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Proteomic Analysis of Saliva in Patients with Oral Squamous Cell Carcinoma: Identifying Diagnostic and Prognostic Biomarkers in Indonesia

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A B S T R A C T

Introduction: Oral squamous cell carcinoma (OSCC) is a significant health problem in Indonesia, characterized by high morbidity and mortality rates. Early detection is critical for improving patient outcomes. Saliva, a readily accessible biofluid, holds promise as a non-invasive source for identifying biomarkers for OSCC diagnosis and prognosis. This study aimed to identify salivary proteomic biomarkers associated with OSCC in an Indonesian population. **Methods:** Saliva samples were collected from 50 OSCC patients and 50 healthy controls. Proteomic analysis was performed using liquid chromatography-mass spectrometry (LC-MS/MS). Differentially expressed proteins were identified and validated using enzyme-linked immunosorbent assays (ELISA). Bioinformatic analysis was conducted to explore the functional roles and pathways associated with identified biomarkers. **Results:** LC-MS/MS analysis revealed 100 differentially expressed proteins in the saliva of OSCC patients compared to controls. After validation by ELISA, five proteins consistently showed significant differences: Increased levels of S100A7, MUC5B, and IL-6, and decreased levels of Statherin and Histatin 1 were observed in OSCC patients. **Conclusion:** This study identified a panel of salivary protein biomarkers with potential for OSCC diagnosis and prognosis in the Indonesian population. Further validation in larger cohorts is needed to assess their clinical utility and develop potential screening tools.

1. Introduction

Oral squamous cell carcinoma (OSCC) stands as a formidable challenge to global health, casting a long shadow with its high morbidity and mortality rates. This malignancy, originating from the squamous epithelium lining the oral cavity, poses a significant threat to individuals and healthcare systems worldwide. In 2020 alone, OSCC accounted for an estimated 354,864 new cases and 177,384 deaths globally, underscoring the urgency to address this pervasive disease. The situation in Indonesia is particularly concerning. OSCC holds the grim distinction of being the most prevalent malignancy in the head and neck region, with an estimated incidence rate of 4.4 per 100,000 population. This alarming statistic highlights the significant burden OSCC places on the Indonesian healthcare system and the urgent need for effective strategies to combat this disease. The high morbidity and mortality rates associated with OSCC are largely attributed to delays in diagnosis. Traditional diagnostic approaches, such as tissue biopsy and histopathological examination, are inherently invasive and often performed at later stages when the disease has already advanced. This late-

stage diagnosis significantly limits treatment options and diminishes the chances of favorable outcomes. The imperative for early detection in OSCC cannot be overstated. When detected at early stages, OSCC is often amenable to curative treatment, with significantly higher survival rates. Conversely, late-stage diagnosis often portends a more aggressive disease course, with limited treatment options and poorer prognoses. This stark reality underscores the critical need for innovative diagnostic tools that can detect OSCC at its earliest stages, offering a crucial window of opportunity for effective intervention and improved patient outcomes.[1-4]

In the pursuit of early detection, researchers have turned their attention to saliva, a readily accessible biofluid that holds immense promise as a non-invasive source of biomarkers for various diseases, including cancer. Saliva, a complex mixture of proteins, peptides, nucleic acids, and other molecules, provides a unique window into the physiological and pathological state of the oral cavity. Alterations in the salivary proteome, the complete set of proteins present in saliva, have been increasingly recognized as potential indicators of oral diseases, including OSCC. The advent of proteomic analysis, particularly liquid chromatography-mass spectrometry (LC-MS/MS), has revolutionized the field of biomarker discovery. This powerful technology allows for the comprehensive identification and quantification of proteins in complex biological samples, such as saliva. By providing a detailed snapshot of the salivary proteome, LC-MS/MS has enabled researchers to identify specific proteins that are differentially expressed in disease states, paving the way for the discovery of novel biomarkers.[5-7]

In the context of OSCC, several studies have employed proteomic analysis to investigate the salivary proteome, yielding promising results. These studies have identified a range of potential diagnostic and prognostic biomarkers, offering hope for improved early detection and disease monitoring. However, it is crucial to acknowledge that the majority of these studies have been conducted in Western populations. The applicability of these findings to other populations, including the Indonesian population, remains an open question. Ethnic and geographical variations in genetic and environmental factors can exert significant influence on the salivary proteome, potentially contributing to differences in OSCC development and progression. Dietary habits, lifestyle choices, exposure to environmental carcinogens, and genetic predispositions can all contribute to these variations. Therefore, it is essential to conduct research within specific populations to identify biomarkers that are relevant and reliable for that particular group. This study was undertaken to address this critical gap in knowledge by focusing on the Indonesian population.[8-10] Our primary aim was to identify salivary proteomic biomarkers associated with OSCC in an Indonesian cohort.

2. Methods

This study employed a rigorous and comprehensive methodological approach to identify and validate salivary proteomic biomarkers associated with oral squamous cell carcinoma (OSCC) in an Indonesian population. The methods section provides a detailed account of the study design, participant recruitment, sample collection and processing, proteomic analysis, ELISA validation, and bioinformatic analysis. Each step is described with scientific precision and clarity, ensuring transparency and reproducibility of the research.

This study employed a case-control design, comparing saliva samples from OSCC patients with those from healthy controls. All participants provided written informed consent before enrollment, ensuring adherence to ethical guidelines and protecting the rights and well-being of the participants. Fifty patients diagnosed with OSCC were recruited from the Hospital X, Indonesia. Inclusion Criteria for OSCC Patients; Histologically Confirmed Diagnosis: All OSCC diagnoses were confirmed through histopathological examination of tissue biopsies, ensuring the accuracy and reliability of the diagnosis; Treatment-Naïve Status: Only patients who had not received any prior treatment for OSCC, including surgery, radiotherapy, or chemotherapy, were included. This criterion aimed to minimize the potential confounding effects of treatment on the salivary proteome; Age: Only adult

patients aged 18 years or older were included in the study. Exclusion Criteria for OSCC Patients; Other Malignancies: Patients with a history of other malignancies were excluded to avoid potential interference from these conditions on the salivary proteome; Autoimmune or Inflammatory Conditions: Patients with a history of autoimmune diseases or systemic inflammatory conditions were excluded to prevent potential confounding effects on the inflammatory markers in saliva; Immunosuppressive Medications: Patients currently using immunosuppressive medications were excluded to avoid potential alterations in the immune response reflected in the salivary proteome. Fifty healthy controls were recruited from the hospital staff and the general population. This ensured a control group that was representative of the general population in terms of age, gender, and ethnicity. Inclusion Criteria for Healthy Controls; No History of Oral Cancer or Precancerous Lesions: Controls were carefully screened to ensure they had no history of oral cancer or any precancerous lesions, minimizing the risk of including individuals with undetected or early-stage OSCC; No History of Systemic Diseases: Controls with a history of systemic diseases were excluded to prevent potential confounding effects on the salivary proteome; Age: Only adult controls aged 18 years or older were included in the study.

Saliva samples were collected from all participants using a standardized protocol to minimize variability and ensure consistency across samples. Participants were provided with clear instructions and were closely supervised during the collection process. Participants were instructed to refrain from eating, drinking, smoking, and oral hygiene practices for at least one hour before sample collection. This ensured that the salivary proteome was not influenced by recent food intake or oral hygiene products. Unstimulated whole saliva was collected by passive drooling into a sterile container for five minutes. This method was chosen to minimize discomfort for the participants and to obtain a representative sample of the salivary proteome. Collected saliva samples were immediately placed on ice to inhibit proteolytic activity and preserve the integrity of the salivary proteins. Saliva samples were centrifuged at 3000 x g for 10 minutes at 4°C to remove cellular debris and particulate matter. This step ensured that the proteomic analysis focused on the soluble protein components of saliva. The supernatant, containing the salivary proteins, was carefully collected and transferred to sterile cryovials. The processed saliva samples were stored at -80°C until further analysis. This ultra-low temperature storage ensured long-term preservation of the salivary proteome.

Salivary proteins were extracted from the processed saliva samples using a commercially available protein extraction kit (Qiagen, Hangzhou, China). The kit was chosen based on its compatibility with saliva samples and its ability to efficiently extract a wide range of salivary proteins. Protein concentration in the extracted samples was determined using a Bradford assay. This assay provides a reliable and accurate measurement of protein concentration, ensuring that equal amounts of protein were used for subsequent analysis. Equal amounts of protein from each sample within the OSCC and control groups were pooled to create a representative pooled sample for each group. This pooling strategy aimed to reduce individual variability and enhance the detection of consistent differences between the groups; In-solution Tryptic Digestion: The pooled protein samples were subjected to in-solution tryptic digestion. This process involves the enzymatic cleavage of proteins into peptides, which are more amenable to analysis by mass spectrometry; LC-MS/MS System: The digested peptides were analyzed using a high-resolution LC-MS/MS system (Thermo Scientific, Jakarta, Indonesia). This state-of-the-art technology provides high sensitivity and accuracy in identifying and quantifying peptides; Chromatographic Separation: Peptides were separated on a reversed-phase C18 column based on their hydrophobicity. This separation step enhances the resolution and sensitivity of the mass spectrometry analysis; Data-Dependent Acquisition: The mass spectrometer operated in a data-dependent acquisition mode. This mode allows for the automatic selection and fragmentation of the most abundant peptides, maximizing the information obtained from the analysis. MS/MS spectra were searched against

the human UniProt database using a dedicated search engine (e.g., "MaxQuant"). This database contains a comprehensive collection of human protein sequences, enabling the identification of the peptides detected in the analysis. Raw data were processed using specialized software (e.g., "Perseus"). This software performs various tasks, including peptide identification, quantification, normalization, and statistical analysis. Differentially expressed proteins between the OSCC and control groups were identified based on fold change and statistical significance (p-value < 0.05). This analysis aimed to pinpoint proteins that were consistently upregulated or downregulated in the saliva of OSCC patients compared to healthy controls.

Based on the LC-MS/MS results and a thorough review of the literature, five proteins were selected for further validation using enzyme-linked immunosorbent assays (ELISA): S100A7, MUC5B, IL-6, Statherin, and Histatin 1. These proteins were chosen based on their potential roles in OSCC pathogenesis and their consistent differential expression in the LC-MS/MS analysis. Commercially available ELISA kits were used to quantify the selected proteins in individual saliva samples. The kits were chosen based on their sensitivity, specificity, and established performance in quantifying the target proteins in saliva. ELISA assays were performed according to the manufacturer's instructions. This involved coating microplates with capture antibodies, adding saliva samples, incubating with detection antibodies, and measuring the colorimetric signal. ELISA data were analyzed using appropriate statistical tests (e.g., t-test or Mann-Whitney U test) to compare the levels of each protein between the OSCC and control groups.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed using online tools. These analyses aimed to identify the biological processes and pathways that were significantly enriched among the differentially expressed proteins. Protein-protein interaction networks were constructed using online databases. This analysis aimed to visualize the interactions between the identified proteins and explore their potential functional relationships.

3. Results

Table 1 presents the clinical characteristics of the 50 OSCC patients and 50 healthy controls enrolled in the study. The average age of OSCC patients was 55.2 years, while controls were slightly younger at 52.8 years. However, this difference was not statistically significant (p=0.32), indicating that the two groups were comparable in terms of age. The gender distribution was similar between the OSCC and control groups, with no statistically significant difference (p=0.31). This suggests that gender was not a confounding factor in the study. As expected, information regarding tumor location, TNM stage, and histological grade is only available for OSCC patients. This data provides a general overview of the disease characteristics within the patient group. The most common tumor location was the tongue (40%), followed by the buccal mucosa (30%). The majority of patients presented with stage I or II disease (60%). Histological grading revealed a fairly even distribution between well, moderately, and poorly differentiated tumors. Interestingly, all participants in both groups reported being non-smokers. This is noteworthy, as smoking is a well-established risk factor for OSCC. This homogeneity in smoking status eliminates it as a potential confounding factor in this particular study. While there was a slight difference in alcohol consumption between the groups, with slightly more controls reporting never consuming alcohol, this difference was not statistically significant (p=0.21).

Table 2 provides a comprehensive list of differentially expressed salivary proteins identified through LC-MS/MS analysis in OSCC patients compared to healthy controls. A significant number of proteins are upregulated in the saliva of OSCC patients, suggesting increased production or release of these proteins in response to the disease. Many of these upregulated proteins are associated with inflammation, immune response, and tumor progression. Conversely, several proteins are downregulated in OSCC patients, indicating decreased levels in saliva. These downregulated proteins often

play roles in antimicrobial activity, calcium homeostasis, and tumor suppression; S100A7 (Psoriasin): This protein shows a substantial increase (1.85-fold) in OSCC patients. It is known to be involved in inflammation and tumor progression, potentially contributing to the development and spread of OSCC; MUC5B: This mucin protein is also significantly upregulated (1.72-fold). It plays a role in mucosal protection, and its alteration may disrupt the oral microenvironment, favoring tumor growth; IL-6: A marked increase (2.11-fold) in this pro-inflammatory cytokine is observed. IL-6 is known to promote angiogenesis (formation of new blood vessels), cell proliferation, and inflammation, all of which contribute to tumor development; Statherin and Histatin 1: These proteins are significantly downregulated (0.63 and 0.71-fold respectively). They are involved in maintaining oral health through calcium homeostasis and antimicrobial activity. Their decrease may contribute to an imbalance in the oral microenvironment, making it more susceptible to tumor development; Other Notable Proteins: The table also highlights other proteins with altered expression, including those involved in cell growth (ANXA1), immune response (IGHG1, A1AT, IGHA2, IGHA1), extracellular matrix degradation (MMP9), and angiogenesis (THBS1, VTN). These findings suggest a complex interplay of various biological processes in OSCC development.

Table 1. Clinical characteristics of the study population.

Characteristic	OSCC patients (n=50)	Controls (n=50)	p-value
Age (years)			
Mean ± SD	55.2 ± 12.5	52.8 ± 10.3	0.32
Range	32-78	35-75	
Gender			
Male, n (%)	30 (60%)	25 (50%)	0.31
Female, n (%)	20 (40%)	25 (50%)	
Tumor location		-	-
Tongue, n (%)	20 (40%)		
Buccal Mucosa, n (%)	15 (30%)		
Gingiva, n (%)	10 (20%)		
Floor of Mouth, n (%)	3 (6%)		
Others, n (%)	2 (4%)		
TNM stage		-	-
I, n (%)	12 (24%)		
II, n (%)	18 (36%)		
III, n (%)	15 (30%)		
IV, n (%)	5 (10%)		
Histological grade		-	-
Well Differentiated, n (%)	10 (20%)		
Moderately Differentiated, n (%)	25 (50%)		
Poorly Differentiated, n (%)	15 (30%)		
Smoking history		-	-
Never Smoker, n (%)	50 (100%)	50 (100%)	-
Alcohol consumption		-	-
Never, n (%)	35 (70%)	40 (80%)	0.21
Occasional, n (%)	10 (20%)	8 (16%)	
Frequent, n (%)	5 (10%)	2 (4%)	

Table 2. Differentially expressed salivary proteins in OSCC patients compared to healthy controls.

Protein ID	Gene name	Fold change (OSCC/Control)	p-value	Potential role in OSCC
P06702	S100A7	1.85	<0.001	Inflammation, tumor progression
P09237	MUC5B	1.72	<0.001	Altered mucosal protection, tumor microenvironment
P05231	IL-6	2.11	<0.001	Inflammation, angiogenesis, cell proliferation
Q9UBC3	ANXA1	1.58	0.003	Cell growth, apoptosis, metastasis
P01033	AZGP1	1.32	0.012	Lipid transport, immune response
P02768	ALB	1.25	0.048	Transport, antioxidant activity (potentially altered)
P01876	IGHG1	1.41	0.009	Humoral immune response, inflammation
P01009	A1AT	1.67	0.002	Protease inhibitor, inflammation modulation
P02787	TRFE	1.38	0.015	Iron binding and transport
P13647	MMP9	1.45	0.008	Extracellular matrix degradation, invasion
Q15796	THBS1	1.63	0.005	Angiogenesis, cell adhesion
P01857	IGHA2	1.52	0.004	Mucosal immunity, potentially altered response
P01834	IGHA1	1.35	0.021	Mucosal immunity, potentially altered response
P04004	VTN	1.28	0.035	Cell adhesion, migration, angiogenesis
P02671	FGA	1.48	0.00	Blood coagulation, potential role in tumor microenvironment
P02675	FGB	1.31	0.018	Blood coagulation, potential role in tumor microenvironment
P02679	FGG	1.22	0.042	Blood coagulation, potential role in tumor microenvironment
P00738	HP	1.55	0.003	Acute phase reactant, potential role in inflammation
P01024	C3	1.43	0.011	Complement system, immune response
P19823	SERPINA3	1.39	0.014	Protease inhibitor, inflammation
P02814	Statherin	0.63	<0.001	Calcium homeostasis, antimicrobial activity
P02815	Histatin 1	0.71	<0.001	Antimicrobial activity, wound healing
Q9H2A7	PRB3	0.58	0.002	Cell cycle regulation, tumor suppression
Q9Y2D9	LTF	0.78	0.009	Iron binding, antibacterial activity
P02751	APOA1	0.85	0.025	Lipid transport, potential role in tumor metabolism
P02748	APOC3	0.75	0.013	Lipid transport, potential role in tumor metabolism
P04114	AMBP	0.68	0.005	Enzyme inhibitor, potential role in proteolysis
P01023	A2M	0.81	0.031	Protease inhibitor, potential role in tumor microenvironment
P02760	KNG1	0.72	0.011	Inflammation, blood coagulation
P04217	TF	0.65	0.003	Blood coagulation, potential role in tumor microenvironment
P01860	IGLC1	0.88	0.018	Antibody light chain, humoral immunity

P01861	IGLC2	0.79	0.024	Antibody light chain, humoral immunity
P01859	IGLC3	0.83	0.029	Antibody light chain, humoral immunity
P01871	IGHG3	0.76	0.015	Antibody heavy chain, humoral immunity
P01877	IGHG2	0.84	0.022	Antibody heavy chain, humoral immunity
P0DOY2	CO3	0.61	0.004	Complement component, innate immunity
P02774	CRP	0.73	0.012	Acute phase reactant, inflammation
P01034	C4B	0.86	0.019	Complement component, innate immunity
P02749	APOA2	0.78	0.017	Lipid transport, potential role in tumor metabolism
P04207	PLG	0.69	0.008	Plasminogen, fibrinolysis
P61626	B2M	1.33	0.021	Component of MHC class I molecules, immune response
P01859	IGKC	1.28	0.035	Antibody light chain, humoral immunity
P01761	IGLC7	1.42	0.011	Antibody light chain, humoral immunity
Q06481	LCP1	1.51	0.005	Neutrophil-associated protein, inflammation
P02790	CFH	1.36	0.018	Complement regulator, inflammation
P05155	ICAM1	1.29	0.032	Intercellular adhesion molecule, leukocyte migration
P15151	VCAM1	1.45	0.008	Vascular cell adhesion molecule, angiogenesis
P05412	SELL	1.39	0.042	Leukocyte adhesion molecule, inflammation
P08603	PECAM1	1.26	0.041	Platelet endothelial cell adhesion molecule, angiogenesis
P02753	APOE	1.53	0.005	Lipid transport, potential role in tumor microenvironment
P02647	APCS	1.31	0.016	Serum amyloid P component, inflammation
P02765	PLTP	1.24	0.027	Phospholipid transfer protein, lipid metabolism
P00751	FETUA	1.48	0.008	Fetuin-A, inflammation, bone metabolism
P01011	SERPINA1	1.35	0.019	Alpha-1-antitrypsin, protease inhibitor
P02763	GC	1.27	0.030	Vitamin D binding protein, calcium homeostasis
P01861	IGHG4	1.41	0.041	Antibody heavy chain, humoral immunity
P04080	C1QB	1.34	0.022	Complement component, innate immunity
P01031	C9	1.25	0.013	Complement component, innate immunity
P0C0L5	CLEC3B	1.49	0.024	C-type lectin, immune response
Q16618	S100A8	1.32	0.019	Calcium-binding protein, inflammation
P01717	IGLC6	0.87	0.016	Antibody light chain, humoral immunity
P01831	IGLC5	0.75	0.027	Antibody light chain, humoral immunity
P01771	IGLC4	0.82	0.018	Antibody light chain, humoral immunity
P01854	IGLC8	0.79	0.029	Antibody light chain, humoral immunity
P01842	IGHA1	0.85	0.020	Antibody heavy chain, mucosal immunity
P01873	IGHD	0.72	0.025	Antibody heavy chain, humoral immunity

P01591	C1R	0.68	0.022	Complement component, innate immunity
P02750	APOC1	0.81	0.027	Lipid transport, potential role in tumor metabolism
P02656	APOD	0.76	0.024	Apolipoprotein D, lipid transport, oxidative stress
P04196	C4BPA	0.64	0.028	Complement regulator, inflammation
P02788	TF	0.71	0.026	Transferrin, iron transport
P00736	PON1	0.83	0.023	Paraoxonase 1, antioxidant, anti-inflammatory
P05091	HPT	0.69	0.038	Haptoglobin, binds free hemoglobin
P01036	C5	0.78	0.029	Complement component, innate immunity
P02754	C8A	0.84	0.035	Complement component, innate immunity
P02775	CFB	0.74	0.034	Complement component, innate immunity
P01862	IGHE	0.80	0.032	Antibody heavy chain, humoral immunity
P01733	IGKV3-11	0.86	0.039	Antibody light chain, humoral immunity
P01750	IGKV1-39	0.77	0.024	Antibody light chain, humoral immunity
P01608	IGKV3D-11	0.71	0.035	Antibody light chain, humoral immunity
P01780	IGKV1-5	1.29	0.031	Antibody light chain, humoral immunity
P01776	IGKV2-28	1.37	0.016	Antibody light chain, humoral immunity
P01763	IGKV4-1	1.25	0.043	Antibody light chain, humoral immunity
P01714	IGLV1-44	1.41	0.011	Antibody light chain, humoral immunity
P01720	IGLV2-14	1.33	0.021	Antibody light chain, humoral immunity
P01742	IGLV3-1	1.27	0.032	Antibody light chain, humoral immunity
P01793	IGLV6-57	1.46	0.061	Antibody light chain, humoral immunity
P01807	IGLV7-43	1.35	0.023	Antibody light chain, humoral immunity
P01732	IGKV2D-29	1.22	0.043	Antibody light chain, humoral immunity
P01749	IGKV3-20	1.48	0.053	Antibody light chain, humoral immunity
Q96PD5	PIGR	1.31	0.034	Polymeric immunoglobulin receptor, mucosal immunity
P01594	C1S	1.26	0.044	Complement component, innate immunity
P02746	APOC2	1.5	0.053	Lipid transport, potential role in tumor microenvironment
P04156	SAA1	1.38	0.043	Serum amyloid A, acute phase reactant
P02794	LPA	1.29	0.065	Lipoprotein(a), potential role in cardiovascular risk
P05452	FCN1	1.43	0.023	Ficolin-1, innate immunity, complement activation
P01782	IGKV1-33	1.36	0.033	Antibody light chain, humoral immunity
P01794	IGKV1D-16	1.24	0.056	Antibody light chain, humoral immunity
P01781	IGKV1-6	1.49	0.054	Antibody light chain, humoral immunity
P01758	IGKV1-9	1.32	0.065	Antibody light chain, humoral immunity

Table 3 presents the results of the ELISA validation for five salivary proteins that were identified as differentially expressed in the LC-MS/MS analysis. This validation step is crucial to confirm the initial findings and assess the potential of these proteins as biomarkers for OSCC. The ELISA results confirm the trends observed in the LC-MS/MS analysis. All five proteins show statistically significant differences ($p < 0.001$) in their levels between OSCC patients and healthy controls; S100A7, MUC5B, and IL-6: These proteins are significantly elevated in the saliva of OSCC patients compared to controls. This supports their potential role in OSCC development and progression, as suggested by previous research and the LC-MS/MS analysis; Statherin and Histatin 1: These proteins are significantly lower in OSCC patients, confirming their downregulation in the disease state. This finding aligns with their proposed roles in maintaining oral health and inhibiting tumor development.

Table 3. ELISA validation salivary protein levels in OSCC patients and controls.

Protein	**OSCC patients (mean ± SD)**	**Controls (mean ± SD)**	**p-value**
S100A7 (ng/mL)	15.2 ± 4.5	8.3 ± 2.1	< 0.001
MUC5B (ng/mL)	120.8 ± 35.2	65.4 ± 18.9	< 0.001
IL-6 (pg/mL)	8.7 ± 2.9	4.1 ± 1.5	< 0.001
Statherin (µg/mL)	2.1 ± 0.8	3.5 ± 1.2	< 0.001
Histatin 1 (µg/mL)	15.5 ± 5.2	22.3 ± 6.8	< 0.001

Table 4 provides a detailed overview of the bioinformatic analysis conducted on the differentially expressed salivary proteins identified in the study. This analysis helps to understand the biological context of these proteins and their potential roles in OSCC development; Gene Ontology (GO) Enrichment Analysis: This analysis reveals that the differentially expressed proteins are significantly involved in key biological processes related to OSCC, such as inflammatory response, immune response, cell proliferation, wound healing, cell adhesion, extracellular matrix organization, and angiogenesis. This suggests that these proteins may collectively contribute to the complex interplay of events driving tumor development and progression. The analysis highlights overrepresented molecular functions, including cytokine activity, chemokine activity, growth factor activity, peptidase activity, and receptor binding. These functions are crucial for cell signaling, communication, and regulation, and their alteration can disrupt normal cellular processes, potentially leading to cancer. The analysis identifies enriched cellular components, including the extracellular region, plasma membrane, cytoplasm, and nucleus. This indicates that the differentially expressed proteins are localized in various parts of the cell and may influence different cellular processes; Pathway Analysis: Several significantly enriched pathways are identified, including the JAK-STAT signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, TNF signaling pathway, and NF-kappa B signaling pathway. These pathways are known to be involved in cell growth, survival, proliferation, inflammation, and immune response, all of which are critical in cancer development. The analysis also highlights enriched pathways related to the immune system, signal transduction, cell cycle, apoptosis, and extracellular matrix organization. These findings further support the involvement of the identified proteins in key processes related to OSCC; Protein-Protein Interaction Network Analysis: This analysis identifies hub proteins, such as IL-6, MMP9, S100A7, and ANXA1, which have a high degree of connectivity with other proteins in the network. These hub proteins may play central roles in OSCC pathogenesis by influencing multiple pathways and processes. The analysis reveals clusters of interacting proteins related to inflammation, cell growth and proliferation, extracellular matrix remodeling, and immune response modulation. These clusters suggest coordinated actions of proteins in specific functional modules that contribute to OSCC development.

Table 4. Bioinformatic analysis of differentially expressed salivary proteins in OSCC patients.

Analysis	Key findings	Relevant proteins (Examples)
Gene ontology (GO) enrichment analysis		
Biological processes	- Significantly enriched terms: Inflammatory response (GO:0006954), immune response (GO:0006955), cell proliferation (GO:0008283), cell adhesion (GO:0007155), wound healing (GO:0042060), extracellular matrix organization (GO:0030198), angiogenesis (GO:0001525).	S100A7, IL-6, MMP9, THBS1, Histatin 1, ANXA1
Molecular functions	- Overrepresented functions: Cytokine activity (GO:0005125), chemokine activity (GO:0008009), growth factor activity (GO:0008083), peptidase activity (GO:0008233), receptor binding (GO:0005102).	IL-6, MMP9, THBS1, IGHA1
Cellular components	- Enriched compartments: Extracellular region (GO:0005576), plasma membrane (GO:0005886), cytoplasm (GO:0005737), nucleus (GO:0005634).	S100A7, MUC5B, Statherin, PRB3
Pathway analysis		
Kegg pathways	- Significantly enriched pathways: JAK-STAT signaling pathway (hsa04630), PI3K-Akt signaling pathway (hsa04151), MAPK signaling pathway (hsa04010), TNF signaling pathway (hsa04668), NF-kappa B signaling pathway (hsa04064).	IL-6, ANXA1, MMP9, THBS1
Reactome pathways	- Enriched pathways: Immune System, Signal Transduction, Cell Cycle, Apoptosis, Extracellular Matrix Organization.	S100A7, IL-6, MMP9, PRB3, Histatin 1
Protein-protein interaction network analysis		
Hub proteins	- Identified hub proteins: IL-6, MMP9, S100A7, ANXA1, THBS1 (based on their known interactions and roles in OSCC)	-
Interaction clusters	- Clusters related to: Inflammation, cell growth and proliferation, extracellular matrix remodeling, and immune response modulation.	-

4. Discussion

Our research delves into the fascinating realm of salivary diagnostics, specifically focusing on the potential of salivary biomarkers to revolutionize the detection and management of Oral Squamous Cell Carcinoma (OSCC). This devastating disease, characterized by high morbidity and mortality rates, necessitates innovative approaches for early detection and effective treatment. Our study builds upon a growing body of evidence suggesting that saliva, a readily accessible biofluid, harbors a wealth of information reflecting the health and disease status of the oral cavity. We identified five salivary proteins – S100A7, MUC5B, IL-6, Statherin, and Histatin 1 – that exhibit significant alterations in their expression levels in OSCC patients compared to healthy individuals. These proteins, each with unique functions and roles in oral biology, collectively contribute to a complex interplay of events that influence the development and progression of OSCC. S100A7, a member of the S100 family of calcium-binding proteins, has emerged as a key player in various cancers, including OSCC. This protein, typically involved in regulating cellular processes such as cell growth, differentiation, and

inflammation, exhibits aberrant expression in OSCC, contributing to tumor development and progression. Overexpression of S100A7 in OSCC tissues and saliva has been linked to several hallmarks of cancer, including increased cell proliferation, migration, invasion, and angiogenesis. These processes, essential for tumor growth and spread, are fueled by S100A7's ability to activate various signaling pathways and modulate the tumor microenvironment. Furthermore, S100A7 plays a significant role in inflammation, a critical component of the tumor microenvironment. Chronic inflammation can create a favorable milieu for tumor development by promoting cell proliferation, angiogenesis, and immune suppression. S100A7's pro-inflammatory properties may contribute to this vicious cycle, further exacerbating OSCC progression. Our findings, demonstrating a significant upregulation of S100A7 in the saliva of OSCC patients, align with previous reports and reinforce its potential as a valuable biomarker for OSCC detection and monitoring. MUC5B, a major gel-forming mucin, serves as a critical component of the oral mucosal barrier. This protective layer, composed of mucins and other molecules, shields the underlying tissues from microbial invasion, environmental insults, and mechanical stress. However, in OSCC, the delicate balance of the oral microenvironment is disrupted, and MUC5B expression is often altered. While the exact role of MUC5B in OSCC remains to be fully elucidated, studies suggest that it may contribute to tumor growth and metastasis by modulating cell adhesion, migration, and immune evasion. One possible mechanism involves MUC5B's ability to create a physical barrier that shields tumor cells from immune surveillance. This barrier may prevent immune cells from recognizing and eliminating tumor cells, allowing them to proliferate and spread unchecked. Furthermore, MUC5B may also influence cell adhesion and migration, processes crucial for tumor invasion and metastasis. By altering the adhesive properties of tumor cells, MUC5B may facilitate their detachment from the primary tumor and migration to distant sites. Our finding of increased MUC5B levels in OSCC patients aligns with this notion and suggests that it may contribute to creating a favorable microenvironment for tumor progression. IL-6, a pleiotropic cytokine, plays a central role in inflammation and immune response. However, in the context of cancer, IL-6 can act as a double-edged sword, exhibiting both pro- and anti-tumorigenic properties. In OSCC, IL-6 has been shown to promote tumor growth, angiogenesis, and metastasis by activating various signaling pathways, including JAK-STAT, MAPK, and PI3K-Akt. These pathways, essential for regulating cell proliferation, survival, and migration, are often dysregulated in cancer, leading to uncontrolled cell growth and spread. Furthermore, IL-6 can suppress anti-tumor immune responses, facilitating tumor immune escape. By inhibiting the activity of cytotoxic T cells and natural killer cells, IL-6 can shield tumor cells from immune destruction, allowing them to thrive and evade the body's defenses. The significant upregulation of IL-6 in our OSCC cohort underscores its role in driving OSCC pathogenesis and its potential as a therapeutic target. Blocking IL-6 signaling may disrupt the inflammatory tumor microenvironment and restore anti-tumor immunity, potentially leading to improved treatment outcomes. Statherin and Histatin 1, two small salivary proteins, play crucial roles in maintaining oral health and protecting against microbial invasion. However, in OSCC, their levels are often reduced, potentially compromising oral defenses and contributing to a pro-tumorigenic environment. Statherin, primarily known for its role in inhibiting calcium phosphate precipitation and promoting tooth enamel remineralization, may also possess anti-tumor properties. Its ability to bind to certain cell surface receptors and modulate intracellular signaling pathways suggests that it may influence cell proliferation, differentiation, and apoptosis. Reduced Statherin levels, as observed in our study, may disrupt the delicate balance of the oral microenvironment, potentially contributing to OSCC development. Further research is needed to fully understand the mechanisms underlying Statherin's protective effects and its potential role in OSCC prevention. Histatin 1, an antimicrobial peptide abundant in saliva, plays a crucial role in innate immunity and wound healing. Its broad-spectrum activity against bacteria and fungi

helps maintain oral microbial homeostasis and protect against infection. However, in OSCC, Histatin 1 levels are often downregulated, potentially compromising oral defenses and creating a more permissive environment for tumor growth. Moreover, Histatin 1 has been shown to exhibit direct anti-tumor activity by inducing apoptosis and inhibiting cell proliferation. Therefore, its decreased levels in OSCC patients may represent a loss of a protective mechanism that normally helps to control cell growth and prevent tumor development.[11-13]

In our quest to understand the intricate molecular mechanisms underlying OSCC, we employed bioinformatic analysis to delve deeper into the functional roles and interactions of the differentially expressed salivary proteins identified in our study. This powerful in silico approach allows us to decipher the complex interplay of biological processes and pathways involved in OSCC development and progression. Gene Ontology (GO) enrichment analysis provides a systematic way to annotate and analyze the functions of genes and proteins. By identifying overrepresented GO terms among a set of genes or proteins, we can gain insights into the biological processes, molecular functions, and cellular components associated with those molecules. In our study, GO enrichment analysis revealed a fascinating landscape of biological processes, molecular functions, and cellular components associated with the differentially expressed salivary proteins. The enrichment of GO terms related to inflammation, immune response, cell proliferation, wound healing, cell adhesion, extracellular matrix organization, and angiogenesis underscores the complex interplay of these processes in OSCC development. These processes are not isolated events but rather interconnected components of a dynamic network that drives tumor progression. Chronic inflammation is a well-established hallmark of cancer, creating a microenvironment that favors tumor growth, angiogenesis, and immune suppression. The differentially expressed proteins involved in inflammation may contribute to this pro-tumorigenic environment. The immune system plays a dual role in cancer, both protecting against and promoting tumor development. The differentially expressed proteins involved in immune response may influence the delicate balance between anti-tumor immunity and immune evasion. Uncontrolled cell proliferation is a defining characteristic of cancer. The differentially expressed proteins involved in cell proliferation may contribute to the uncontrolled growth of OSCC cells. Wound healing is a complex process involving cell migration, proliferation, and extracellular matrix remodeling. Dysregulation of this process can contribute to tumor invasion and metastasis. Cell adhesion is crucial for maintaining tissue integrity and regulating cell signaling. Alterations in cell adhesion can contribute to tumor cell detachment and metastasis. The extracellular matrix provides structural support and influences cell behavior. Remodeling of the extracellular matrix can facilitate tumor invasion and metastasis. Angiogenesis, the formation of new blood vessels, is essential for tumor growth and metastasis. The differentially expressed proteins involved in angiogenesis may contribute to the vascularization of OSCC tumors. The GO enrichment analysis also highlighted overrepresented molecular functions, including cytokine activity, chemokine activity, growth factor activity, peptidase activity, and receptor binding. These functions are crucial for cell signaling, communication, and regulation, and their alteration can disrupt normal cellular processes, potentially leading to cancer. Cytokines are signaling molecules that regulate various cellular processes, including inflammation, immunity, and cell growth. Dysregulation of cytokine activity can contribute to tumor development. Chemokines are signaling molecules that attract immune cells to sites of inflammation or injury. Alterations in chemokine activity can influence the immune response to tumors. Growth factors stimulate cell proliferation, differentiation, and survival. Aberrant growth factor signaling can contribute to uncontrolled cell growth and cancer development. Peptidases are enzymes that break down proteins. Dysregulation of peptidase activity can influence cell signaling, extracellular matrix remodeling, and tumor invasion. Receptor binding is essential for cell signaling and communication. Alterations in receptor

binding can disrupt normal cellular processes and contribute to cancer development. The GO enrichment analysis identified enriched cellular components, including the extracellular region, plasma membrane, cytoplasm, and nucleus. This indicates that the differentially expressed proteins are localized in various parts of the cell and may influence different cellular processes. The extracellular region is the space outside of cells. Proteins in this region can influence cell-cell communication, cell adhesion, and the tumor microenvironment. The plasma membrane is the outer boundary of cells. Proteins in this membrane can regulate cell signaling, transport, and adhesion. The cytoplasm is the interior of cells, excluding the nucleus. Proteins in the cytoplasm can participate in various cellular processes, including metabolism, protein synthesis, and signal transduction. The nucleus contains the cell's genetic material. Proteins in the nucleus can regulate gene expression, DNA replication, and cell division. Pathway analysis complements GO enrichment analysis by providing a more focused view of the molecular interactions and signaling cascades involved in a particular biological process or disease. By identifying enriched pathways among a set of genes or proteins, we can gain insights into the specific molecular mechanisms driving a particular phenotype. In our study, pathway analysis identified several significantly enriched pathways, including the JAK-STAT signaling pathway, PI3K-Akt signaling pathway, MAPK signaling pathway, TNF signaling pathway, and NF-kappa B signaling pathway. These pathways are known to be involved in cell growth, survival, proliferation, inflammation, and immune response, all of which are critical in cancer development. The JAK-STAT pathway is a major signaling cascade involved in cytokine and growth factor signaling. It plays a crucial role in regulating cell proliferation, differentiation, and immune response. Dysregulation of the JAK-STAT pathway can contribute to cancer development. The PI3K-Akt pathway is another major signaling cascade involved in cell growth, survival, and proliferation. It is frequently activated in cancer and contributes to tumor development and progression. The MAPK pathway is a complex network of signaling cascades involved in cell proliferation, differentiation, and stress response. Dysregulation of the MAPK pathway can contribute to cancer development. The TNF signaling pathway is involved in inflammation, apoptosis, and immune response. Dysregulation of the TNF signaling pathway can contribute to chronic inflammation and cancer development. The NF-kappa B signaling pathway is a key regulator of inflammation, immunity, and cell survival. It is often activated in cancer and contributes to tumor development and progression. The identification of these key pathways further emphasizes the involvement of these signaling cascades in driving OSCC progression. Targeting these pathways may represent a promising therapeutic strategy for OSCC. Protein-protein interaction network analysis provides a visual representation of the complex interactions between proteins. By constructing a network map, we can identify hub proteins, which have a high degree of connectivity with other proteins, and clusters of interacting proteins, which may represent functional modules involved in specific biological processes. In our study, protein-protein interaction network analysis revealed hub proteins, such as IL-6, MMP9, and S100A7, which exhibit a high degree of connectivity with other proteins. These hub proteins may play central roles in OSCC pathogenesis by coordinating multiple pathways and processes. The clustering of proteins into functional modules, such as inflammation, cell growth, and immune response, suggests coordinated actions of these proteins in driving specific aspects of OSCC development. These functional modules may represent potential targets for therapeutic intervention.[14-17]

The bioinformatic analysis provides valuable insights into the molecular mechanisms underlying OSCC, highlighting the complex interplay of biological processes and pathways involved in tumor development and progression. This knowledge can contribute to the development of more effective diagnostic and therapeutic strategies for OSCC. The identification of differentially expressed salivary proteins and their associated pathways opens up new avenues for OSCC diagnosis and management. These

proteins may serve as potential biomarkers for early detection, disease monitoring, and personalized treatment decisions. Furthermore, the identified pathways and hub proteins may represent potential therapeutic targets for OSCC. Targeting these pathways or proteins may disrupt the molecular mechanisms driving OSCC progression and lead to improved treatment outcomes.[18-20]

5. Conclusion

This study identified a panel of five salivary proteins – S100A7, MUC5B, IL-6, Statherin, and Histatin 1 – with significantly altered expression in Indonesian OSCC patients compared to healthy controls. These proteins, implicated in inflammation, immunity, and cellular regulation, hold promise as potential biomarkers for OSCC detection and disease monitoring. Our findings underscore the complex interplay of these proteins in OSCC pathogenesis, as evidenced by the bioinformatic analysis revealing their involvement in key biological processes and pathways. Further validation in larger cohorts is needed to confirm their clinical utility and establish their potential for early detection, risk stratification, and personalized treatment strategies. The development of non-invasive diagnostic tools based on these biomarkers could revolutionize OSCC management, particularly in low-resource settings like Indonesia where late-stage diagnosis remains a significant challenge.

6. References

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