ORIGINAL ARTICLE

Exploratory Analyses of STIM1, NF-κB, and ORAI1 Salivary Protein Biomarkers in Nasopharyngeal Carcinoma: Insights from Proteomic and Bioinformatics Approaches

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ABSTRACT

Introduction: Nasopharyngeal carcinoma (NPC) is a major health concern in Southeast Asia due to its aggressive nature and frequent late-stage diagnosis, which leads to poor prognosis and limited treatment options. Research has increasingly focused on identifying non-invasive biomarkers for improving early detection and diagnosis of NPC. This exploratory study aims to evaluate the expression of salivary proteins Stromal interaction molecule 1 (STIM1), Nuclear Factor Kappa B (NF-κB), and Calcium Release-Activated Calcium Channel Protein 1 (ORAI1) in NPC patients and healthy subjects. **Methodology:** The extracted proteins were analysed using western blotting to detect STIM1, NF-κB, and ORAI1 proteins. Additionally, bioinformatics tools, including Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) enrichment analysis, were employed to explore the roles of these proteins in various biological processes. **Results:** STIM1 and NF-κB levels in saliva from NPC patients were slightly higher than in healthy subjects, though not statistically significant, likely due to small sample size. In contrast, ORAI1 protein levels were significantly reduced in NPC saliva, potentially reflecting underlying pathological changes. Bioinformatics analyses further indicated that these proteins are involved in critical processes such as calcium signaling, membrane raft dynamics, cell migration, and immune regulation, highlighting their potential roles in NPC progression. **Conclusion:** This exploratory study identifies salivary STIM1, NF-κB, and ORAI1 as potential biomarkers for NPC. However, the small sample size limits the statistical power of the findings. Further studies with larger cohorts are needed to validate their diagnostic potential and investigate their roles in NPC pathogenesis and therapeutic development.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a highly aggressive cancer that originates in the nasopharynx, a region deep within the nasal cavity near the Eustachian tube (1). The initial symptoms of NPC are often subtle and can be mistaken for common colds or rhinitis, complicating early detection and leading to late-stage diagnoses (2). In 2022, Asia had the highest incidence rates of NPC, with the hotspots region in southern China, Indonesia, and Malaysia (3, 4).

Current diagnostic methods for NPC include CT (Computed Tomography), MRI (Magnetic Resonance Imaging), PET-CT (Positron Emission Tomography-

Computed Tomography), endoscopy, and biopsy, which are effective but sometimes invasive and challenging for early detection (5, 6). In some clinical settings, Epstein–Barr virus (EBV)-based diagnostics provide the advantage of non-invasive early detection. However, EBV serology is limited by cross-reactivity leading to false positives, variable sensitivity, and an inability to differentiate between past and current infections, restricting its application in certain scenarios (7, 8).

EBV-based diagnostics are limited to EBV-associated NPC, while in Southeast Asia, dietary and lifestyle factors also significantly contribute to NPC development (9, 10). Therefore, there is a need to identify broader, non-invasive biomarkers for NPC to enhance diagnostic strategies for both EBV-associated and non-EBV-associated cases. This study explores other potential non-invasive biomarkers by analyzing saliva samples from NPC patients and healthy subjects.

Saliva has become a valuable tool for non-invasive diagnosis and monitoring due to its content of biomolecules such as Deoxyribonucleic acid (DNA), Ribonucleic acid (RNA), and protein, which can serve as cancer biomarkers (11). Saliva testing offers several advantages over traditional methods like blood or tissue biopsies, including a reduced risk of infection and less patient discomfort (12). Additionally, saliva testing holds promise for diagnosing NPC, monitoring disease progression, and identifying therapeutic targets.

This study focuses on the roles of Stromal interaction molecule 1 (STIM1), Nuclear Factor Kappa B (NF-κB), and Calcium Release-Activated Calcium Channel Protein 1 (ORAI1) in NPC, given their involvement in regulating cellular calcium signaling and inflammatory responses. STIM1 and ORAI1 manage intracellular calcium levels, influencing cell proliferation and survival (13, 14), while NF-κB is a key transcription factor linked to inflammation, immune response, and cancer progression (15, 16). Investigating these proteins will help elucidate NPC's molecular mechanisms and identify potential biomarkers (17). The study extracted proteins from saliva samples to assess the expression of STIM1, ORAI1, and NF-κB in NPC. Bioinformatics platforms were used to understand the roles of these proteins and gain insights into the signaling networks involved in NPC.

By exploring these salivary biomarkers, the study aims to address the limitations of current diagnostic methods and improve detection and management of NPC across diverse populations. This approach could provide a more comprehensive understanding of NPC and enhance diagnostic strategies for both EBV-associated and non-EBV-associated cases, ultimately improving patient outcomes.

MATERIALS AND METHODS

Salivary Protein Analysis by Western Blot

This exploratory study included six NPC patients and six healthy subjects from Pusat Perubatan USM Bertam (PPUSMB) in Pulau Pinang, Malaysia, following protocols approved by the Human Research Ethics Committee USM (HREC) under reference number 22040244. Consistent with previous small-sample studies (Talungchit et al., 2018; Bastнas et al., 2024), the use of optimized methods and precise analysis led to the identification of salivary protein biomarkers with potential diagnostic and prognostic value. Healthy volunteers were required to be free from chronic diseases such as cancer, ischemic heart disease, or chronic obstructive pulmonary disease, and must be aged 18 or older with a normal BMI. Exclusion criteria for healthy volunteers included pregnancy, breastfeeding, and chronic infections like hepatitis B, hepatitis C, or the human immunodeficiency virus (HIV). For NPC subjects, the inclusion criteria were newly diagnosed patients, while exclusion criteria included a history of other malignancies (past or present) and prior treatments such as surgery, chemotherapy, or radiotherapy. The collected saliva samples were then centrifuged at 3000 revolutions per minute (RPM), 4°C for 15 minutes to spin down the cells. The saliva supernatant was separated from the pellet and stored at −80°C, following the protocol described by Lirong et al. (2019) (18).

Protein extraction was performed using the acetone / trichloroacetic acid (TCA) / dithiothreitol (DTT) method as described by Emsies (2019)(13). Specifically, 200 µL of saliva was mixed with 5 mM DTT, incubated for 30 minutes, and then treated with 10% TCA. After cooling at 4°C, the samples were centrifuged, washed with acetone, dried, and resuspended in phosphate-buffered saline (PBS) for analysis. Protein concentrations of 10 µg per sample, as referenced by Liang, Chen, et al. (2024) (19), were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, following the method outlined by SMN Mydin, R. B., et al. (2017)(20). Protein bands were visualized using chemiluminescence with a VersaDoc imaging system (Bio-Rad, USA), and densitometry analysis was conducted with ImageLab software. Primary antibodies used were STIM1 (1:1000, Cell Signaling Technology, catalog no. 5668S), ORAI1 (1:1000, Bio-Rad, catalog no. AHP1494), NF-kB (1:1000, Cell Signaling Technology, catalog no. 8242S) and Beta-Actin (β-Actin) as loading control (1:1000, Cell Signaling Technology, catalog no. 4970S). The secondary antibody was anti-rabbit Immunoglobulin G (IgG) H+L (horseradish peroxidase conjugate) (1:5000, Cell Signaling Technology, catalog no. 7074). Data analysis employed Cohen's formula, with effect sizes categorized as small ($0.2 \leq d < 0.5$),

medium (0.5 ≤ d < 0.8), or large (d ≥ 0.8), according to Gallo-Oller, Ordonez, et al. (2018)(21).

In silico and Bioinformatic Analysis

The study utilized the Cancer Genome Atlas (TCGA)- head and neck squamous cell carcinoma (HNSC) Microarray dataset, which includes 44 normal and 519 tumor samples, all obtained from the TCGA database (https://cancergenome.nih.gov/). The dataset was selected based on inclusion criteria, which encompassed raw data, protein expression profiling, and human case and control studies. Using R programming, the data were pre-processed, standardized, and analysed for differential protein expression, with criteria set as an adjusted P-value < 0.05 and |log2 fold change| > 2 to identify biomarker candidates. Gene Set Enrichment Analysis (GSEA) and Gene Ontology (GO) were employed to predict the biological roles of target proteins, with conditions of a normalized enrichment score (NES) > 1, P-value < 0.05, and false discovery rate (FDR) < 0.25. Further, GO enrichment analysis via Metascape provided insights into the biological functions of differentially expressed proteins. Pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG) database and R language explored the significant biological pathways related to NPC, with statistical significance defined by the false discovery rate (FDR) < 0.05. These analyses provide valuable insights into potential biomarkers and their molecular mechanisms.

RESULTS

The Western blot analysis of saliva samples showed that protein expression levels of STIM1 and NF-κB were increased pattern in the NPC compared to healthy subjects, while ORAI1 protein expression was decreased pattern (Figure 1A and Figure 1B). Specifically, STIM1

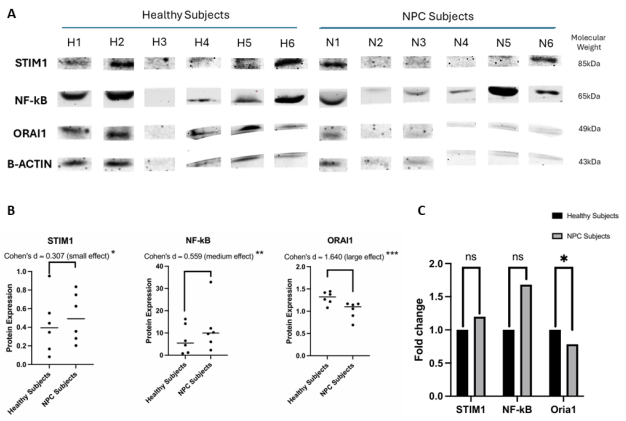

Figure 1: The comparison of STIM1, NF-κB, and ORAI1 protein expression NPC and healthy individuals. (A) Western blot analysis of targeted proteins. (B) Cohen's d analysis for the targeted proteins, which is used to measure effect sizes. (C) Histogram showed the fold-change of targeted proteins. The range of effect sizes was defined as follows: when 0.2 ≤ d < 0.5, it indicates a small effect (*); when 0.5 ≤ d < 0.8, it indicates a medium effect (**); and when 17 d ≥ 0.8, it indicates a large effect (***).

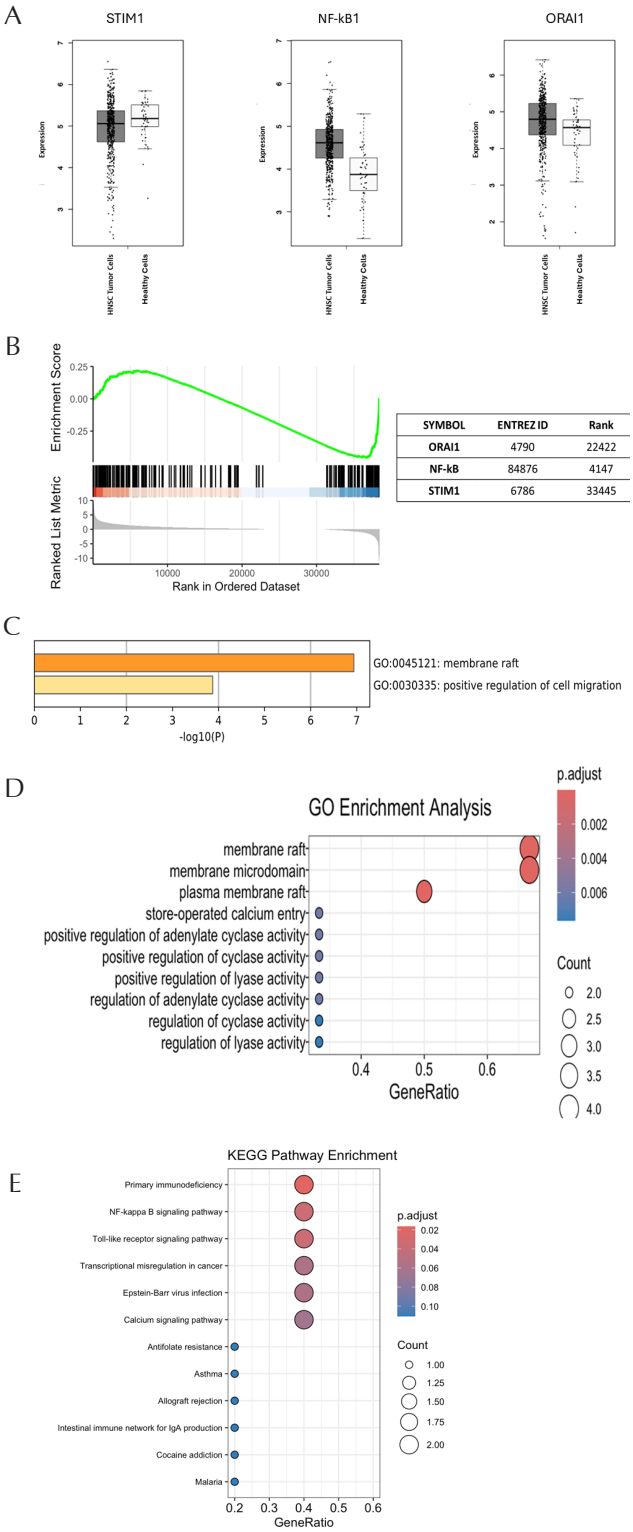

Figure 2: Bioinformatics Analysis. (A) The expression levels of STIM1, NF-κB, and ORAI1 were analyzed in HNSC using the GEPIA tool. (B) GSEA was performed to evaluate the functional enrichment and pathway activity of NF-κB and ORAI1 in NPC. (C) GO term enrichment analysis for genes related to NF-κB and ORAI1 was conducted using the Metascape platform. (D) GO enrichment results were visualized as bubble plots using R language tools. (E) GG Pathway enrichment analysis identified key signaling pathways.

expression was elevated by 1.2-fold and NF-κB by 1.6-fold in NPC relative to healthy groups (Figure 1C). In contrast, ORAI1 expression was reduced to 0.79-fold in the NPC compared to healthy groups, with this decrease

being statistically significant ($p < 0.05$). Furthermore, the sample effect size analysis using Cohen's d revealed that the change in STIM1 expression corresponded to a small effect ($d \approx -0.307$), NF-κB showed a medium effect ($d \approx 0.559$), and ORAI1 exhibited a large effect ($d \approx 1.64$) (Figure 1B).

Further analysis using public datasets showed that the expression pattern of STIM1, NF-κB, and ORAI1 were tabulated higher range in 519 HNSC tumours compared to 44 healthy tissues (Figure 2A), indicating a strong association with tumor growth and progression. However, median expression analysis revealed that STIM1 was actually lower in HNSC tumors (Figure 2A), whereas NF-κB and ORAI1 had higher median expression levels in HNSC tumors compared to healthy tissues (Figures 2B and 2C).

GSEA analysis predicted the roles of STIM1, NF-κB, and ORAI1 in NPC, with higher enrichment scores indicating greater gene activity in target pathways (Figure 2). Specifically, ORAI1 ranked 22,422, NF-κB ranked 4,147, and STIM1 ranked 33,445 (Figure 2B), where lower ranks indicate higher relative importance or expression in the analysis. The higher ranking of NF-κB suggests its critical role in NPC, with the analysis indicating significant enrichment in NPC-related pathways, particularly those involving apoptosis, immune responses, and cell proliferation.

Gene Ontology (GO) enrichment analysis (Figure 2D) identified biological processes (BP), cellular components (CC), and molecular functions (MF) significantly enriched in NPC. The Metascape analysis highlighted two significantly enriched GO terms: GO:0045121 (membrane rafts) and GO:0030335 (positive regulation of cell migration), with higher -log10(p-values) indicating greater significance. The dot plot further displayed several significantly enriched terms, including membrane rafts, membrane microdomains, plasma membrane rafts, and store-operated calcium entry.

DISCUSSION

Previous studies have identified STIM1 as a potential biomarker for NPC, demonstrating notably high expression levels in formalin-fixed paraffin-embedded (FFPE) NPC tissues (23). In the current study, protein expression levels of STIM1 and NF-κB in saliva samples from NPC patients were slightly higher than those in healthy groups. Elevated STIM1 expression has been linked to advanced tumor characteristics, such as increased regional lymph node metastasis (22–24). Similarly, NF-κB is associated with aggressive tumor features, including deep tumor invasion, lymph node metastasis, higher TNM stages, and poorer disease-free survival rates (25, 26). However, the differences in STIM1 and NF-κB expression observed in this study were not statistically significant, likely due to the relatively small sample size, which limited the ability to detect subtle changes.

In contrast, ORAI1 protein levels were significantly lower in saliva samples from NPC patients compared to healthy groups. ORAI1 has been implicated in poor prognosis and aggressive behavior in various cancers (27, 28), suggesting its downregulation in saliva may reflect important pathological changes in NPC.

Further comparative analysis using public datasets revealed that NF-κB and ORAI1 are significantly elevated in HNSC tumors (Figure 2A). Since NPC is a major subtype of HNSC, these findings provide valuable insights into the expression patterns of STIM1, NF-κB, and ORAI1 in larger cohorts, reinforcing their strong association with tumor growth and progression. GSEA demonstrated that NF-κB plays a central regulatory role in NPC-related signaling pathways (Figure 2B), underscoring its critical involvement in NPC pathogenesis.

Our detailed bioinformatics analysis identified significant enrichment of pathways related to membrane rafts, membrane microdomains, plasma membrane rafts, and migration regulation (Figures 2C and 2D). Membrane rafts serve as platforms for the aggregation of signalling molecules, enhancing the efficiency of signal transmission (29). The enrichment of migration-related pathways suggests that these proteins facilitate signal transduction processes involved in cell motility and invasion. STIM1, ORAI1, and NF-κB are implicated in membrane raft dynamics and the regulation of cell migration through their roles in store-operated calcium entry (SOCE) and downstream signaling pathways. Specifically, STIM1 clusters within lipid rafts to facilitate SOCE via ORAI1, which influences cytoskeletal dynamics and cellular migration (30, 31). NF-κB, modulated by calcium signaling, regulates genes involved in cell migration (32, 33). These interactions highlight the complex interplay between calcium signaling, membrane rafts, and transcription factors in controlling cellular migration processes, emphasizing their importance in NPC pathophysiology.

Additionally, KEGG pathway analysis highlights NF-κB's potential role in immune regulation within NPC, particularly through pathways such as Toll-like receptor signaling, Epstein-Barr virus (EBV) infection, and calcium signaling (Figure 2E). Toll-like receptor signaling is crucial for innate immunity, recognizing pathogen-associated molecular patterns and activating downstream signaling cascades (34). In NPC, especially EBV-associated cases, these pathways may be activated by EBV infection, potentially impairing immune function. This impairment could lead to immunodeficiency characterized by defects in T-cell or natural killer (NK) cell function or disruptions in pathways essential for controlling EBV replication and preventing its oncogenic effects (35, 36). Calcium signaling may further modulate these immune

responses, as previously discussed.

In summary, this study underscores the important roles of calcium signaling components-particularly the STIM1-ORAI1 axis-and inflammatory mediators like NF-κB in the development and metastasis of NPC. The findings support the potential of STIM1 and NF-κB as biomarkers associated with NPC aggressiveness, while also identifying a significant decrease in ORAI1 levels in NPC saliva. Future research should prioritize larger sample sizes and investigate the molecular mechanisms by which these proteins contribute to NPC progression. These efforts are crucial to validate their clinical utility, especially for developing non-invasive diagnostic methods. Although the observed differential protein expression patterns are promising, saliva-based detection will require studies with larger and more diverse cohorts to achieve robust statistical validation.

CONCLUSION

The exploratory study offers promising insights into the use of salivary proteomic biomarkers for NPC detection. It was found that STIM1, NF-kB and ORAI1 proteins could serve as potential salivary biomarkers for NPC. Additional bioinformatics analyses revealed that these proteins are involved in key immune responses, including calcium signalling. Therefore, future research is needed, particularly with larger sample sizes, to enhance statistical significance and reliability. Exploring the therapeutic potential of these biomarkers could provide valuable insights for developing precise diagnostic and therapeutic approaches for NPC. These advancements could improve diagnostic strategies and ultimately lead to better patient outcomes.

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Ethical clearance

This study protocol has received human ethical approval from the Human Research Ethics Committee USM (HREC). Universiti Sains Malaysia, approval no: 22040244.

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