomes [c]; Felipe Paiva Fonseca [c]; Ana Luisa Sirotheau Corrêa Pontes [b]; Sue Ann Lavareda Corrêa Uchoa [b]; Samara Silveira da Cruz [d]; Rommel Mario Rodriguez Burbano* [d] and Hélder Antônio Rebelo Pontes* [a,b].

a- Department of Oral Diagnosis (Pathology and Semiology Areas), Faculdade de Odontologia de Piracicaba, Universidade de Campinas, Piracicaba, Brazil;

b- Department of Oral Surgery and Pathology, Hospital Universitário João de Barros, Universidade Federal do Pará, Belém, Brazil;

c- Department of Pathology, Biological Sciences Institute, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil;

d- Laboratory of Molecular Biology, Hospital Ophir Loyola , Belém, Brazil;

*These authors contributed equally to this work

CORRESPONDING AUTHOR:

Glauce Guimarães Pereira

Department of Oral Diagnosis (Pathology and Semiology Areas), Faculdade de Odontologia de Piracicaba, Universidade de Campinas, Piracicaba, Brazil;

Address: Av. Limeira, 901 - Areião, Piracicaba - SP, 13414-903.

E-mail: glaucepereira94@gmail.com Telephone number: +55 91 980450417

ABSTRACT

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity. It can be preceded by Oral leukoplakia (OL), the most prevalent potentially malignant disorder, which presents a malignant transformation rate of 3,5%, found most commonly in tongue lesions. In this study we aimed to investigate loss of heterozygosity (LOH) as well as the frequency and patterns of the mutations in the PTEN, AKT and TP53 genes in OL and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients. Tissue specimens were obtained from 73 patients: 35 cases of TSCC and 38 cases of high grade OL occurring in the lateral border of the tongue. Slight differences between the genetic changes studied in the TP53, PTEN and AKT genes in smoking and non-smoking patients were detected. Changes in the TP53 genes did not occur early, in the OL stage, while changes in the PTEN gene occurred in both OL and carcinomas. Furthermore, changes in the AKT gene did not occur with high frequency in either OL or carcinomas, demonstrating that changes in the important PI3K pathway are primarily due to genetic changes in PTEN rather than in the AKT gene.

Keywords: *TP53, PTEN, AKT*, Loss of Heterozygosity and Smoking behavior.

INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity [1]. A significant number of OSCC can be preceded by asymptomatic clinical lesions collectively referred to as oral potentially malignant disorders (OPMD). Oral leukoplakia (OL) is the most prevalent OPMD, with a global prevalence of 2–3%. The reported rate of OL malignant transformation is 3.5%, with tongue lesions being the most commonly affected site[2].

PTEN is a tumor suppressor gene, which frequently shows loss of heterozygosity (LOH) or mutations in human cancer. Its encoded protein, PTEN, plays an important role in the PI3K/Akt signaling pathway of cell survival by negatively regulating Akt (protein kinase B) activity. Akt is a major downstream target of growth factor receptor tyrosine kinase that signals via phosphatidylinositol -3 kinase (PI3K) and controls a variety of critical cellular pathways during the carcinogenic process, including those leading to apoptosis inhibition and increased cell proliferation, as well as enhanced tumor cell invasion, angiogenesis and cell metabolism. In addition, loss of PTEN function and increased Akt activity has been associated with poor prognosis in a number of malignancies, including OSCC [3-5].

TP53 is a tumor suppressor gene and it is the most frequently altered gene in human cancer. It is involved in a group of cell biology events, such as cell cycle control, apoptosis and the preservation of genomic stability. In oral carcinogenesis, *TP53* encoded protein, p53, has been pointed out as a biomarker for predicting the malignant transformation of OL [6].

Oral carcinogenesis is a multistep process and a combination of environmental risk factors, viral infection, genetic and epigenetic alterations might give rise to OSCC [7]. In this sense, several studies have focused on molecular alterations of OL in an attempt to identify predictive biomarkers for malignant transformation. Several molecular markers, used alone or in combination, have been recognized as important in the study of oral carcinogenesis [8]. However, the frequency of mutation in *PTEN, AKT* and *TP53* genes in both OL and OSCC remains to be investigated. Additionally, it is not clear if such frequencies are similar in smokers and non-smokers. Therefore, in this study, we aimed to investigate LOH, as well as the frequency and patterns of the mutations in the *PTEN, AKT* and *TP53* genes in OL and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients.

MATERIALS AND METHODS

Tissue samples

The Formalin-fixed, paraffin-embedded (FFPE) tissue specimens were obtained from 73 patients: 35 cases of TSCC and 38 cases of high grade OL occurring in the lateral border of the tongue. Histological sections (5 μm) of all samples were routinely stained with haematoxylin and eosin (H&E) and analyzed under light microscopy. Two independent oral pathologists without prior knowledge of the clinical data assessed the stained sections to classify the histological grades of oral dysplasia according to previously proposed binary system criteria of classification [9]. In the cases of disagreement, pathologists discussed the findings to achieve a final agreement.

Ethical aspects

This study was approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas, Piracicaba, Brazil (process number 6.010.777).

Nucleic acid extraction

The genomic DNA (gDNA) was extracted using the QIAamp DNA Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Total RNA was extracted with Tri-reagentH (Life Technologies, Carlsbad, California, USA) according to the manufacturer's protocol. DNA and RNA concentration and quality were determined using the NanoDrop spectrophotometer (Kisker, Germany). RNA integrity was determined by gel electrophoresis (1% agarose gels). All samples were stored at -80ºC until use.

PTEN, AKT and TP53 copy number

FISH was used to evaluate *PTEN*, *TP53* and *AKT* copy number in a subset of 74 samples. FISH was performed according to the protocol of Pinkel et al. [10] with modifications introduced by Calcagno et al [11]. Cells were hybridized with Spectrum Orange Probe (LSI Vysis/Abbott, Inc., IL) and nuclei were counterstained with 49,6-diamidino-2-phenylindole antifade. Fluorescence was detected using an Olympus BX41 fluorescence microscope (Olympus, Japan) with excitation filters for 49,6- diamidino-2-phenylindole (260 nm) and rhodamine (570 mn). For each case, 200 interphase nuclei were analyzed using an ASI image analysis system (Applied Spectral Imaging, Israel). Positive *PTEN*, *TP53* and *AKT* gene signals appeared as red spots in nuclei and were scored using the criteria of Hopman et al. [12] To avoid misinterpretation due to technical error, normal lymphocyte nuclei and normal oral tissue were used as controls. The FISH results were presented as the percentage of *PTEN, TP53* and *AKT* amplification by a cell, in which the percentage of cells showing 3 or more signals for *PTEN, TP53* and *AKT* probes by cell were calculated.

PTEN, AKT and TP53 genotyping

Exons of *PTEN, TP53* and *AKT* genes were selected for mutation analysis in all 74 OSCC/OL samples. The PCR reactions were carried out with 0.1 mmol/L of dNTPs, 2 mmol/L of MgCl2, 0.5 mmol/L of primers, 1 U of Taq polymerase, and 100 ng of DNA. The PCR reactions followed standard conditions. The amplicons were separated on a 2% agarose gel stained with SYBRH Safe DNA Gel Stain (Life Technolgies, USA) and directly visualized under UV illumination.

Amplicons were sequenced using the Sanger method. Direct sequencing was carried out using the Big DyeH Terminatorv3.1 Cycle Sequencing kit (Life Technologies, USA) and analyzed on an ABI PRISMH 3130 Genetic Analyzer (Life Technologies, USA) using Pop 7 polymer.

The sequencing chromatograms were inspected using the Chromas Pro 1.5 (Technelysium Pty Ltd, Australia). The reference sequences were Gene ID: 5728 (NCBI), ID: 7157 (NCBI), ID: 207 (NCBI) . Variants with less than 1% minor allele frequency were reported. The predicted pathogenicity of missense mutations was assessed by *in silico* analysis using PolyPhen (http://genetics.bwh.harvard.edu/ pph/) and SIFT (http://sift.jcvi.org).

Statistical analysis

To determine any significant differences in allelic loss and mutation frequency of *PTEN, TP53* and *AKT* among the groups investigated, a Fisher's Exact test was applied. Statistical analysis was conducted using RStudio Team 2023 (RStudio Team, Boston, MA, USA), with a significance level set at $p < 0.05$ and 95% confidence intervals.

RESULTS

Sample characterization

This study included 35 patients (20 male and 15 female) with TSCC. Mean age of 63.5 years was observed, ranging from 27 to 100 year-old. Eighteen (51.4%) were smokers, and 17 (48.6%) were non-smokers. Regarding the OL cases, 38 (11 male and 27 female) high grade cases in the lateral border of the tongue were retrieved. Seventeen (44.7%) were smokers and 21(55.3%) were non-smokers. Mean age of 57.5 years was observed, ranging from 32 to 83 year-old.

Tongue squamous cell carcinoma molecular results

Table 1 shows the results and specific mutations by sample. In tobacco-associated OSCC, *TP53* LOH was detected in 14/18 (77.8%) of the cases. The most frequent mutation was missense (12/14 - 85.7%), followed by frameshift deletion (2/14 - 14.3%).

In non-smokers, *TP53* LOH was detected in 9/17 (52.9%) of the cases. The most frequent mutation was missense (6/9 - 66.7%), followed by frameshift deletion (2/9 - 22.2%) and frameshift insertion (1/9 - 11.1%). One out of seventeen (5.9%) patients demonstrated allele deletion but the remaining gene was not mutated.

PTEN LOH was detected in 11/18 (61.1%) of smokers cases. The most frequent pattern of mutation was missense (8/11 - 72.7%), followed by nonsense mutation (2/11 - 18.2%) and exon deletion (1/11 - 9.1%). Two patients (11.1%) exhibited homozygous mutations, indicated by exon deletion and nonsense mutation. In two patients (2/18 - 11.1%), allele deletion was identified without mutations in the remaining gene.

PTEN LOH was detected in 6 of the 17 cases from non-smokers (35.3%). The most frequent mutation was missense (4/6 - 66.7%), followed by exon deletion (2/6 - 33.3%).

AKT LOH in smokers was detected in two patients (2/18 - 11.1%) and these cases showed AKT p.E17K. There also was one case (1/18 - 5.6%) of homozygous mutation, showing the activating mutation AKT p.E17K. In three patients (3/18 - 16.7%), we identified allele deletion but the remaining gene was not mutated.

We identified LOH for *AKT* in 1 of the 17 cases from non-smokers (5.9%). The mutation in the remaining allele was activating mutation AKT p.Q79K. Three (3/17 - 17.7%) of the non-smoker cases demonstrated allele deletion without mutations in the remaining gene. One case showed homozygous missense mutation (AKT p.E17K).

Samples from two smokers simultaneously harboured *PTEN*, *TP53* and *AKT* mutation, 8 out of 18 (44.4%) had a *PTEN/TP53* concomitant mutation, one (1/18 - 5.9%) had a *PTEN/AKT* concomitant mutation.

Two non-smoker patients' cases (2/17 - 11.8%) had *TP53/PTEN* co-occurring mutations, one (1/17 - 5.9%) had a *TP53/AKT* co-occurring mutations and one sample (1/17 - 5.9%) had a *PTEN/AKT* concomitant mutation.

Oral Leukoplakia molecular results

Table 2 shows the results and specific mutations by sample. Considering smokers patients, LOH at *TP53* was not observed. In 3/17 cases, both alleles were mutated and the most frequent mutation was frameshift deletion (2/3 - 66.7%), followed by missense (1/3 - 33.3%). Meanwhile in non-smokers, one sample (1/21 - 4.8%) showed LOH for *TP53* (nonsense mutation). Homozygous mutations were detected in four cases (4/21 - 19.1%), and the most frequent pattern of mutation was frameshift deletion (3/4 - 75%), followed by nonsense mutation (1/4 - 25%).

7/17 (41.2%) of the smokers samples showed LOH at *PTEN*, all missense mutations. Four cases (4/17 - 23.5%) had homozygous mutation, of which two (2/4 - 50%) showed missense mutation and the other two cases (2/4 - 50%), exon deletion. In non-smokers, LOH at *PTEN* was found in 3 out of 21 cases (14.3%). Missense mutation was detected in all remaining alleles. In addition, three cases (3/21 - 14.3%) demonstrated homozygous missense mutation.

LOH at *AKT* was not observed in the OL samples from smokers. One out of the 17 smokers (5.9%) showed *PTEN* and *AKT* mutation, the last one being the activating mutation AKT p.L52R. In non-smokers, LOH at AKT was detected in one case (1/21 - 4.8%) and the remaining allele showed the activating mutation AKT p.E17K. In three cases (3/21 - 14.3%), homozygous mutation was detected, two demonstrating activating mutant p.L52R and one demonstrating activating mutant AKT p.E17K.

Regarding smokers patients, two samples (11.8%) exhibited simultaneous mutations in *PTEN* and *TP53*. In non-smokers, one sample (1/21 - 4.8%) demonstrated co-occurrence of *TP53* and *AKT* mutations, while another sample (1/21 - 4.8%) displayed a double mutation in *PTEN* and *AKT*.

DISCUSSION

OSCC remains a significant health problem [13], leading to a poor survival rate, high morbidity and mortality [14]. Considering that the smoking habit is a well-established risk factor for both OPMD and OSCC [13], in this study we investigated LOH, the frequency and patterns of the mutations in the *TP53*, *PTEN* and *AKT* genes in both TSCC and OL tissues of smokers and non-smokers.

Regarding tobacco-associated TSCC, our results show *TP53* as the most frequently mutated gene (77.8%), consistently with previous reports [15]. In agreement with findings by Nagakagi et al and Hyodo et al [16, 17], this study demonstrates that the *TP53* mutation site is mostly localized in the DNA binding Domain (DBD), and that missense pattern of mutation is predominant (85.7%). Although truncating mutations are more common outside the DBD [16], two patients exhibited missense mutations at codon 45, in the Transactivation Domain 2 (TAD2). In the DBD, the TP53 mutations found on codons 193, 220 and 248 had been formerly demonstrated on OSCC [16].

In non-smokers TSCC patients, *TP53* was also the most recurrently mutated gene, but the frequency of mutation was lower than in tobacco-associated TSCC. All cases exhibited LOH in the DBD region on codons 193, 203, 220 and 248 and missense was the most common mutation. Therefore, LOH for *TP53* was more frequent in smokers and missense mutations were predominant in both smokers and non-smokers.

PTEN was the second most frequently mutated gene in tobacco-associated TSCC, with a frequency of 72.2%. LOH at *PTEN* was detected in 61.1% of cases. Missense mutation was identified as the most frequent pattern of mutation in the remaining allele, differently to the findings of Kato et al [18], in which nonsense pattern was predominant. In the cases where homozygous mutation was detected, frameshift deletion and missense patterns were identified.

Similarly to tobacco-associated TSCC, *PTEN* was the second most frequently mutated gene in non-smoker patients with TSCC. However, the frequency of mutation was lower compared to smokers. All cases showed LOH and the missense pattern of mutation was predominant in the remaining gene.

AKT1 was found to be the least frequently mutated gene in smoking TSCC patients. Although past studies have pointed to high expression of p-AKT as correlated with a poor prognosis in OSCC, there is insufficient evidence to support *AKT1* gene mutation in OSCC [19]. Deletion not followed by mutation was detected in 16.7% of cases, and LOH occurred in an equal proportion of cases. In all cases of LOH, we detected activating mutant AKT p.E17K, which is a rare event in oral carcinogenesis, though it is the most common hotspot mutation in other malignancies [20]. No patient showed *AKT1* mutation alone, either it was a double (AKT/PTEN) or a triple mutation.

In non-smoker TSCC patients, *AKT1* was the least mutated gene as well, with a lower frequency of mutation compared to smokers. Missense mutation was the only pattern present. Similarly, any case showed *AKT1* mutation alone, only concomitant *AKT/PTEN* and *AKT/P53* mutations. Therefore, LOH was found to be infrequent in both smoking and non-smoking patients, suggesting it is not a highly prevalent event in oral carcinogenesis.

Regarding simultaneous mutations, smoking patients exhibited a greater number of double mutations in *PTEN* and *TP53* when compared to non-smoking patients (8 out of 18 patients and 2 out of 17 patients respectively). Moreover, smoking patients presented two cases with a triple mutation (*TP53*, *PTEN* and *AKT*), while non-smoking patients did not present any. This discrepancy suggests a possible difference in oral carcinogenesis between smokers and non-smokers.

Concerning tobacco-associated OL, *PTEN* was the most frequently mutated gene (64.7%). Although loss of *PTEN* function in OL has been previously reported [3,21], our findings

are in disagreement with the literature on this subject, as *TP53* and *KMT2C* have been described as the most frequently mutated genes in OL [22]. LOH was significantly more frequent than homozygous mutation, and missense pattern of mutation was predominant.

PTEN was the most recurrent mutated gene in OL of non-smokers as well (28.6%). However, the frequency of LOH and homozygous mutation was the same. Missense was the only pattern of mutation identified. Therefore, in non-smoking patients, allelic loss in the *PTEN* gene in both carcinomas and OL occurs less frequently compared to smoker patients. This difference reveals a distinction in oral carcinogenesis between these patient groups. However, a common thread is the predominance of missense mutations.

Considering that *PTEN* LOH in smoking patients occurs both in OL (41.2% of cases) and in TSCC (61.1% of cases), it is clearly an early event in oral carcinogenesis. Additionally, the frequency of the missense characterizes it as the main mutation in the process of acquiring the oral malignant phenotype from leukoplakia.

In this research, *TP53* was the second most recurrently mutated gene in OL of smokers (17.6%) and non-smokers (23.8%). Its frequency of mutation was significantly lower than PTEN in smokers and nearly the same as PTEN in non-smokers. In tobacco-associated OL, homozygous mutations were unanimous, with frameshift deletion being more frequent than missense mutation. Conversely, in non-smokers, homozygous mutations were predominant, with frameshift deletion being the most frequent mutations, followed by nonsense.

In both smokers and non-smokers OL patients, *TP53* LOH was infrequent, with only one case observed in non-smokers and none detected in smokers. Furthermore, only a few cases were mutated in the two groups. Therefore, it can be concluded that LOH and the presence of mutation in the studied samples do not represent an event that occurs early in oral carcinogenesis via OL in both smokers and non-smokers.

AKT1 was the least frequently mutated gene in OL of both smokers (5.9%) and non smokers (19%). In tobacco associated OL, only one homozygous missense mutation was present, which was a concomitant *AKT/PTEN* mutation. However, in non-smokers OL, although *AKT1* mutations were less frequent, their frequency was higher than in smokers, leading to the conclusion that AKT1 may play a stronger role in OL not associated with tobacco. Furthermore, the non-smoking group was the one in which we identified samples where *AKT1* was the only mutated gene, all of them presenting homozygous missense mutations. LOH for *AKT* in OL was not observed in smokers, and only one case was detected in the non-smoker group. Overall, as few mutations for *AKT1* were detected, it is deduced that the main alteration in the PI3K pathway in oral carcinogenesis in the cases studied arises especially from alterations in the *PTEN* gene.

In conclusion, the present study suggests that there are slight differences between the genetic changes studied in the *TP53, PTEN* and *AKT* genes in smoking and non-smoking patients. Changes in the *TP53* genes did not occur early, in the OL stage, while changes in the *PTEN* gene occurred in both OL and carcinomas, suggesting that such alterations occur early in oral carcinogenesis. Furthermore, changes in the *AKT* gene did not occur with high frequency in either OL or carcinomas, demonstrating that changes in the important PI3K pathway are primarily due to genetic changes in *PTEN* rather than in the *AKT* gene.

ACKNOWLEDGEMENTS

No acknowledgements.

CONFLICT OF INTEREST

The authors state that they have no potential conflict of interest that could bias the results obtained in the current study.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethical Committee of the Piracicaba Dental School, University of Campinas, Piracicaba, Brazil (process number 6.010.777).

FUNDING

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DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table 1 - PTEN, AKT and RP63 mutations in Oral Squamous Cell Carcinoma patients.

Smoker	**PTEN alleles**	**PTEN mutations[A]**	**AKT alleles**	**AKT mutations**	**P53 alleles**	**TP53 Mutation (codon)**	**AA change**
No	2	not mutated	2	not mutated	91%-2 9%-1	193	His → leu
No	95%-2 5% -1	c.209T>C p.(Leu70Pro)	2	not mutated	49%-2 51%-1	198–200 9-bp deletion	Frameshift
No	96%- 24%-1	c.697C>T p.(Arg233Ter)	90%-2 10% -1	not mutated	2	not mutated	wild
No	85%-2 15%-1	c.528T>G p.(Tyr176Ter)	80%-2 20%-1	Q79K	2	not mutated	wild
No	50%-2 50%-1	exons 6-9del	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	62%-2 38%-1	203	Val → Ala
No	2	not mutated	92%-2 8% -1	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	92%-2 8% -1	not mutated	54%-2 46%-1	not mutated	wild
No	2	not mutated	2	E17K	60%-2 40%-1	248	Arg → Glu

No	2	not mutated	2	not mutated	90%-2 10% - 1	248	Arg → Glu
No	2	not mutated	2	not mutated	83%-2 17% -1	220	Tyr → Cys
No	80%-2 20 % -1	exons 6-9del	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	79%-2 21%-1	237/238 3-bp insertion	Framesh ift
No	2	not mutated	2	not mutated	82%-2 18%-1	198–200 9-bp deletion	Framesh ift
No	88%-2 12%-1	c.395G>A p.(Gly132Asp)	2	not mutated	65%-2 35%-1	248	Arg → Glu
Yes	54%-2 46%-1	c.395G>A p.(Gly132Asp)	2	not mutated	87%-2 13%-1	248	Arg → Glu
Yes	2	not mutated	2	not mutated	57%-2 43 % -1	45	Leu → Met
Yes	52%-2 42% - 1	c.388C>T p.(Arg130Ter)	79%-2 21%-1	E17K	90%-2 10%-1	45	Leu → Met
Yes	83%-2 14%-1	not mutated	67%-2 33%-1	not mutated	91%-2 9%-1	184	Asp → Gly
Yes	64%-2 36% - 1	c.359T>G Stopp codon	70%-2 30%-1	not mutated	50%-2 50%-1	220	Tyr → Cys
Yes	92% - 2 8% - 1	c.388C>T p.(Arg130Ter)	2	not mutated	2	not mutated	wild
Yes	89% - 2 11% - 1	c.406T>C p.(Cys136Arg)	2	not mutated	63%-2 37%-1	248	Arg → Glu
Yes	63% - 2 37% - 1	Exon 2 deletion	2	not mutated	53%-2 47%-1	184	Asp → Gly
Yes	70% - 2 30% - 1	c.1003C>T p.(Arg335Ter)	2	not mutated	2	not mutated	wild
Yes	60% - 2 40% - 1	c.697C>T p.(Arg233Ter)	2	not mutated	2	not mutated	wild
Yes	47% - 2 53% - 1	not mutated	2	not mutated	92%-2 8%-1	203	Val → Ala
Yes	46%-2 54% - 1	c.359T>G Stopp codon	2	E17K	2	not mutated	wild
Yes	2	not mutated	2	not	87%-2	198–200	Framesh

				mutated	13%-1	9-bp deletion	ift
Yes	2	c.359T>G Stopp codon	2	not mutated	50%-2 50%-1	220	Tyr → Cys
Yes	2	not mutated	2	not mutated	90%-2 10%-1	250–253 10-bp deletion	Framesh ift
Yes	70% - 2 30% - 1	c.368A>G p.(His123Arg)	2	not mutated	88%-2 12%-1	248	Arg → Leu
Yes	78%-2 22%-1	c.406T>C p.(Cys136Arg)	95%-2 5% - 1	not mutated	60%-2 40%-1	193	His → Arg
Yes	2	Exon 2 deletion	72%-2 28% - 1	E17K	95%-2 5%-1	248	Arg → Glu

A- Numbering of the bases indicating the alteration is given relative to the cDNA sequence.

Table 2 - PTEN, AKT and TP53 mutations in Oral Leukoplakia patients

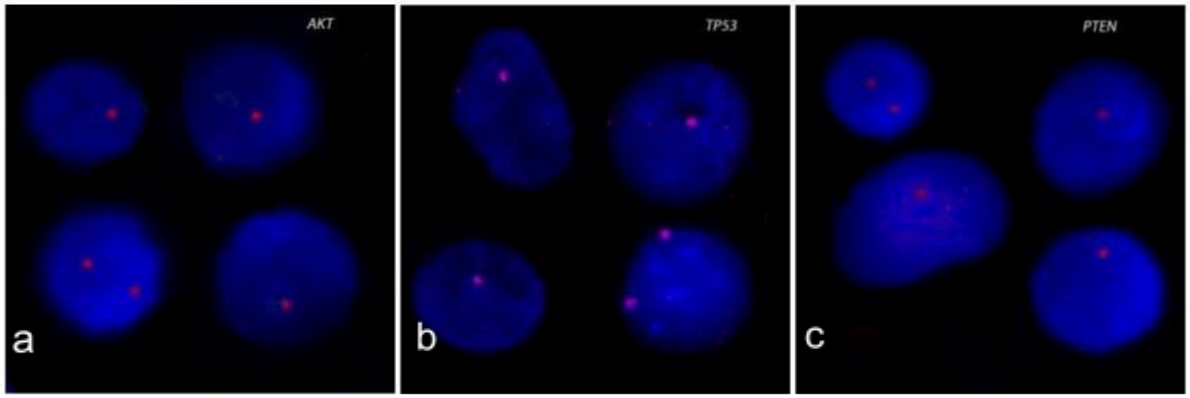
Smoker	**PTEN alleles**	**PTEN mutations[A]**	**AKT Alleles**	**AKT mutations**	**P53 alleles**	**TP53 mutation(codon)**	**AA change**
Yes	2	not mutated	2	not mutated	2	not mutated	wild
Yes	92%-2 8% - 1	c.386G>A p.(Gly129Glu)	2	not mutated	2	not mutated	wild
Yes	2	c.388C>T p.(Arg130Ter)	2	not mutated	2	not mutated	wild
Yes	2	not mutated	2	not mutated	2	not mutated	wild
Yes	2	exons 6-9del	2	not mutated	2	not mutated	wild
Yes	74%-2 26%-1	c.209T>C p.(Leu70Pro)	2	not mutated	2	not mutated	wild
Yes	2	not mutated	2	not mutated	2	not mutated	wild
Yes	2	not mutated	2	not mutated	2	45	Leu → Met
Yes	2	c.528T>G p.(Tyr176Ter)	2	not mutated	2	not mutated	wild
Yes	57%-2 43% - 1	c.388C>T p.(Arg130Ter)	2	not mutated		not mutated	wild
Yes	88%-2 9% - 1	c.388C>T p.(Arg130Ter)	2	not mutated	2	not mutated	wild
Yes	62%-2	c.1003C>T	2	not mutated	2	198–200 9-bp	Framesh

	38%-1	p.(Arg335Ter)				deletion	ift
Yes	80%-2 20% -1	c.388C>T p.(Arg130Ter)	2	L52R	2	not mutated	wild
Yes	2	not mutated	2	not mutated	2	not mutated	wild
Yes	47% - 2 53% - 1	c.386G>A p.(Gly129Glu)	2	not mutated	2	not mutated	wild
Yes	2	Exon 2 deletion	2	not mutated	2	250–253 10-bp deletion	Framesh ift
Yes	2	not mutated	2	not mutated	2	not mutated	wild
No	2	c.697C>T p.(Arg233Ter)	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	91	Trp → stop
No	82%-2 18% -1	c.388C>T p.(Arg130Ter)	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	88%-2 12% - 1	91	Trp → stop
No	2	c.697C>T p.(Arg233Ter)	75%-2 25 % -1	E17K	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	L52R	2	not mutated	wild
No	2	not mutated	2	not mutated	2	198–200 9-bp deletion	Framesh ift
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	E17K	2	198–200 9-bp deletion	Framesh ift
No	2	not mutated	2	not mutated	2	not mutated	wild
No	98%-2 2% -1	c.368A>G p.(His123Arg)	2	not mutated	2	not mutated	wild
No	75%-2 25% - 1	c.386G>A p.(Gly129Glu)	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild

No	2	not mutated	2	not mutated	2	198–200 9-bp deletion	Framesh ift
No	2	not mutated	2	L52R	2	not mutated	wild
No	2	c.697C>T p.(Arg233Ter)	2	not mutated	2	not mutated	wild
No	2	not mutated	2	not mutated	2	not mutated	wild

A- Numbering of the bases indicating the alteration is given relative to the cDNA sequence.

Figure 1. Fluorescence in situ hybridization (FISH) for *AKT* (a)*, TP53* (b) and *PTEN* (c).



3 CONCLUSÃO

O presente estudo sugere que existem pequenas diferenças entre as alterações genéticas estudadas nos genes *TP53, PTEN* e *AKT* em pacientes fumantes e não fumantes. Alterações nos genes TP53 não ocorreram precocemente, no estágio OL, enquanto alterações no gene PTEN ocorreram tanto nos OL quanto nos carcinomas, sugerindo que tais alterações ocorrem precocemente na carcinogênese oral. Além disso, alterações no gene *AKT* não ocorreram com alta frequência em OL ou carcinomas, demonstrando que alterações na importante via PI3K são principalmente devido a alterações genéticas no *PTEN* e não no gene *AKT*.

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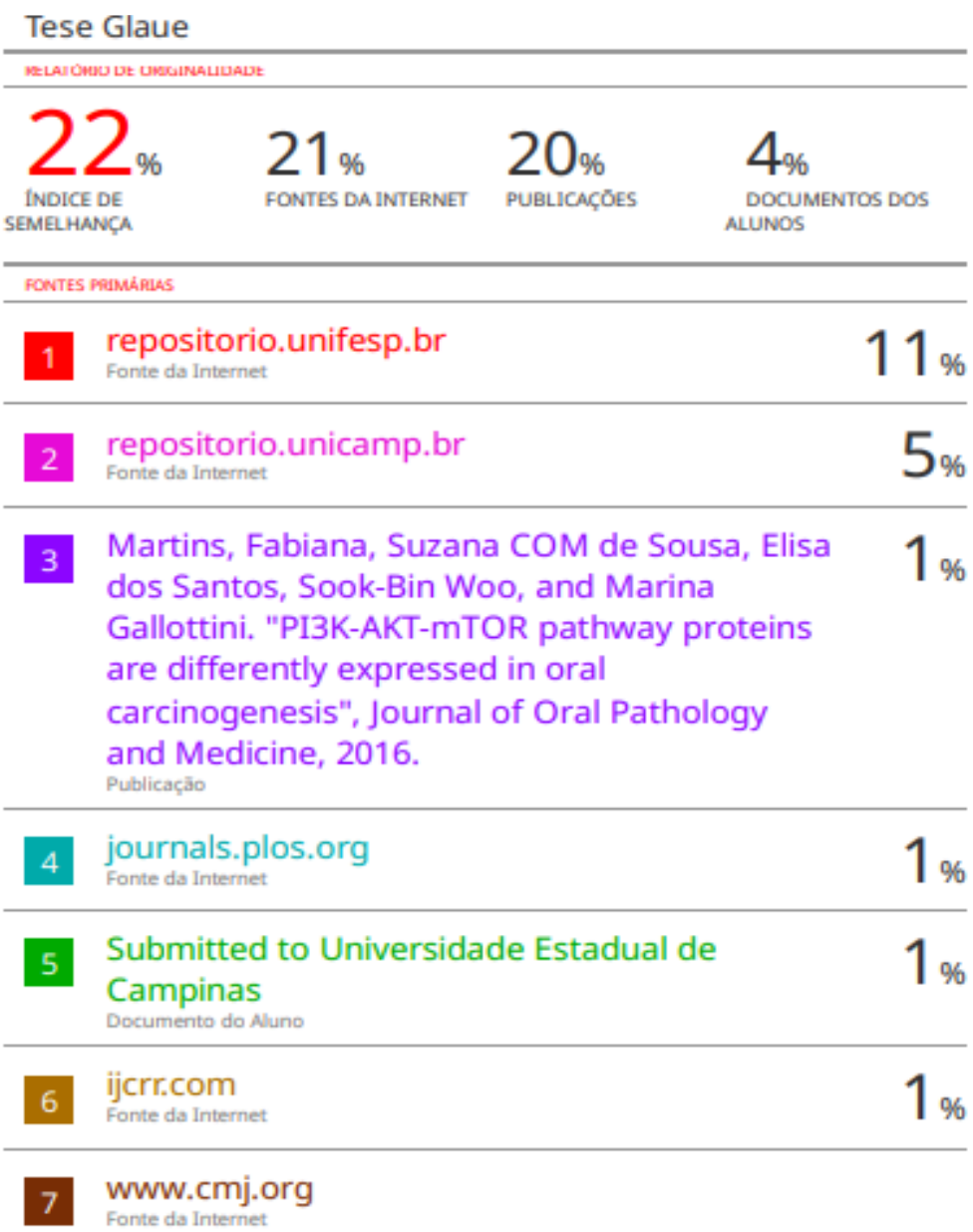
ANEXOS

Anexo 1 - Relatório de similaridade



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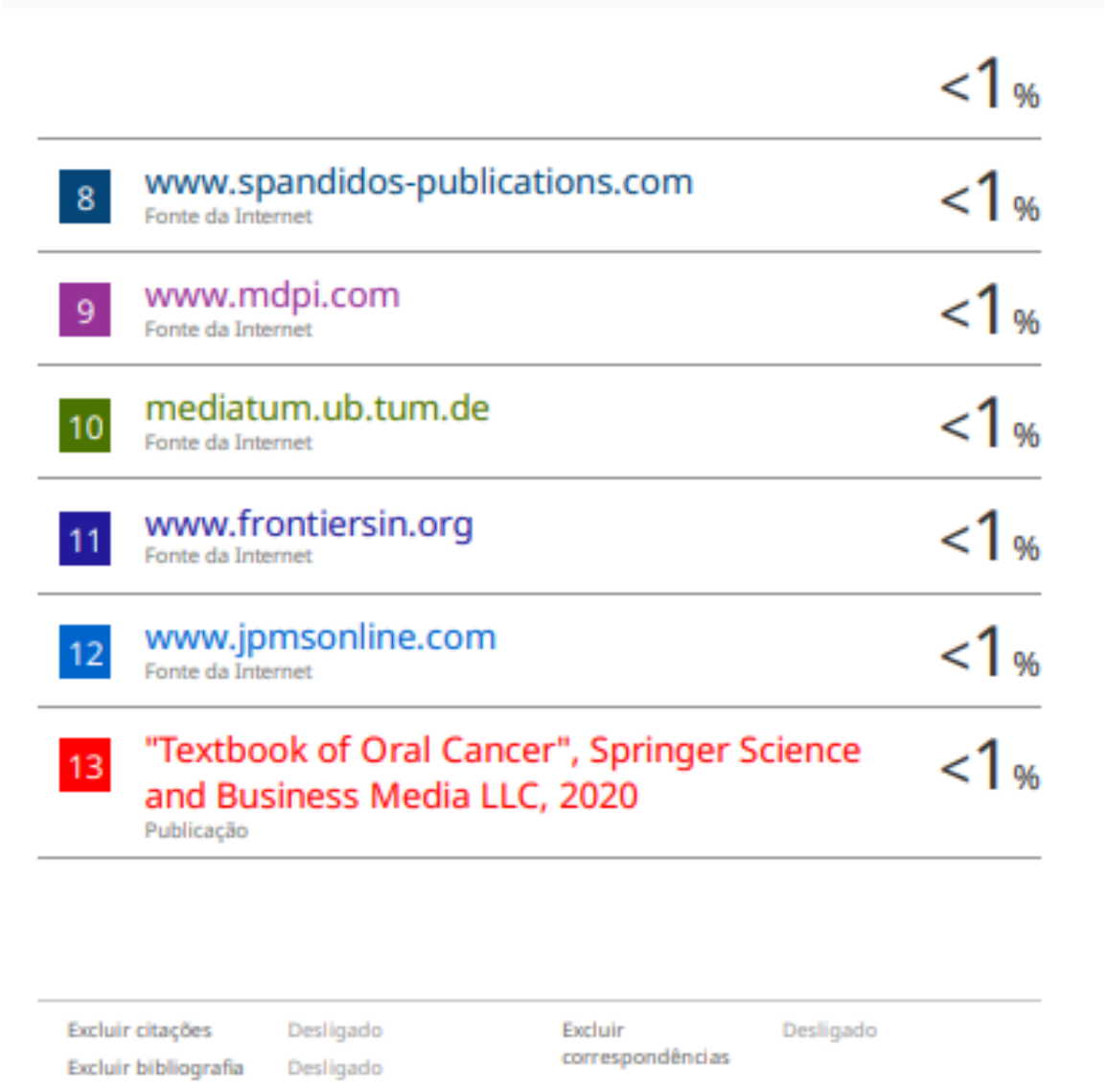

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Anexo 2 - Certificado do Comitê De Ética em Pesquisa

FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DA TRANSLOCAÇÃO DOS GENES C-ERBB 2, P-53, MYC, PTEN, BCL2 e BCL6 E AKT PELO MÉTODO DE FISH E DA EXPRESSÃO IMUNOHISTOQUÍMICA DAS PROTEÍNAS C-ERBB 2, P-53, C-MYC, PTEN, BCL2, BCL6 E AKT EM AMOSTRAS DE LEUCOPLASIAS E DE CARCINOMA ESPINOCELULARES DE BOCA EM PACIENTES TABAGISTA E NÃO TABAGISTA

Pesquisador: HÉLDER ANTÔNIO REBELO PONTES

Área Temática:

Versão: 4

CAAE: 64568722.6.1001.5418

Instituição Proponente: Faculdade de Odontologia de Piracicaba - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 6.010.777

Apresentação do Projeto:

O parecer inicial é elaborado com base na transcrição editada do conteúdo do registro do protocolo na Plataforma Brasil e dos arquivos anexados à Plataforma Brasil. Os pareceres de retorno, emendas e notificações são elaborados a partir do último parecer e dos dados e arquivos da última versão apresentada.

A EQUIPE DE PESQUISA citada na capa do projeto de pesquisa inclui HÉLDER ANTÔNIO REBELO PONTES (Cirurgião Dentista, Docente no PPG em Estomatopatologia da FOP-UNICAMP, Professor Associado da Universidade Federal do Pará, Pesquisador responsável), DANIEL HABER OLIVEIRA (Cirurgião dentista, Mestrando no PPG em Estomatopatologia da FOP-UNICAMP), ROSA HIOLANDA ABREU DE SOUSA (Cirurgiã dentista, Doutoranda no PPG em Estomatopatologia da FOP-UNICAMP), IGOR MESQUITA LAMEIRA (Cirurgião dentista, Mestrando no PPG em Estomatopatologia da FOP-UNICAMP), CAROLINA ALMEIDA PARADELA (Cirurgiã dentista, Mestranda no PPG em Estomatopatologia da FOP-UNICAMP), ANDERSON MAURICIO PAIVA E COSTA (Cirurgião dentista, Doutorando no PPG em Estomatopatologia da FOP-UNICAMP), GLAUCE GUIMARÃES PEREIRA

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Bairro: Areião **CEP:** 13.414-903
UF: SP **Município:** PIRACICABA
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FACULDADE DE ODONTOLOGIA DE PIRACICABA DA UNIVERSIDADE DE CAMPINAS - FOP/UNICAMP

Continuação do Parecer: 6.010.777

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

PIRACICABA, 19 de Abril de 2023

Assinado por:
jacks jorge junior
(Coordenador(a))

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Bairro: Areião **CEP:** 13.414-903
UF: SP **Município:** PIRACICABA
Telefone: (19)2106-5349 **Fax:** (19)2106-5349 **E-mail:** cep@unicamp.br

Anexo 3 - Documento de submissão do artigo

Oral Oncology

ASSESSMENT OF TP53, PTEN AND AKT GENE MUTATIONS IN TONGUE SQUAMOUS CELL CARCINOMA AND ORAL LEUKOPLAKIA OF SMOKERS AND NON-SMOKERS

--Manuscript Draft--

Manuscript Number:	
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Corresponding Author:	Glauce Guimarães Pereira Universidade Estadual de Campinas Faculdade de Odontologia de Piracicaba BRAZIL
First Author:	Glauce Guimarães Pereira
Order of Authors:	Glauce Guimarães Pereira
	Flávia Sirotheau Corrêa Pontes
	Carolina Cavalieri Gomes
	Felipe Paiva Fonseca
	Ana Luisa Sirotheau Corrêa Pontes
	Sue Ann Lavareda Corrêa Uchoa
	Samara Silveira da Cruz
	Rommel Mario Rodriguez Burbano
	Hélder Antônio Rebelo Pontes
Abstract:	Oral Squamous Cell Carcinoma (OSCC) is the most common malignant tumor of the oral cavity. It can be preceded by Oral leukoplakia (OL), the most prevalent potentially malignant disorder, which presents a malignant transformation rate of 3,5%, found most commonly in tongue lesions. In this study we aimed to investigate loss of heterozygosity (LOH) as well as the frequency and patterns of the mutations in the PTEN, AKT and TP53 genes in OL and tongue squamous cell carcinoma (TSCC) samples of smokers and non-smokers patients. Tissue specimens were obtained from 74 patients: 35 cases of TSCC and 38 cases of high grade OL occurring in the lateral border of the tongue. Slight differences between the genetic changes studied in the TP53, PTEN and AKT genes in smoking and non-smoking patients were detected. Changes in the TP53 genes did not occur early, in the OL stage, while changes in the PTEN gene occurred in both OL and carcinomas. Furthermore, changes in the AKT gene did not occur with high frequency in either OL or carcinomas, demonstrating that changes in the important PI3K pathway are primarily due to genetic changes in PTEN rather than in the AKT gene.
Suggested Reviewers:	Manoj Mahimkar mmahimkar@actrec.gov.in
	Silvia Franchesi francheschis@iarc.fr
	Ulrich Keilholz ulrich.keilholz@charite.de