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ORIGINAL ARTICLE

Clinicopathological correlations and cellular dynamics in a murine model of oral carcinogenesis

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Abstract:

Objective: To investigate clinicopathological correlations, cell proliferation, and immortalization during induced oral carcinogenesis. **Methods:** Forty-three Wistar rats were divided into a control group (n=10) or a 4-Nitroquinoline 1-oxide (4NQO) group (n=33). Control animals were euthanized after 20 weeks, and 4NQO-treated animals after 4 (n=10), 12 (n=10), or 20 weeks (n=13). Oral lesions were classified macroscopically and histologically, with Ki-67 and BMI-1 immunolabeling used to assess cell proliferation and immortalization. **Results:** Histological alterations, including hyperplasia/hyperkeratosis (n=4) and severe dysplasia (n=2), were observed in clinically normal mucosa. Leukoplakic lesions exhibited varying severity, ranging from hyperplasia/hyperkeratosis (n=3) to squamous cell carcinoma (SCC, n=2). Most SCCs appeared as ulcers (n=3) or nodules (n=4). Ki-67 expression increased progressively with histopathological changes, while BMI-1 levels rose significantly in later stages. A positive correlation was found between Ki-67 and BMI-1 (R=0.33, p=0.03). **Conclusion:** Cellular alterations often precede visible clinical lesions. Clinical appearances, particularly of leukoplakic lesions, frequently did not align with histopathological findings. Proliferation and immortalization were interconnected but occurred at distinct stages of carcinogenesis.

Keywords: Mouth neoplasms; Carcinogenesis; Cell proliferation; Cell transformation; Neoplastic; Biomarkers.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is one of the most prevalent malignancies worldwide, characterized by rapid and invasive growth[1,2]. With 389,485 new cases and 188,230 deaths reported annually, OSCC is a significant global public health concern[1]. The primary risk factors associated with OSCC are tobacco and alcohol consumption[2]. OSCC exhibits a high mortality rate, largely influenced by the disease stage at diagnosis, emphasizing the critical role of early detection in reducing mortality[1]. In many cases, OSCC is preceded by oral potentially malignant disorders (OPMDs)[3], such as leukoplakia, erythroplakia, and oral lichen planus[3]. These conditions exhibit varying risks of malignant transformation, driven by molecular alterations such as dysregulation of tumor suppressor genes (e.g., TP53 mutations), activation of oncogenic pathways, and increased cell proliferation. Epithelial dysplasia, commonly observed in OPMDs, remains the most reliable finding for predicting progression to OSCC, as it reflects underlying genetic and epigenetic instability[4].

Statement of Clinical Significance
This study highlights that cellular alterations often precede visible clinical lesions in oral carcinogenesis. The findings emphasize the importance of histopathological evaluation of oral potentially malignant disorders, as visual inspection alone may underestimate disease severity. Cell proliferation increases gradually from early stages of oral carcinogenesis and could be used as a target for chemopreventive therapies.

Animal models, such as those using 4-nitroquinoline 1-oxide (4NQO), have proven invaluable for studying the progression of oral carcinogenesis from subclinical

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stages to clinical manifestation. These models allow for histopathological assessments at different stages and mimic the stepwise development of human oral carcinogenesis[5]. Although widely used to investigate factors accelerating cancer progression[6] or the effects of chemopreventive agents[7], few studies have explored clinicopathological correlations during 4NQO-induced carcinogenesis. Furthermore, this model provides a robust platform for biomarker investigation.

Among the key molecular events driving tumor progression, cell proliferation and immortalization are particularly crucial. The oncoprotein BMI-1 plays a pivotal role in regulating proliferation through chromatin structure modulation and gene silencing[8]. It is overexpressed in OSCC compared to normal oral mucosa and is implicated in the proliferation and immortalization of epithelial cells during oral carcinogenesis[9]. Another critical marker, Ki-67, is expressed in the G1, S, G2, and M phases of the cell cycle and reflects the total growth fraction in tissues[10]. Its expression correlates with the grade of epithelial dysplasia and histological grading of OSCC[11]. Clinically, BMI-1 overexpression is linked to tumor aggressiveness and poor prognosis[12], while high Ki-67 levels are associated with increased recurrence and lower survival rates[13]. Both markers may aid in risk stratification, patient monitoring, and treatment planning.

This study aimed to analyze clinicopathological correlations and evaluate cell proliferation and immortalization during 4NQO-induced oral carcinogenesis in Wistar rats, providing insights into tumor progression that may inform biomarker development and early detection strategies in clinical practice.

METHODS

Animals and ethics approval

This study followed the ARRIVE 2.0 guidelines (Animal Research: Reporting of In Vivo Experiments) to ensure transparency and reproducibility in animal research[14]. Forty-three male Wistar rats (2 months old, weighing ~300 g at study initiation) were procured from the Laboratory Animal Breeding Center (LABC) at the Federal University of Pelotas. The study was approved by the Animal Use Ethical Committee of the Federal University of Rio Grande do Sul (protocol #140049) and adhered to the National Institutes of Health guidelines for the care and use of laboratory animals.

Experimental design

Animals were randomly assigned to four groups using a statistical randomization tool (randomization.com): Control Group (n=10): Fed standard chow and water ad libitum; Test Groups (4NQO-treated): Received standard chow with drinking water containing 25 ppm of 4NQO (Sigma Aldrich, St. Louis, MO, USA) in amber bottles to protect from light. The test groups were divided into: T4 (n=10): Euthanized after 4 weeks; T12 (n=10): Euthanized after 12 weeks; and T20 (n=13): Euthanized after 20 weeks. No specific inclusion criteria, apart from sex and weight, were used and no specific measures to avoid confounders during animal allocation and treatment were needed. Only one researcher (CCB), responsible for changing the water, was aware of group allocation.

Animals were housed in specific pathogen-free facilities at LABC, in transparent polypropylene boxes (65×25×15 cm) with four animals per box. Environmental conditions were controlled: temperature (20°C±2°C), humidity (50–60%), and a 12-hour light/dark cycle. Animals were weighed weekly, and their health was monitored daily. Humane endpoints for euthanasia included severe weight loss (>20% of initial body weight), significant signs of distress (lethargy, persistent piloerection, or labored breathing), or inability to eat or drink. One rat in the T20 group (3%) died at week 17 due to 4NQO-related adverse effects. Euthanasia was performed via exsanguination following cardiac puncture under general anesthesia (isoflurane 5% in 0.5 L/min O_2).

Sample size Estimation

Sample size was determined based on previous studies using 4NQO-induced carcinogenesis models[15,16]. Each group included 10 animals, with three additional rats allocated to T20 to account for the reported 28% mortality rate in extended 4NQO exposure[17]. The primary outcome measure was the development of microscopic alterations compatible with oral carcinogenesis progression.

Macroscopic evaluation

After euthanasia, tongues were excised and macroscopically examined for clinical lesions, including leukoplakia, nodules, and ulcers. Photos were captured and independently graded by two blinded examiners (IPK and VCC). Samples were categorized as (a) clinically normal, (b) leukoplakic lesion, (c) nodule, or (d) ulcer. In cases of multiple lesion types, all were analyzed, and the most severe classification was used for clinicopathological correlation.

Histopathological analysis

Tongue tissues were fixed in 10% neutral formalin and paraffin-embedded. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and evaluated independently by three blinded pathologists (VCC, IPK, and CCB). Tissues were classified into the following categories: no alterations, hyperplasia with/without hyperkeratosis, epithelial dysplasia (graded as mild, moderate, or severe), or squamous cell carcinoma (SCC), based on Reibel et al.[18]. Consensus was reached for discrepant cases. Tissue sections were selected based on the most representative lesion area, ensuring consistent evaluation across samples.

Immunohistochemical analysis

Paraffin sections (3 µm) were deparaffinized, rehydrated, and subjected to antigen retrieval. For Ki-67, low-pH solution incubation at 90°C for 18 h was performed, while Tris-HCl buffer (pH 8.5) at 98°C for 20 min was used for BMI-1. Slides were incubated with primary antibodies: Ki-67 (MIB-1, DAKO, 1:50) and BMI-1 (ab14389, Abcam, 1:100). Detection was achieved using the EnVision system (DakoCytomation, Carpinteria, CA, USA) with DAB as a chromogen and Mayer's hematoxylin for counterstaining. Negative controls excluded the primary antibody, and human appendix and lymph node tissue served as positive controls.

Images were captured using an Olympus CX41RF microscope with a QColor 5 camera and analyzed with QCapture software. Ki-67 labeling index (LI) was calculated as the percentage of positive nuclei per 1000 cells. BMI-1 staining was scored semi-quantitatively based on positive cell percentages: very low (0%-30%), low (30%-50%), moderate (50%-80%), and high (>80%).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) software, version 20.0 (IBM Corporation, Armonk, USA). Data normality was tested using the Shapiro-Wilk test. Weight, food, and water consumption were analyzed using ANOVA with Tukey's post hoc test, while non-normally distributed weight gain data were analyzed with the Kruskal-Wallis test followed by Dunn's multiple comparisons. Ki-67 and BMI-1 staining results were analyzed using Kruskal-Wallis and Dunn's tests. For clinic-pathological correlations the experimental unit considered was the lesion, with the possibility of more than one lesion by animal. Hyperplasia/hyperketosis and mild dysplasia were gathered as initial changes and moderate and severe dysplasia were gathered as intermediate changes. Correlations between Ki-67 and BMI-1 were assessed with Spearman's correlation. Statistical significance was set at $p<0.05$.

RESULTS

Weight gain and chow diet consumption

Weight gain was significantly lower ($p<0.05$, data not shown) in the groups treated with 4NQO for 4 and 20 weeks compared to the control group. Chow diet consumption was reduced in animals treated with 4NQO for 4 weeks. Among the 43 rats exposed to 4NQO, one animal from the 20-week treatment group died at the 17th week of the experiment, resulting in a mortality rate of 3%.

Clinical and histopathological analyses

Clinically, lesions were categorized as normal mucosa, leukoplakic lesions, nodules, or ulcers (Figure 1). Table 1 provides the distribution of clinically detected lesions across experimental groups and their association with histopathological changes. The control group showed no clinical alterations, and the dorsal tongue mucosa was used as a reference for morphologically normal epithelia. In the T4 and T12 groups, the lesions were predominantly leukoplakic. In contrast, the T20 group displayed more pronounced lesions, such as nodules and ulcers, with an average of 2.8±0.8 lesions per animal (range: 2–4).

In total, 65 histopathological alterations were observed (Figure 2). Even in animals exposed to 4NQO for only 4 weeks, significant histological changes, including moderate dysplasia, were noted (Table 2, Figure 3). Some sites (n=5 in the T4 group and n=1 in the T12 group) exhibited no histopathological changes and were excluded from further assessments.

Histopathological evaluation of lesions diagnosed as leukoplakic revealed a spectrum of changes, ranging from hyperplasia/hyperkeratosis (H/H) to squamous cell carcinoma (SCC). Nodular lesions exhibited severe epithelial dysplasia to SCC, while ulcers demonstrated changes ranging from moderate epithelial dysplasia to SCC. Notably, even tongues that were clinically classified as normal mucosa occasionally exhibited histological alterations, including epithelial hyperplasia (n=4) and various grades of epithelial dysplasia (n=16).

Immunohistochemical analysis

The immunohistochemical analysis revealed increased Ki-67 expression in intermediate changes (moderate/severe dysplasia) and SCC compared to morphologically normal epithelia (ANOVA/Tukey, $p<0.05$) (Table 3, Figure 4). However, initial changes, such as H/H and mild dysplasia, did not differ significantly from either morphologically normal epithelia or intermediate changes.

BMI-1 expression was significantly elevated in SCC compared to morphologically normal epithelia and epithelia with initial changes (hyperplasia/hyperkeratosis) ($p<0.05$) (Table 3, Figure 4). A positive correlation was observed between BMI-1 and Ki-67 expression (Spearman's correlation, $R=0.36$, $p<0.05$).

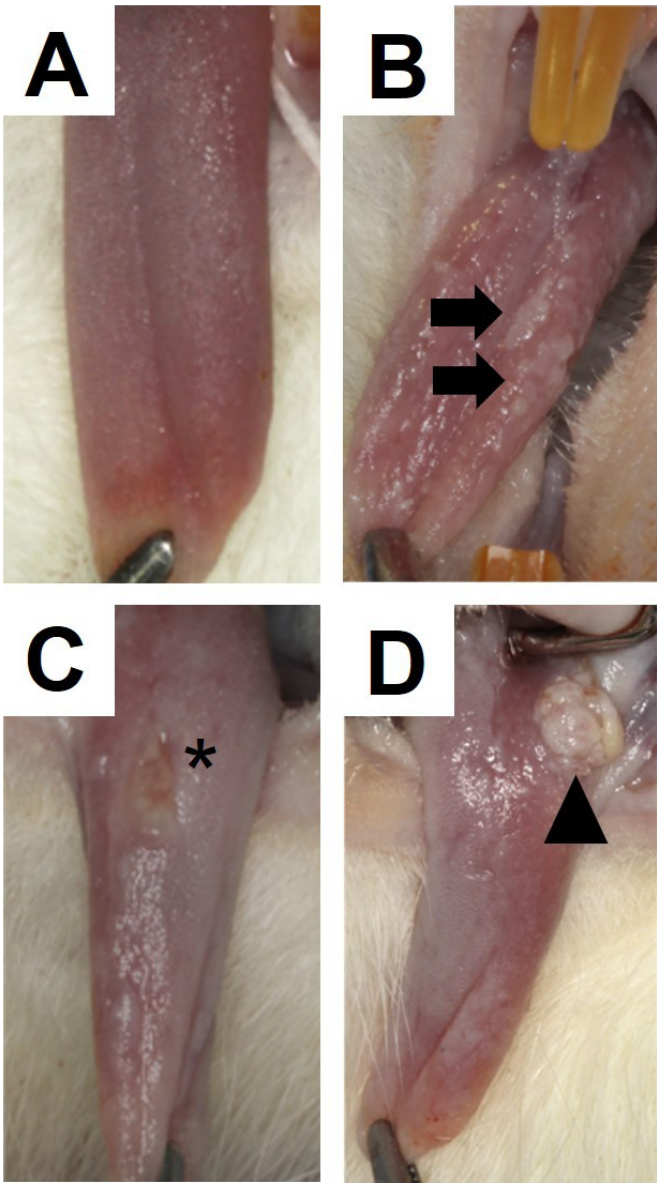

Figure 1. Clinical presentation of the lesions, which were classified as clinically normal **(A)** leukoplakic; **(B)** arrow, ulcerative; **(C)** asterisk or nodular lesions; **(D)** arrow head.

DISCUSSION

This study presents a thorough analysis of chemically induced oral carcinogenesis using the 4NQO model, emphasizing its utility in understanding the progression of oral SCC and the associated morphological and molecular changes. The findings delineate distinct stages of lesion development, from hyperplasia to SCC, and reveal cellular-level alterations, such as moderate dysplasia, even in tissues clinically classified as normal. These observations highlight the critical importance of microscopic evaluation in detecting early changes, as reliance on clinical assessments alone risks underestimating disease burden.

Oral carcinogenesis arises from a complex interplay of genetic and epigenetic factors. Tobacco-induced carcinogenesis, for example, can lead to a phenomenon known as field cancerization. This concept, introduced by Slaughter et al.[19], suggests that patients with OPMDs or previous SCC diagnoses have an elevated likelihood of developing malignant transformation or new primary tumors in the oral cavity. These characteristics allow for the identification of high-risk patients and present opportunities for early diagnostic strategies or chemoprevention. However, clinical experience in oral medicine reveals that most SCC cases are diagnosed at advanced stages, even though some are preceded by leukoplakia.

Table 1. Frequency of oral mucosal lesions by experimental group.

	Clinically normal	Leukoplakic	Ulcer	Nodule	Total
Treatment					
Control	10	-	-	-	10
4NQO – 4 weeks	10	3	-	-	13
4NQO – 12 weeks	10	3	-	-	13
4NQO – 20 weeks	-	19	4	10	33

NQO: Nitroquinoline 1-oxide.

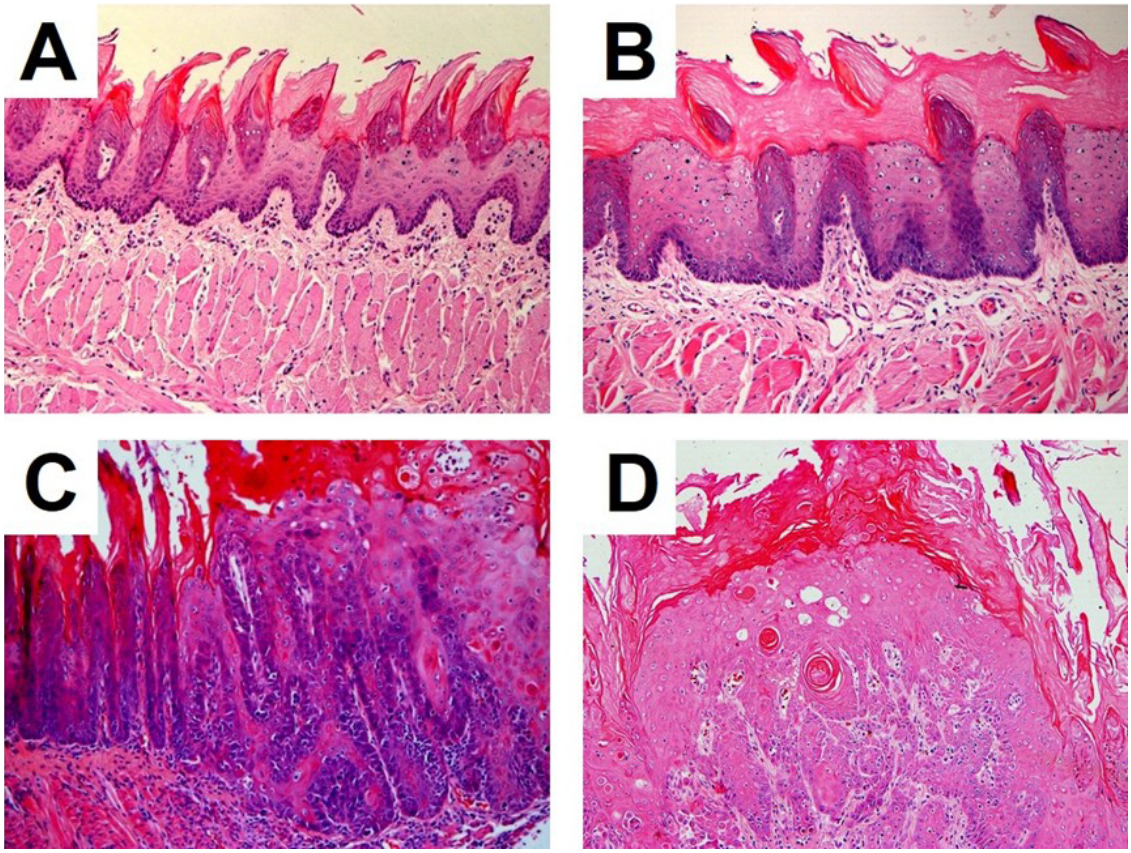

Figure 2. **(A)** Histopathological analysis showed tongue mucosa presenting no morphological changes; **(B)** epithelial hyperplasia/hyperkeratosis; **(C)** epithelial dysplasia, and **(D)** squamous cell carcinoma.

Table 2. Clinic-pathological correlations

	Clinicallynormal	Leukoplakic	Ulcer	Nodule	Total
No morphological changes	10	-	-	-	10
Hyperplasia/Hiperkeratosis	4	3	-	-	7
Epithelial dysplasia					
Mild	8	5	-	-	13
Moderate	6	8	1	-	15
Severe	2	7	6	6	21
Squamous cell carcinoma	-	2	3	4	9

This delays opportunities for conservative treatment and significantly impacts prognosis[20].

The 4NQO-induced carcinogenesis model effectively replicates the histopathological progression seen in human SCC[5,21], making it a reliable tool for studying SCC development and its underlying mechanisms. This model induces apoptosis via a p53-dependent pathway, reflecting the frequent mutation of the p53 gene in oral SCC patients[22]. The progression of lesions in this model is proportional to the duration of carcinogen exposure, closely mirroring patterns observed in human SCC[23]. The combination of this classic model with innovative molecular techniques has led to significant discoveries. For instance, genomic and single-cell transcriptomic analyses have identified key driver mutations (TP53, NOTCH1, FAT1) and cellular subpopulations linked to tumor progression and therapy resistance[24,25]. Emerging approaches, such as spatial transcriptomics and lineage tracing of long-lived epithelial stem cells, provide deeper insights into clonal evolution and tumor microenvironment interactions[26]. However, despite extensive use of the 4NQO model, clinicopathological correlations have not been deeply explored.

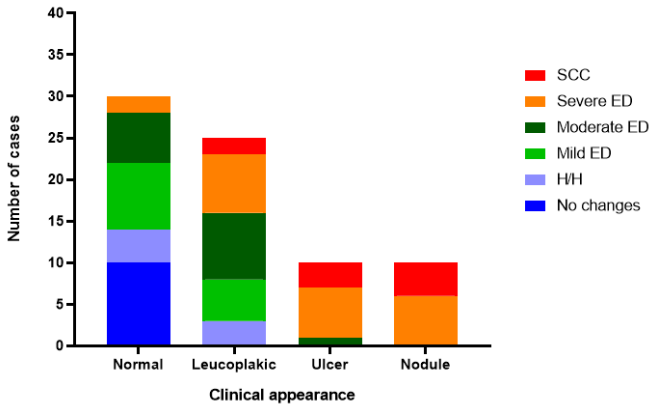

Figure 3. Clinic-pathological correlations presented as number of cases.

In this study, animals exposed to 4NQO for as little as four weeks exhibited histopathological changes, ranging from epithelial hyperplasia and hyperkeratosis to severe dysplasia, even in mucosa classified as clinically normal. Lesions identified as leukoplakic showed a spectrum of histopathological changes, with milder alterations like hyperplasia and mild dysplasia observed in short-term exposure and more severe changes, including carcinoma in situ and invasive SCC, after 20 weeks. This variability in histopathological changes among leukoplakic lesions parallels findings in human leukoplakias[27], highlighting the model's relevance for studying oral carcinogenesis. Furthermore, the detection of histopathological changes in clinically normal

Table 3. Ki67 and BMI-1 immunolabeling according to histopathological diagnosis.

	Ki67		BMI-1	
	Median	**P25–75**	**Median score**	**P25–75**
No morphological changes	37.0	32.8–43.0	2	1–3
Hyperkeratosis/epithelial hyperplasia and mild dysplasia	48.8	37.8–62.8	2	1–3
Moderate to severe dysplasia	54.3	48.2–64.8	2	1.5–3
SCC	66.2	58.1–71.3	3	3–4

Ki67: percentage of positive cells; BMI-1 scores: 1: 0–30% positive cells; 2: 30–50% positive cells; 3: 50–80% positive cells; and 4: more than 80% positive cells. P25–75–25th percentile and 75th percentile. SCC: squamous cell carcinoma.

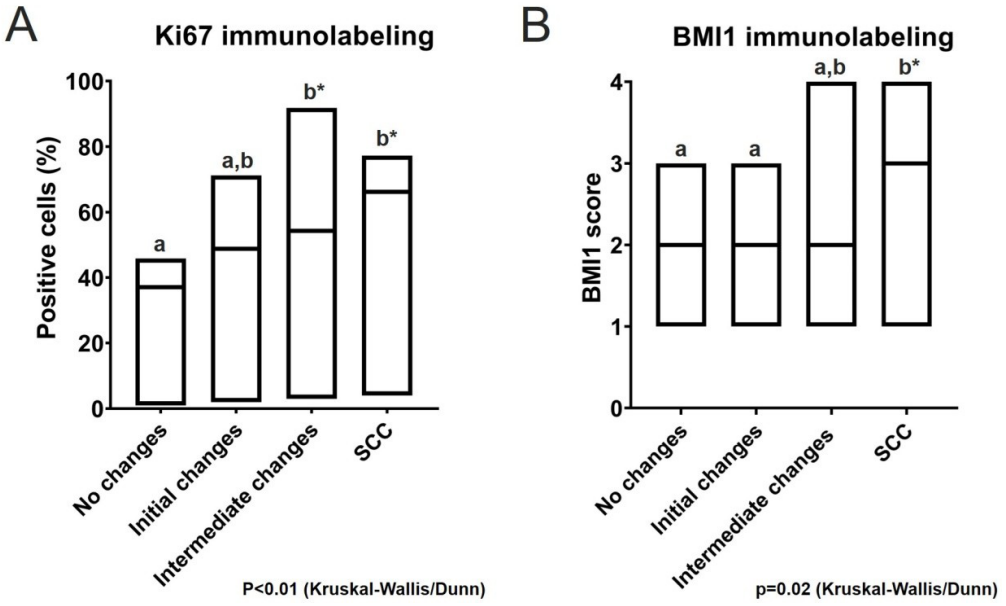

Figure 4. (A) Ki67 immunolabeling did not differ in the epithelium with no morphological changes compared to initial or intermediate changes. SCC and intermediate changes group presented an increase in immunolabeling when compared to group without morphological changes (Kruskal Wallis/Dunn's test; $p<0.01$). **(B)** BMI1 immunolabeling did not differ in the epithelium with no morphological changes compared to initial or intermediate changes. BMI1 immunolabeling was higher in SCC than groups with none or initial changes (Kruskal Wallis/Dunn's test; $p<0.01$). Different lowercase letters indicate differences statistically significant.

mucosa supports the concept of field cancerization, where molecular and cellular changes precede visible clinical manifestations. These findings emphasize the importance of monitoring high-risk individuals, such as heavy smokers, and performing histopathological analyses of any clinical changes to facilitate early diagnosis and intervention.

The study also sheds light on key molecular events during carcinogenesis, specifically cell proliferation and immortalization. Ki-67 immunostaining revealed a gradual increase in cell proliferation from early to advanced stages, reinforcing its role as a hallmark of tumor progression. These findings align with those of Kaplan et al.,[28] and Silva et al.[29], who studied cell proliferation in similar models. Conversely, BMI-1 expression was predominantly observed in the SCC stage, suggesting its involvement in later tumor development. While our findings suggest a gradual increase in cell proliferation, immortalization appears to become evident only in the later stages. This contrasts with prior studies that reported rapid increases in telomerase activity, another marker of immortalization, in earlier stages of carcinogenesis[30,31]. These results indicate that BMI-1's role in cellular immortalization is complex and potentially indirect. Additionally, BMI-1 may contribute to other aspects of carcinogenesis, such as migration and invasion, further validating its role as a marker of poor prognosis[32]. The observed correlation between Ki-67 and BMI-1 in this study suggests that proliferation and immortalization are interconnected. This is supported by Kang et al., who demonstrated a reduction in epithelial proliferation following BMI-1 protein knockout[9].

From a translational perspective, these findings highlight the value of experimental models in advancing early detection and chemoprevention strategies. Our results underscore the importance of evaluating chemopreventive drugs capable of slowing cell proliferation in the early stages of carcinogenesis. While clinical trials face challenges such as patient recruitment[33], animal models provide a controlled environment for assessing the mechanisms, safety, and efficacy of potential therapies. These preclinical studies help bridge the gap between laboratory research and clinical applications, paving the way for developing interventions with higher success rates in human studies. Despite its strengths, this study has its limitations. One possible limitation is the sample size, which, while sufficient for detecting histopathological and molecular changes, may not fully capture the variability of lesion progression observed in human OSCC. Additionally, although the 4NQO model effectively mimics many aspects of human oral carcinogenesis, it does not entirely replicate the complexity of the tumor microenvironment.

CONCLUSION

In conclusion, our study demonstrates that cellular changes can precede clinical manifestations during 4NQO-induced carcinogenesis. The lack of a direct correlation between clinical and microscopic findings mirrors observations in human SCC. Cell proliferation and immortalization are interconnected events, with proliferation increasing gradually from early stages and immortalization becoming more pronounced in advanced stages. These findings underscore the importance of early detection, comprehensive histopathological evaluations, and the exploration of chemopreventive interventions to improve outcomes for individuals at risk of oral SCC. Furthermore, animal models remain indispensable for studying the molecular mechanisms underlying OSCC development, providing a controlled platform to investigate tumor biology and identify potential molecular targets for therapeutic intervention. The integration of advanced molecular techniques into these models will enhance our understanding of key pathways driving carcinogenesis and support the development of targeted therapies.

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AUTHORS' CONTRIBUTIONS

CCB: data curation, formal analysis, methodology, writing – original draft. IPK: data curation, investigation, methodology, writing – original draft. MEA: investigation, methodology. LM: investigation, methodology. RMC: formal analysis, investigation, methodology. CPK: data curation, investigation, methodology. VPW: formal analysis, writing – original draft, writing – review & editing. MDM: investigation, methodology. VCC: conceptualization, data curation, funding acquisition, methodology, supervision, writing – original draft, writing – review & editing.

CONFLICT OF INTEREST STATEMENT

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Competing interests: The authors have no relevant financial or non-financial interests to disclose.

Ethics approval: Animal Use Ethical Committee of the Federal University of Rio Grande do Sul protocol #140049.

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