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ARTIKEL PENELITIAN

MMP9 mRNA expression in oral squamous cell carcinoma according to smoking status and histopathological grade

Nabella Deby Ramadhani[1], Aliffala Widianaputri Setya Pangastuti[1], Kurnia Jelang Ramadian[1], Choirum Minannisa Imma Mahardika[1], Helmi Hirawan[2], Dwi Nur Indah Sari[3], A. Haris Budi Widodo[4], Tirta Wardana[5]

[1] *Undergraduate Program, School of Dentistry, Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.*

[2] *Department of Oral Surgery, School of Dentistry, Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.*

[3] *Department of Biomedicine, School of Dentistry, Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.*

[4] *Department of Oral Biology, School of Dentistry, Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.*

[5] *Department of Genetics and Molecular Medicine, Faculty of Medicine, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia.*

Korespondensi: *Tirta Wardana;* tirta.wardana@unsoed.ac.id*; +6282326136343.*

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Abstrak

Tujuan: Menganalisis perbedaan ekspresi mRNA MMP9 berdasarkan status merokok dan derajat histopatologi pada karsinoma sel skuamosa mulut (OSCC). **Metode:** Penelitian observasional analitik potong lintang ini mencakup 39 spesimen jaringan OSCC yang difiksasi formalin dan ditanam dalam parafin dari RSUD Prof. Dr. Margono Soekarjo, Purwokerto. Ekspresi mRNA MMP9 diperiksa menggunakan reverse transcription quantitative PCR, dinormalisasi terhadap GAPDH, dan dihitung menggunakan metode $2^{-}\Delta\Delta Ct$. Perbandingan antarkelompok menggunakan uji t independen. **Hasil:** Sampel mencakup 19 perokok dan 20 nonperokok, serta 20 tumor derajat rendah dan 19 derajat tinggi. Analisis awal melaporkan ekspresi relatif MMP9 sebesar 2,64 kali pada perokok dibandingkan nonperokok (p=0,04) dan 2,51 kali pada tumor derajat tinggi dibandingkan derajat rendah (p=0,01). Perbandingan berdasarkan usia, jenis kelamin, dan ukuran tumor menghasilkan p masing-masing 0,26; 0,11; dan 0,90. **Simpulan:** Analisis menunjukkan perbedaan ekspresi mRNA MMP9 menurut status merokok dan kelompok derajat histopatologi. Temuan ini bersifat eksploratori, belum mempertimbangkan perancu, dan tidak membuktikan hubungan sebab akibat atau nilai prognostik.

Kata kunci: karsinoma sel skuamosa mulut; MMP9; merokok; derajat histopatologi; RT-qPCR

Abstract

Objective: To examine differences in MMP9 mRNA expression according to smoking status and histopathological grade in oral squamous cell carcinoma (OSCC). **Methods:** This analytical cross-sectional study included 39 formalin-fixed paraffin-embedded OSCC tissue specimens from Prof. Dr. Margono Soekarjo Hospital, Purwokerto, Indonesia. MMP9 mRNA expression was measured using reverse transcription quantitative PCR, normalized to GAPDH, and quantified using the $2^{-}\Delta\Delta Ct$ method. Between-group comparisons used independent-samples t tests. **Results:** The sample included 19 smokers and 20 nonsmokers, with 20 low-grade and 19 high-grade tumors. The original analysis reported 2.64-fold MMP9 expression in smokers relative to nonsmokers (p=0.04) and 2.51-fold expression in high-grade relative to low-grade tumors (p=0.01). Comparisons by age, sex, and tumor size yielded p values of 0.26, 0.11, and 0.90, respectively. **Conclusion:** The analysis indicated differences in MMP9 mRNA expression by smoking status and histopathological grade. These exploratory, unadjusted findings do not establish causality or prognostic value.

Keywords: oral squamous cell carcinoma; MMP9; smoking; histopathological grade; RT-qPCR

INTRODUCTION

Oral squamous cell carcinoma (OSCC) accounts for most oral malignancies. Tobacco exposure and host factors contribute to its development, while variation in tumor pathology and molecular profiles supports investigation of exposure-related pathways.[1,2]

Anatomical subsite matters when interpreting molecular findings, as studies of p16-positive oral cancers illustrate.[3] Genetic susceptibility studies in head and neck carcinoma address a different question from measured tumor transcript abundance.[4]

Tobacco-related oxidative stress can alter cellular signaling, inflammatory responses, and cancer metabolism.[5] Analyses of human cancers have identified mutational signatures associated with tobacco smoking, providing evidence of exposure-related molecular changes across tumor types.[6] These observations support a biological rationale for studying smoking-associated expression differences without establishing the direction or cause of a particular transcript change in established OSCC.

Matrix metalloproteinase-9 (MMP-9), encoded by MMP9, is a zinc-dependent protease involved in extracellular matrix remodeling. Its substrates include basement-membrane components, and its activity depends on enzyme activation and regulation by tissue inhibitors of metalloproteinases.[7] Studies of oral tongue carcinoma have implicated MMP9 in molecular patterns associated with invasion and treatment outcomes.[8] A tissue RT-qPCR study also reported higher MMP9 expression in OSCC than in normal tissue.[9]

Molecular research on OSCC spans tissue, cell, and fluid-based approaches.[10] Investigators have examined salivary cytokines and MMP-9 as potential diagnostic measurements, but salivary protein concentration and tumor mRNA expression are different biological endpoints.[11,12] The present study compared MMP9 mRNA expression by smoking status and histopathological grade in 39 patients with OSCC. Comparisons by age, sex, and tumor size provided additional clinicopathological context.

METODE

Study design and participants

We conducted an observational analytical cross-sectional study using 39 formalin-fixed paraffin-embedded (FFPE) OSCC specimens from Prof. Dr. Margono Soekarjo Hospital, Purwokerto, Indonesia. Eligibility criteria comprised clinical and pathological confirmation of OSCC, age 20-60 years, absence of another malignancy, and no previous cancer treatment. All comparisons were within the OSCC sample; the study did not include cancer-free controls or longitudinal outcome assessment.

We obtained age, sex, tumor size, smoking history, and histopathological grade from patient records. Age categories were >40 and ≤40 years, and tumor-size categories were >4 and ≤4 cm. The analysis used the recorded smoking categories and the low-grade and high-grade tumor groups.

Ethical considerations

The Medical Ethics Committee of the Faculty of Medicine, Jenderal Soedirman University, approved the study under approval number 026/KEPK/PE/V/2022. All participants provided written informed consent before inclusion. The study followed the Declaration of Helsinki.

RNA extraction and complementary DNA synthesis

We extracted total RNA using the Monarch® Total RNA Miniprep Kit and synthesized complementary DNA using the ProtoScript® First Strand cDNA Synthesis Kit according to the manufacturers' instructions. The reverse-transcription program comprised incubation at 42°C for 60 minutes, heating at 80°C for 5 minutes, and a final hold at 4°C.

RT-qPCR and relative expression

We measured MMP9 transcripts using SensiFAST SYBR Lo-ROX reagents on an Applied Biosystems 7500 Fast Real-Time PCR system. The amplification program comprised initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 10 seconds and 58°C for 10 seconds. The MMP9 primers were forward 5'-ACGACGTCTTCCAGTACCGA-3' and reverse 5'-TGGTCCACCTGGTTCAACTC-3'. The GAPDH primers were forward 5'-CCATGGGGAAGGTGAAGGTC-3' and reverse 5'-TGAAGGGGTCATTGATGGCA-3'.
Instrument software provided threshold-cycle (Ct) values and melting-curve analysis.

We used GAPDH as the reference gene and calculated relative expression using the comparative Ct approach: ΔCt = Ct(MMP9) − Ct(GAPDH), ΔΔCt = ΔCt(sample or comparison) − ΔCt(calibrator), and relative expression = 2^{-}ΔΔCt. This approach assumes suitable amplification efficiencies; the calculation itself does not correct unequal efficiencies. Transparent reporting of assay performance, controls, and normalization is necessary for interpreting relative-expression estimates.[13]

Reference-gene suitability depends on the biological material and experimental conditions. Studies in an OSCC animal model, drug-treated oral carcinoma cells, and human head and neck tumors illustrate this requirement.[14,15,16] Investigators have also evaluated internal controls using tumor expression datasets and prospectively collected salivary samples.[17,18] We therefore interpret GAPDH-normalized results in the context of the present tissue assay rather than assuming universal reference-gene stability.

FFPE tissue allows analysis of archived pathological material, although fixation and storage may affect RNA recovery and Ct measurements.[19] Wimmer and colleagues also demonstrated the importance of functional RNA-quality assessment in FFPE expression profiling.[20] The available methodological record did not include amplification-efficiency estimates, sample-level reference-gene stability results, or the number of technical replicates; these reporting limitations constrain assessment of analytical reproducibility.

Statistical analysis

We summarized categorical characteristics as frequencies and percentages. The original 39-patient analysis reported qPCR subgroup summaries as means ± standard errors (SE), relative-expression ratios, and independent-samples t-test p values. GenEx version 6.0 supported expression calculations, and GraphPad Prism 9 supported visualization. We retained the reported inferential results and interpreted $p \leq 0.05$ as the original significance threshold.

The comparisons were unadjusted for covariates and multiple testing. We treated the findings as exploratory, with smoking status and tumor grade as the principal comparisons of interest. Confidence intervals were not available in the original analysis. A p value above the threshold does not establish equivalence, and a p value below it does not measure the

size or clinical importance of an association.[21]

HASIL DAN PEMBAHASAN

Participant characteristics

The study included 39 patients with OSCC. Fourteen were men (35.9%) and 25 were women (64.1%). Thirty-two patients (82.1%) were older than 40 years. Nineteen patients (48.7%) had a smoking history, while 20 (51.3%) were nonsmokers. Twenty tumors (51.3%) belonged to the low-grade group and 19 (48.7%) to the high-grade group. Seventeen tumors (43.6%) measured >4 cm and 22 (56.4%) measured ≤4 cm. Each two-group comparison accounted for all 39 participants (Table 1).

Table 1. Demographic and clinicopathological characteristics of the 39 patients with OSCC

Characteristic	Category	n	%
Age (years)	>40	32	82.1
	≤40	7	17.9
Sex	Male	14	35.9
	Female	25	64.1
Tumor size (cm)	>4	17	43.6
	≤4	22	56.4
Histopathological grade	Low	20	51.3
	High	19	48.7
Smoking history	Smoker	19	48.7
	Nonsmoker	20	51.3

Noted: OSCC, oral squamous cell carcinoma. Percentages use N=39 as the denominator. Categories follow those recorded in the original 39-patient dataset.

MMP9 expression and clinicopathological comparisons

In the original 39-patient analysis, smokers had 2.64-fold MMP9 expression relative to nonsmokers (p=0.04), while high-grade tumors had 2.51-fold expression relative to low-grade tumors (p=0.01). Table 2 presents the corresponding reported qPCR summaries. The Ct values were lower in smokers than nonsmokers (33.61 ± 1.1 versus 35.01 ± 1.4) and in high-grade than low-grade tumors (33.80 ± 1.1 versus 34.78 ± 1.6). Under comparable assay conditions, a lower target Ct is consistent with greater starting template abundance; the normalized expression ratio also depends on reference-gene measurements and the aggregation of individual values.

Table 2. MMP9 qPCR summaries and unadjusted comparisons in the 39-patient analysis

Group	n	MMP9 Ct, mean ± SE	p value[1]	Reported relative-expression ratio[2]
Age >40 years	32	34.31 ± 1.2	0.26	—
Age ≤40 years	7	34.35 ± 2.4		—
Male	14	34.76 ± 1.4	0.11	—
Female	25	34.09 ± 1.4		—
Tumor >4 cm	17	34.47 ± 1.7	0.90	—
Tumor ≤4 cm	22	34.15 ± 1.1		—
Low grade	20	34.78 ± 1.6	0.01	1.00 (reference)
High grade	19	33.80 ± 1.1		2.51
Smoker	19	33.61 ± 1.1	0.04	2.64
Nonsmoker	20	35.01 ± 1.4		1.00 (reference)

Noted: Ct, threshold cycle; SE, standard error. [1]Independent-samples t-test p values from the original analysis, shown once for each two-group comparison; no adjustment for covariates or multiple testing. [2]Ratios for the main comparisons follow the original normalized-expression analysis: high versus low grade and smoker versus nonsmoker. They were not recalculated from the displayed group target-Ct means. A dash indicates that a

relative-expression ratio is not presented here. Mean ± SE labeling follows the original subgroup table.

Age, sex, and tumor-size comparisons yielded p values of 0.26, 0.11, and 0.90, respectively. These results did not meet the original significance threshold. They do not exclude differences that a larger study might detect, particularly given the small younger subgroup. Figure 1 reproduces the author-supplied relative-expression plot.

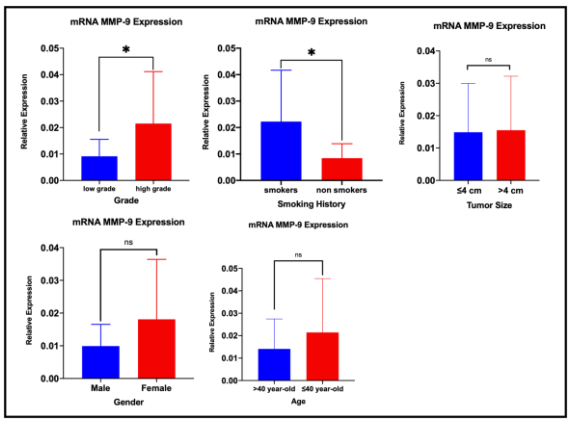


Figure 1. Author-supplied MMP9 relative-expression plot by histopathological grade (upper left), smoking history (upper middle), tumor size (upper right), sex (lower left), and age (lower middle). The total sample comprises 39 patients; subgroup sizes are provided in Table 1. *p<0.05; ns, not significant at the original threshold. The original plot is reproduced without recalculating bar heights or error bars.

Relationship with tumor grade

The observed direction of the tumor-grade comparison is compatible with the involvement of MMP9 in matrix remodeling, but it does not establish a mechanism. Thangaraj and colleagues linked MMP9 expression with molecular and pathological features in oral tongue carcinoma.[8] Their subsequent work examined a panel including MMP9 in relation to treatment outcomes.[22] Sowmya and colleagues assessed both gene and protein expression of invasion-related markers in metastatic and nonmetastatic OSCC.[23] Patil and colleagues also reported grade-related differences using MMP-9 immunohistochemistry.[24] These studies provide context for the present finding while involving different samples, assays, and clinical endpoints.

Histopathological grade concerns microscopic differentiation, whereas clinical stage concerns anatomical extent. The grade comparison cannot establish nodal involvement, metastasis, or survival, none of which this study evaluated. The finding represents an exploratory association rather than a validated prognostic result.

Relationship with smoking history

The smoking comparison is biologically plausible in view of the oxidative and inflammatory effects of tobacco exposure.[5,6] Foki and colleagues studied cigarette-smoke effects on oral keratinocytes and identified changes involving MMP-1; their experiment supports exposure-related pathway research but does not directly validate an MMP9 result.[25] The present study measured transcript expression in established OSCC. It cannot establish exposure-expression temporality or determine whether MMP9 mediated disease progression.

Smoking history may differ by sex, age, alcohol exposure, tumor subsite, or grade. The unadjusted comparison cannot isolate smoking from these potential confounders. Without cancer-free controls, the study also cannot estimate OSCC risk among smokers.

Tissue expression and biomarker interpretation

Bulk-tissue mRNA measurements reflect the mixture of tumor, stromal, and inflammatory cells in the specimen.

Expression differences may therefore arise from changes in cellular composition as well as changes within malignant cells. Silva and colleagues evaluated epithelial and stromal staining, illustrating the importance of the cellular compartment in interpreting MMP-9 measurements.[26] Miguel and colleagues studied MMP-9 protein in oral epithelial dysplasia, a disease setting distinct from established carcinoma.[27] Their findings cannot directly confirm a smoking-associated tissue mRNA difference in the present sample.

Salivary studies address a related but separate question. Peisker and colleagues measured MMP-9 in OSCC and healthy-control saliva.[12] Smriti and colleagues compared salivary MMP-9 across OSCC, potentially malignant disorders, tobacco users, and controls.[28] Shin and colleagues examined salivary MMP-9 in a case-control study with postoperative measurements.[29] These studies support investigation of MMP-9 across biological compartments; they do not make salivary protein, tissue mRNA, and active enzyme concentration interchangeable.

Technical factors also affect comparability. RT-qPCR reproducibility depends on the full workflow, including reverse transcription, normalization, and analysis.[30,31] Work comparing qPCR with droplet digital PCR illustrates the challenge of quantifying low-abundance targets under varying assay conditions.[32] In the present study, the Ct values warrant attention to assay sensitivity and precision, but they cannot alone establish whether a sample was near the limit of quantification. That assessment requires target-specific validation.

Clinical implications and future research

MMP9 expression could contribute to future molecular characterization of OSCC, but clinical use requires evidence beyond a between-group expression difference. Diagnostic research can involve molecular measurements or approaches such as image-based detection, each of which requires its own performance assessment.[33] The current study did not estimate sensitivity, specificity, calibration, or incremental clinical value and should not change diagnostic or treatment decisions on its own.

A transcript difference does not establish suitability for MMP-9-directed treatment. Enzyme activity is regulated at several levels, while inhibitor development faces specificity and safety challenges.[7,34] Therapeutic conclusions require functional evidence.

Future studies should use a defined recruitment period, reproducible smoking and grading definitions, and a prespecified analysis plan. Larger samples should support estimates with confidence intervals and a limited set of clinically justified covariates. If a prognostic model is later developed, its sample-size requirements should be determined for that prediction task rather than inferred from the present molecular comparison.[35]

Study limitations

This single-center study included 39 patients, with unequal subgroup sizes and only seven patients aged ≤40 years. The cross-sectional design prevents temporal or causal inference. Detailed smoking exposure, recruitment procedures, and the grading framework were not fully documented in the available study report, limiting assessment of selection and exposure classification. The analysis did not adjust for confounding or multiple comparisons, and confidence intervals were not reported.

FFPE processing, RNA quality, and tissue composition may have influenced transcript measurements. The available

report did not document sample-specific GAPDH stability, amplification efficiencies, or technical-replicate performance. These omissions limit analytical appraisal. The study also lacked normal-tissue comparisons, protein or enzyme-activity measurements, and clinical follow-up. The findings require replication before clinical application.

CONCLUSION

The original analysis of 39 OSCC specimens indicated higher relative MMP9 mRNA expression in smokers and in the high-grade tumor group. These findings represent exploratory, unadjusted associations. They do not establish a causal effect of smoking, diagnostic performance, or prognostic value. Further work should confirm assay reproducibility and evaluate the associations in a larger, clinically characterized cohort.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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