

Original article

Evaluation of MDM2 mRNA expression according to clinicopathological characteristics in patients with oral squamous cell carcinoma

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Abstract

Background: The murine double minute 2 (MDM2) gene encodes an E3 ubiquitin ligase that targets p53 for degradation, which influences the pathogenesis of oral squamous cell carcinoma (OSCC).

Objective: This study determined MDM2 mRNA expression and evaluated its association with the clinical and pathological characteristics thereof in patients with OSCC.

Methods: We performed an observational-analytical study using a cross-sectional design involving 39 patients with OSCC. RNA extraction, complementary DNA synthesis, and quantitative real-time polymerase chain reaction were performed. The relative MDM2 mRNA expression was quantified using the Livak method ($2^{-\Delta\Delta CT}$) with GenEX MultiD software version 6.0. Statistical significance was assessed using unpaired Student *t*-tests.

Results: MDM2 mRNA expression was 1.64-fold lower in patients with OSCC and tumor volumes >5 cc compared with those ≤ 5 cc (CT mean $\pm$ SE: 32.0 ± 2.6 vs. 32.7 ± 1.8 , respectively; $P = 0.34$). In high-grade cases, the expression was 0.92-fold lower than in low-grade cases (32.1 ± 2.3 vs. 32.2 ± 2.7 , respectively; $P = 0.9$). The expression thereof was also 2.12-fold higher in smokers than in non-smokers (31.8 ± 2.4 vs. 32.9 ± 2.1 , respectively; $P = 0.30$). None of these differences were significant. Network analysis identified MDM2, TP53, and CDKN1A as central hub genes, thus bridging p53 signaling, cell cycle regulation, and cellular senescence in OSCC. Gene Ontology enrichment revealed significant overrepresentation of p53-mediated DNA damage response and apoptotic pathways among the candidate genes.

Conclusion: Although MDM2 mRNA expression exhibited no significant clinical associations, in silico profiling identified MDM2 as a central molecular hub linking p53 signaling and cell cycle regulation in OSCC, thereby suggesting a mechanistic role that warrants further functional validation.

Keywords: Livak's method, MDM2 mRNA, oral cancer, OSCC, real-time qPCR.

Oral squamous cell carcinoma (OSCC) is a malignancy of the oral cavity, affecting the tongue, floor of the mouth, soft palate, and lower lip.⁽¹⁾ OSCC accounts for 90.0% of oral cancer cases worldwide.⁽²⁾ Globocan

2020 data indicate the 5-year prevalence of oral cancer at 12.6 per 100,000 in Asia and 5.2 per 100,000 in Indonesia. This demonstrates a considerable impact of oral cancer in Indonesia.⁽³⁾

OSCC stems from multiple factors, including genetic, epigenetic, and environmental influences. These factors may interact; for example, smoking, an environmental influence, disrupts epigenetics in cancer development.^(1, 4) In Southeast Asia, there are more than 180,000 new oral cancer cases annually, with 90.0% associated with smoking and betel nut use.^(5, 6)

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The International Agency for Research on Cancer reports that cigarettes contain over 60 carcinogens. These carcinogens increase cancer risk through oxidative tissue stress, persistent free radicals, DNA mutations, and compromised immune function.⁽⁷⁾

The body reacts to cigarette-induced DNA damage by pausing the cell cycle and activating DNA repair, which are processes that are orchestrated by the tumor protein p53 (TP53).⁽⁸⁾ The function of TP53 is regulated by murine double minute 2 (MDM2) to maintain physiological balance. Upon DNA damage, proteasome-mediated degradation occurs. MDM2, a proto-oncogene, stimulates cell proliferation and serves as an anti-apoptotic agent by inhibiting TP53. Increased MDM2 mRNA suppresses TP53 activity, thus hindering DNA repair.⁽⁹⁾ MDM2's association with Nibrin (NBS1) further impedes DNA damage repair, thereby fostering genomic instability and tumorigenesis.⁽¹⁰⁾ Nevertheless, the association between MDM2 mRNA expression and the clinical and pathological characteristics of OSCC remains insufficiently investigated. Therefore, this study aimed to determine MDM2 mRNA expression and evaluate its association with the clinical and pathological characteristics thereof in patients with OSCC.

Materials and methods

Study population and data collection

The study protocol was reviewed and approved by the Health Research Ethics Committee of the Faculty of Medicine, Jenderal Soedirman University (approval letter number: 026/KEPK/PE/V/2022). An observational–analytical study with a cross-sectional design was performed. Formalin-fixed paraffin-embedded (FFPE) OSCC samples were collected from Prof. Dr. Margono Soekarjo Hospital between 2017 and 2020. Clinical data, including age, gender, tumor location, smoking status, and histological grading, were obtained from the patients' medical records. All participants reviewed the research protocol and provided written informed consent before recruitment. All patients fulfilled the inclusion and exclusion criteria for this study.

Inclusion criteria required participants to be aged 21–60 years, with a primary OSCC diagnosis confirmed by histological biopsy. Exclusion criteria included prior cancer therapy and a family history of cancer. Thirty-nine patients with OSCC who had complete clinical data and met the study criteria were included. All participants provided written informed consent before study initiation.

RNA isolation

RNA was isolated from the FFPE samples using the Monarch® NEB #T2010S kit. The supernatant was transferred to a 1.5-mL tube, labeled with a code, and stored at -80°C . The A_{260}/A_{280} purity ratio of all RNA samples ranged from 1.8 to 2.0. The lowest concentration measured was 2.2 ng/ μL . RNA samples with concentrations greater than 2.2 ng/ μL were diluted to normalize the concentration before the two-step real-time polymerase chain reaction (RT-PCR).

cDNA synthesis

Reverse transcriptase PCR was performed using the ProtoScript® NEB #E6300S/L kit, according to the manufacturer's protocol. This stage converts RNA into complementary DNA (cDNA), and the Veriti™ 96-Well Fast Thermal Cycler was used. The run program included 5 min at 70°C for denaturation, 5 min at 25°C for incubation, 1 h at 42°C , and 5 min at 80°C to inactivate the reverse transcriptase enzyme. cDNA samples were stored at -20°C . The A_{260}/A_{280} purity ratio of all cDNA samples ranged from 1.8 to 2.0.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed using the Applied Biosystems 7500 Real-Time PCR System. MDM2 mRNA expression was normalized to that of *GAPDH* as a reference gene using sequence-specific primers. The primers for MDM2 were 5'-GACT CCAAGCGCGAAAACCC-3' (forward) and 5'-ACCAGGGTCTCTTGTTCCGA-3' (reverse), producing a 124 bp product. The primers for *GAPDH* were 5'-AAGACGGGCGGAGAGAAACC-3' (forward) and 5'-GTTGACTCCGACCTTCACCTT-3' (reverse), producing a 139 bp product. The 2×SensiFAST SYBR® Lo-ROX #BIO-94005 reagent was used for qRT-PCR. All procedures were performed according to the manufacturer's protocol. For each reaction, 2 μL cDNA (50 ng/ μL) was added to achieve a total volume of 20 μL . The run program consisted of 2 min at 95°C for polymerase activation, followed by 40 cycles of 5 sec at 95°C for denaturation and 30 sec at 60°C for annealing. Melt curve analysis was performed to confirm the amplification of a single, specific product and the absence of primer dimers. All experimental samples, including non-template controls as negative controls, were analyzed in technical duplicates. Primer amplification efficiencies

for MDM2 and GAPDH were validated to be within the acceptable range of 90.0% – 110.0% before final quantification. The real-time qPCR instrument registered the cycle threshold (CT) values at the point where fluorescence intensity crossed the threshold.

Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SE). Data analysis was performed using Applied Biosystems' 7500 RT-PCR software to obtain the CT values and melting curves. Relative quantification of MDM2 mRNA expression was performed using Livak's method.⁽¹¹⁾ GenEX MultiD software version 6.0 was employed to calculate the fold change and differential MDM2 mRNA expression. Data normality and homogeneity of variance were assessed using the Shapiro-Wilk test and Levene's test, respectively, to confirm suitability for parametric analysis. Statistical significance of the relative quantification (Δ Ct values) between groups was evaluated using an independent *t*-test, with a significance threshold of $P < 0.05$. The 95% confidence intervals and effect sizes were recorded where applicable. All statistical computations and graphical visualizations were performed using GenEX MultiD software version 6.0 and GraphPad Prism 9. A $P < 0.05$ was considered statistically significant.

In silico functional enrichment and protein–protein interaction (PPI) network analysis

To elucidate the broader molecular landscape of MDM2 in OSCC, a core set of candidate genes that directly interact with MDM2 and TP53 was selected based on established literature and cancer-related databases. Functional PPI networks were constructed using the STRING database (<https://string-db.org/>) with a strict high-confidence interaction score threshold of > 0.70 ⁽¹²⁾ and visualized utilizing GeneMANIA within Cytoscape software (v3.9.1). The network analysis incorporated experimentally derived interactions and verified literature-derived co-expressions. To identify the biological pathways associated with these hub nodes, Gene Ontology (GO) biological processes and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analyses were performed using the DAVID and ClueGO platforms. Statistical stringency was maintained by applying the Benjamini-Hochberg false discovery rate (FDR < 0.05) correction.

Results

Sample's characteristics

Thirty-nine samples were obtained from patients aged 31–60 years, with a mean age of 49 at diagnosis. There were 16 male and 23 female patients, and most tumors were low grade (well- or moderately differentiated) rather than high grade (poorly differentiated). Patients who smoked one cigarette per day or had smoked 100 cigarettes in their lifetime are considered smokers. Among the male patients, 16 had smoked for more than two decades during their lifetime, in the range of 21–29 years, with a mean smoking duration of 24.2 years.⁽¹³⁾

MDM2 mRNA expression

MDM2 mRNA expression in patients with OSCC older than 40 years revealed a 2.35-fold increase in expression compared to younger patients (CT mean $\pm$ SE of MDM2 mRNA were 33.2 ± 1.4 and 31.3 ± 2.5 , respectively; $P = 0.21$), **Figure 1**. MDM2 mRNA expression in patients with OSCC and a tumor size > 5 cc was 1.64-fold lower than that of those with ≤ 5 cc (CT mean $\pm$ SE of MDM2 mRNA were 32.0 ± 2.6 and 32.7 ± 1.8 , respectively; $P = 0.34$), as visualized in **Figure 1B**. Comparing the MDM2 mRNA expression in patients with low-grade (well- to moderately differentiated) and high-grade (poorly differentiated) OSCC was not significantly different (CT mean $\pm$ SE of MDM2 mRNA were 32.1 ± 2.3 and 32.2 ± 2.7 , respectively; $P = 0.90$), as shown in **Figure 2A**. MDM2 mRNA expression was also not significantly different across the smoking history groups (**Table 1**).

Construction of PPI network, enrichment analysis, and GO

Central hub proteins, including *MDM2*, *TP53*, and *CDKN1A*, exhibited high connectivity, bridging multiple functional modules and suggesting their crucial roles in coordinating oncogenic signaling, cell fate determination, and proliferative control (**Figure 3**). The p53 signaling cluster was enriched for canonical regulators (e.g., MDM2, MDM4, and GADD45A), while the cell cycle module included critical effectors such as RB1, E2F1, and RBL2. The cellular senescence cluster was represented by TP53 and CDKN1A, thereby highlighting their involvement in growth arrest and tumor suppression.⁽¹⁴⁾

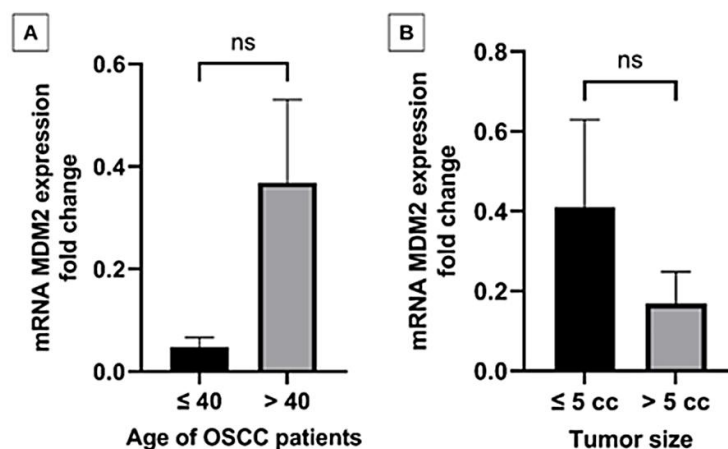


Figure 1. Expression of mRNA MDM2. (A) OSCC patients older than 40 showed higher mRNA MDM2 expression than younger patients, (B) expression of mRNA MDM2 in OSCC patients with tumor size ≤ 5 cc and those with > 5 cc. mRNA MDM2 expression was not significantly different between the two tumor size groups. MDM2, murine double minute 2; OSCC, oral squamous cell carcinoma.

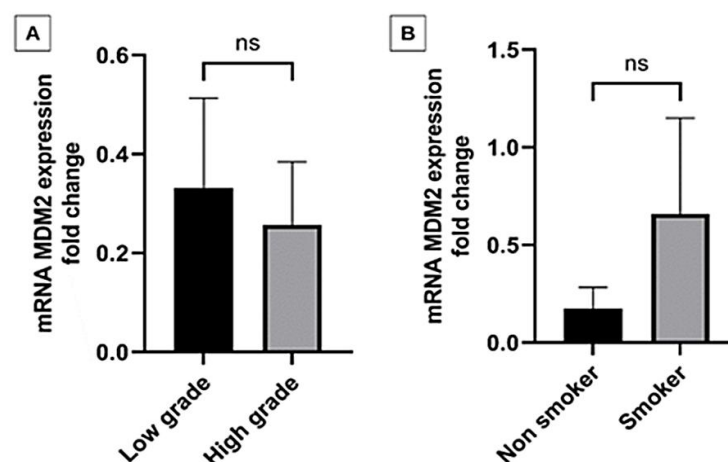


Figure 2. Correlation of mRNA MDM2 expression with histological grading and smoking history of OSCC patients. (A) mRNA MDM2 expression was not significantly different in low-grade and high-grade OSCC patients, (B) smoker OSCC patients showed higher mRNA MDM2 expression than non-smoker patients. MDM2, murine double minute 2; OSCC, oral squamous cell carcinoma.

Table 1. Clinical Characteristics of OSCC Patients (39 patients) and the correlation with mRNA MDM2 expression.

Variables	N	MDM2 mRNA expression (mean ± SE)	Fold change	P-value
Age				
≤ 40-year-old	7	33.2 ± 1.4	2.35	0.21
> 40-year-old	32	31.9 ± 2.5		
Tumor size				
≤ 5 cc	14	32.0 ± 2.6	0.61	0.34
> 5 cc	25	32.7 ± 1.8		
Histological grading				
Low grade	28	32.1 ± 2.3	0.92	0.90
High grade	11	32.2 ± 2.7		
Smoking history				
Non-smoker	23	32.9 ± 2.1	2.12	0.30
≥ 20 years smoker	16	31.8 ± 2.4		

MDM2, murine double minute 2; OSCC, oral squamous cell carcinoma; SE, standard error of the mean.

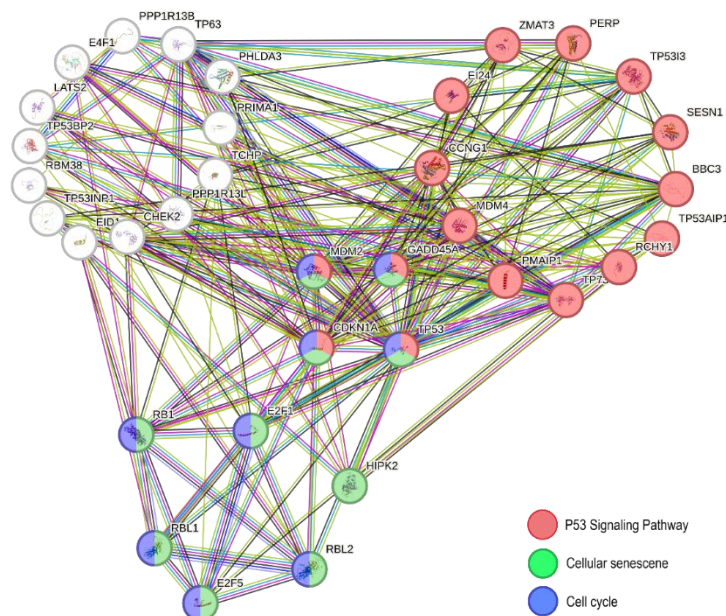


Figure 3. Protein-protein interaction network of MDM2 and associated genes in OSCC. Nodes are colored by function: p53 signaling pathway (red), cellular senescence (green), and cell cycle (blue). Hub proteins (MDM2, TP53, CDKN1A) demonstrate extensive connectivity across modules, suggesting key regulatory roles in OSCC. MDM2, murine double minute 2; OSCC, oral squamous cell carcinoma; TP53, tumor protein p53

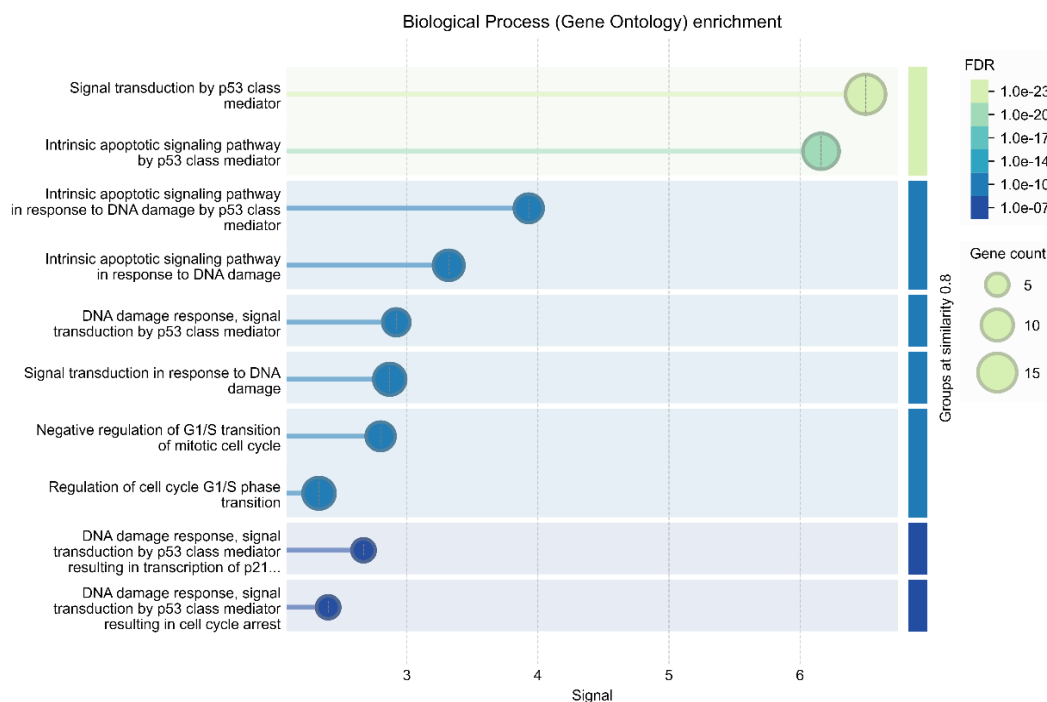


Figure 4. Gene Ontology (GO) enrichment analysis of biological processes. The bubble plot shows the top enriched GO terms, with bubble size representing gene count and color indicating FDR significance. p53-mediated signal transduction and DNA damage response pathways are most prominently enriched, underscoring their relevance to the studied gene set.

GO enrichment analysis of the candidate gene set revealed a substantial overrepresentation of processes related to p53-mediated signal transduction and DNA damage response (**Figure 4**). The most significantly enriched terms included “signal transduction by p53 class mediator,” “intrinsic apoptotic signaling pathway by p53 class mediator,” and “DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest”

p53 class mediator,” all with an extremely low FDR ($< 1.0e-20$). Additional enrichment was observed for pathways involved in cell cycle regulation, notably “negative regulation of G1/S transition of mitotic cell cycle” and “regulation of cell cycle G1/S phase transition.” These findings underscore the central role of p53-dependent DNA damage signaling and cell cycle control in the molecular landscape under investigation.⁽¹⁵⁾

Discussion

MDM2 prevents cells from dying by breaking down the TP53 proteasome.⁽¹⁶⁾ MDM2 can influence tumorigenesis independently via several mechanisms, including ubiquitination and proteasome degradation of tumor suppressor proteins, genomic instability through DNA damage, and stabilization of mRNA coding for pro-tumorigenesis and metastasis.⁽¹⁷⁾ Furthermore, MDM2 can interact with NBS1 and delay the phosphorylation of histone H2AX, which leads to inefficient DNA damage repair.⁽¹⁸⁾ Genomic instability and delayed repair of damaged DNA due to increased MDM2 expression and its oncogenic activity are causes of cancer development, including OSCC.⁽¹⁹⁾

This study used FFPE samples from patients with OSCC and performed qRT-PCR to evaluate the mRNA expression of MDM2. No significant differences in MDM2 mRNA expression were observed according to the clinical characteristics of patients with OSCC. The MDM2 mRNA expression was 2.35-fold higher in patients with OSCC who were older than 40 years compared to younger patients, a finding that is commonly associated with aggressive tumor biology and poor prognosis.⁽²⁰⁾ In addition, MDM2 mRNA expression in patients with OSCC who smoked was 2.12-fold higher than that in non-smoking patients. These findings are consistent with Gołabek K, *et al.* (2022), who reported a significant negative correlation between the age of patients with OSCC and MDM2 mRNA expression in non-smoking patients.⁽²¹⁾

Cigarette smoking affects MDM2 mRNA expression by inducing DNA damage from polycyclic aromatic hydrocarbons (PAHs). PAHs, consisting of benzo (a) pyrene (BaP) and dibenzo (a, l) pyrene (DBP), can induce DNA damage and phosphorylate MDM2.⁽²²⁾ Recent studies have demonstrated that exposure to BaP can induce the phosphorylation of MDM2 and thereby interfere with DNA repair in the cell cycle. The mRNA expression of MDM2 also increases in epithelial cells exposed to BaP.^(18, 23)

Wallace SJ, *et al.* reported that environmental factors, such as cigarette smoking and the combustion of fossil fuels, significantly release hazardous compounds such as BaP and PAHs, which subsequently induce DNA damage.⁽²⁴⁾ Beyond inhalation, human dermal layers can also absorb PAHs derived from the smoke of grilled or charred foods.⁽²⁵⁾ Mechanistically, PAHs are generated when dietary macronutrients, specifically lipids, carbohydrates,

and proteins, undergo thermal pyrolysis during high-temperature cooking or grilling processes.^(26, 27) This thermal processing promotes the pyrolytic decomposition of organic matter into highly reactive carcinogenic intermediates.⁽²⁸⁾

Saputro E, *et al.* revealed that the food consumption trend of roasting, burning, and frying in hot oil by people in Indonesia is one of the sources by which the body is exposed to PAHs.⁽²⁹⁾ PAH contamination in food can originate from incomplete combustion of organic matter, direct deposition from the air, and food processing at high temperatures because PAHs are significantly formed at temperatures of 400°C.⁽²⁹⁾ Although further study is required, exposure to PAHs obtained from cigarettes and food processing at high temperatures may explain their effect on MDM2 mRNA expression in smoking and non-smoking patients with OSCC.

Integrated network and enrichment analyses demonstrate that MDM2, TP53, and CDKN1A function as the central hubs within the OSCC regulatory landscape, mediating extensive crosstalk between p53 signaling, cell cycle control, and the senescence pathways. GO enrichment further confirmed that p53-mediated DNA damage response and apoptotic signaling are the most significantly overrepresented biological processes among the candidate genes, thus emphasizing the crucial role of p53 pathway dysregulation in OSCC pathogenesis. These findings are consistent with previous reports demonstrating the centrality of p53 signaling in tumor suppression as well as therapeutic response across diverse cancers.^(2, 11, 17) Collectively, these results highlight the therapeutic potential of targeting p53 network components in OSCC and provide a robust framework for future functional studies.

Several critical limitations must be acknowledged when interpreting the findings of this study. First, the relatively modest sample size and single-center design restrict the broader generalizability of the results to heterogeneous OSCC populations. Second, the cross-sectional design precludes establishing a direct causal relationship between MDM2 expression dynamics and clinical outcomes. Third, the reliance on FFPE tissue samples introduces inherent challenges regarding RNA fragmentation and quality degradation, which may influence the quantification consistency compared to fresh-frozen specimens. Furthermore, this study evaluated MDM2 strictly at the transcriptional level; the lack of protein-level validation via western blot analysis or immunohistochemistry limits our understanding of the post-translational modifications

and functional protein stability. Lastly, the absence of adjacent healthy tissue or benign oral mucosal controls prevents the determination of a true baseline of normal physiological MDM2 expression. In addition, functional assays and mechanistic studies in cellular and animal models are warranted to clarify the causal relationship between MDM2-mediated p53 regulation and OSCC progression as well as to explore the therapeutic potential of targeting this pathway. Such efforts are essential to translate these molecular insights into clinically actionable strategies, as emphasized in recent literature on p53 pathway-targeted therapies in cancer.⁽³⁰⁻³²⁾

Conclusion

This study reveals distinct trends in MDM2 mRNA expression across various clinicopathological demographics in patients with OSCC, notably demonstrating elevated transcriptional tendencies in older individuals and chronic smokers, alongside suppressed expression in tumors with larger volumes. Although these differences were not significant, the integration of bioinformatic network modeling substantiates the central hub role of the p53–MDM2 axis in cell cycle progression and apoptosis during oral carcinogenesis. Future multi-center prospective investigations with larger cohorts and protein-level validations are warranted to clarify the precise prognostic efficacy of MDM2.

Author contributions

AWSP and TW contributed substantially to the study concept and design, methodology, and writing of the manuscript. AWSP, NDR, KJR, and CMIM contributed substantially to investigation and data acquisition. NDR contributed to data curation and data analysis. KJR, AHBW, and DN contributed to providing resources. KJR contributed to data validation. CMIM contributed to visualization and project administration. AHBW, DN, DJW, and TW provided overall supervision and contributed to writing, reviewing, and editing the manuscript. TW was responsible for funding acquisition. All authors critically revised the manuscript for important intellectual content, approved the final version to be published, and agreed to be accountable for all aspects of the work.

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Data sharing statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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