CHARACTERIZATION OF CTDNA MUTATIONS AND VALIDATION OF CIRCULATING MICRORNA AS PROGNOSTIC BIOMARKERS IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

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Introduction:

Head and Neck Squamous Cell Carcinoma (HNSCC) is the seventh most common cancer worldwide and remains a major public health concern, particularly in developing countries where tobacco use, alcohol consumption, and HPV infection are prevalent risk factors. Despite advances in cancer genomics and targeted therapies, survival rates remain limited due to delayed diagnosis, tumor heterogeneity, and frequent recurrence. Conventional tissue biopsy, though considered the diagnostic gold standard, is invasive and provides only a static snapshot of tumor biology.

Liquid biopsy has emerged as a transformative alternative that enables minimally invasive monitoring of tumor-derived molecular alterations through body fluids such as blood and saliva. Circulating tumor DNA (ctDNA) reflects tumor-specific mutations released during apoptosis and necrosis, offering insights into tumor burden and disease progression. Similarly, circulating microRNAs (miRNAs) function as post-transcriptional regulators and play critical roles in cancer development and metastasis.

Among them, the miR-196 family has gained attention due to its oncogenic role in regulating tumor suppressor pathways and promoting epithelial–mesenchymal transition. Their stability in circulation makes them promising non-invasive biomarkers. Integrating ctDNA mutational profiling with circulating miRNA analysis provides a comprehensive molecular understanding of HNSCC progression and prognosis, supporting precision oncology approaches. This study focuses on identifying and validating these biomarkers through systematic literature analysis and bioinformatic investigation

Objectives:

1. To perform comprehensive bioinformatics analyses on publicly available head and neck cancer (HNSCC) datasets to identify ctDNA alterations associated with disease progression.

2. To detect and quantify selected miRNAs (miR-196a and miR-196b) from plasma samples of HNSCC patients, and evaluate their expression patterns, clinical relevance, and potential as prognostic biomarkers.

Methodology:

Review of Literature: A systematic PRISMA-based literature review identified prognostic ctDNA and miRNA biomarkers in HNSCC from PubMed and Google Scholar. Of 9,385 screened articles, 77 ctDNA and 14 miRNA studies were included. Excluding HPV-focused and non-human research, consistent genetic alterations were extracted to establish the foundation for subsequent bioinformatic analyses.

Circulating Tumor DNA (ctDNA) Analysis: Mutational Analysis and Gene Expression Profiling: Somatic mutations in ctDNA genes were analyzed across 523 HNSCC samples using cBioPortal (TCGA), targeting substitutions, indels, and splice-site variants. mRNA expression was compared between 520 tumors and 40 controls using log2-transformed RSEM/FPKM values. Results were visualized via cBioPortal profiles and Clustergrammer heatmaps to identify differential expression patterns.

Protein–Protein Interaction (PPI) Network Construction: To investigate functional connectivity, PPI networks were constructed using Cytoscape. Interaction data were retrieved from STRING, HIPPIE, IntAct, BioGRID, and GeneMANIA. Self-loops and duplicate interactions were removed to ensure high-confidence associations. Separate networks were generated for tumor suppressors, oncogenes, and dual-role genes to identify hub proteins central to HNSCC progression.

Gene Ontology (GO) and Pathway Enrichment: Functional enrichment of 53 selected ctDNA genes was performed to identify biological significance with Metascape Analysis and KEGG Pathway Analysis

Circulating MicroRNA Analysis: miRNet was utilized for *in silico* analysis to identify miRNAs interacting with HNSCC, highlighting the miR-196 family (hsa-miR-196a and has-miR-196b). Seed sequences for the 3p and 5p isoforms were analyzed for target recognition properties. High-confidence target genes were predicted using miRDB (MirTarget algorithm), with the top 20 ranked genes per miRNA selected for Gene Ontology (GO) enrichment via Metascape to determine biological significance.

Result and Conclusion:

Circulating Tumor DNA (ctDNA) Analysis: Mutational analysis of the 523-sample TCGA-HNSCC cohort revealed a heterogeneous mutational landscape, dominated by TP53 (75%), CDKN2A (56%), and PIK3CA (47%). mRNA expression profiling confirmed the upregulation of oncogenes (SOX2, EGFR) and silencing of tumor suppressors (PTEN).

PPI networks identified TP53, MYC, and EGFR as primary hubs regulating cell cycle and DNA repair. Metascape and KEGG analyses showed an 18-fold enrichment in core oncogenic pathways, specifically the PI3K/EGFR axis, WNT, and Notch signaling. Over 90%

of the gene set mapped to critical biological processes like stimulus response and protein binding, confirming these ctDNA markers as robust indicators of HNSCC progression.

Circulating MicroRNA Analysis: miRNet analysis confirmed a strong association between the miR-196 family and HNSCC. Both miR-196a and miR-196b isoforms (3p and 5p) share high-similarity seed sequences but regulate distinct high-confidence targets identified via miRDB (scores: 80–100), including oncogenic factors like HMGA2 and regulators like ZMYND11.

GO enrichment via Metascape revealed that 5p isoforms primarily regulate the mitotic cell cycle and intracellular signalling, while 3p isoforms target stress responses, VEGFA-VEGFR2 angiogenesis, and TORC1 signaling. Collectively, these microRNAs act as master regulators of proliferation, DNA metabolism, and epithelial-mesenchymal transition, highlighting their potential as potent prognostic biomarkers in HNSCC.

In conclusion, this study identified ctDNA and miR-196a/b as potent prognostic biomarkers for HNSCC. Integrated bioinformatic analyses—including PPI networks, mutational profiling, and pathway enrichment—confirmed their critical roles in oncogenic signalling and molecular dysregulation. These findings validate the clinical relevance of these markers for non-invasive risk stratification and personalised treatment strategies.

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Future scope:

1. Experimental validation of identified circulating microRNAs (miR-196a and miR-196b) will be performed using quantitative real-time PCR (qPCR) in plasma samples from HNSCC patients.
2. Total RNA will be isolated from patient-derived plasma, followed by cDNA synthesis using miRNA-specific primers.
3. Relative expression levels of miRNAs will be quantified using SYBR Green–based qPCR assays.
4. Comparative expression analysis between cancer patients and healthy controls will be conducted to evaluate diagnostic and prognostic significance.

The study will bridge in-silico predictions with experimental validation to support liquid biopsy–based biomarker development. Ultimately, the findings may enable integration of ctDNA and miRNA biomarkers into multiplex liquid biopsy panels for improved early prognosis detection and recurrence monitoring.