



## Differential Gene Expression Analysis of TCGA mRNA Sequencing Data from Male Patients with and without Lymph Node Metastasis in Tongue Cancer

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### ABSTRACT

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Gene expression plays a crucial role in progression, invasion, and metastasis in many groups of oral squamous cell carcinoma. Based on extensive patient data, no research has been done so far on the differences in gene expression between groups of male patients with tongue squamous cell carcinoma who drink alcohol and those who do not, about lymph node metastasis. Our study analyzed differential gene expression in male patients with and without lymph node metastasis in tongue cancer, utilizing mRNA sequencing data from The Cancer Genome Atlas Program (TCGA). Analysis was performed using R and bioinformatics tools to process mRNA sequencing data, compare differential gene expression, and examine functional signaling pathways and survival analysis. After screening the database, 38 out of 74 cases with metastasis were selected for the analysis. The computational analysis identified a significant increase in cancer invasion transcription factors, such as GATA2, SP1 and MYC in the metastatic group, whereas CREB1 was more associated with the non-metastatic group. Functional analysis suggested new prognostic biomarkers for unfavorable outcomes (TRIML2 and EXOSC4) and favorable outcomes (HS3ST4 and SFTPA1). New suggested markers for oral and tongue cancer such as TRIML2, EXOSC4 are known for their role in non-oral cancer including lung and liver cancer. Especially, HNSCC survival analysis and protein atlas investigation confirmed the indirect biological roles of found markers. The study indicates that TRIML2 and EXOSC4 could serve as biological markers for predicting lymph node metastasis in tongue squamous cell carcinoma among male patients who consume alcohol. These findings suggest that gene expression profiles may play a role in the progression and metastasis of tongue cancer. Further research is needed to validate these results and to develop targeted therapies for patients with tongue cancer.

**Keywords:** *Metastasis; mRNA sequencing; oral squamous cell carcinoma; TCGA; tongue cancer.*

## INTRODUCTION

Tongue cancer is characterized by malignant growth of the mucosa or connective tissue of the tongue. It is the most common type of oral cavity cancer, accounting for 30%-50% of all oral cavity cancers (De Berardinis *et al.*, 2022). In the USA, it is projected that there will be 3,320 mortality cases from tongue cancer in 2024, along with 19,360 new cases. Tongue cancer has been associated with elderly men who had smoked and/or used alcohol the estimated frequency of the disease varies greatly with geographic region, with a small male predominance (SEER, 2024). Surgery or radiation therapy can be an effective single-modality treatment for early-stage tongue cancer (T1 or T2). Conversely, individuals with advanced cancer (T3 or T4) do not respond well to treatment with a single modality. Improved survival was observed after postoperative adjuvant chemoradiation, according to two separate clinical trials (Kirtane & Rodriguez, 2018).

The long-term prognosis for patients with advanced-stage tongue squamous cell carcinoma is generally poor, with a five-year survival rate of approximately 50% (Zwetyenga *et al.*, 2003). The tongue is an easily accessible organ, allowing for early detection and diagnosis. However, early-stage tongue cancer often lacks symptoms or presents with subtle signs, leading to underdiagnosis (Kozioł-Wójcik & Chloupek, 2023). As a result, patients are often admitted at later stages (Stage III, IV), accounting for up to 61.6% (Wagle *et al.*, 2014). Despite significant advancements in diagnosis and treatment in modern medicine, the majority of patients have a poor prognosis, leading to challenges and high costs in their management. Therefore, it is crucial to identify biological markers associated with the invasion and metastasis of cancer to accurately predict lymph node metastasis.

RNA sequencing (RNA-seq) helps identify the set of transcripts and their respective quantities at different stages of development or under specific physiological conditions. Understanding transcriptional regulation is essential for deciphering the function of the gene network and uncovering the molecular components of cells and tissues, as well as gaining insights into developmental processes and diseases (Wang *et al.*, 2009). TCGA is a project conducted by the National Institutes of Health (NIH) to catalog and discover major genetic changes driving cancer in a large cohort of over 30 human tumor types through large-scale genome sequencing and multidimensional integrated analysis. It offers cancer genome data that is available to the public, which can enhance cancer prevention by raising standards for treatment and diagnosis (Tomczak *et al.*, 2015). Within TCGA, oral cancer falls under the category of head and neck cancers. There are a total of 16,861 datasets and studies on 531 cases with 14 primary tumor sites for head and neck cancers on TCGA.

To the best of our knowledge, there have been no studies on the differential gene expression between the lymph node metastasis and non-metastatic groups in male patients with tongue squamous cell carcinoma who consume alcohol, based on large-scale patient data. To shed light on the connection between gene expression and lymph node metastases in male patients with alcohol-consuming tongue cancer and suggest new biomarkers for lymph node metastatic tongue squamous cell carcinoma, we conducted a study to assess the differences in gene expression between the metastatic and non-metastatic groups in male patients with tongue squamous cell carcinoma who consume alcohol, based on publicly available bulk mRNA sequencing data from TCGA.

## MATERIAL AND METHODS

### *Tongue Cancer mRNA Sequencing Data Collection from the TCGA Database*

The study was approved by the Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City (No 478/HDDD-DHYD, 2023). This cross-sectional descriptive study collected data from RNA-seq data obtained from TCGA in male patients with tongue cancer who consume alcohol, including both lymph node metastasis and non-metastasis cases. Patient with tongue cancer, the base of the tongue, other and unspecified parts of the tongue, squamous cell neoplasms and primary tumor. All samples that met the criteria were selected.

In the Cases section of the TCGA searching tool, we selected the tumor location (primary site: base of tongue, other and unspecified parts of tongue), the disease type (squamous cell neoplasms), and the sample type (primary tumor). Then, in the Clinical section, we chose Gender as male, AJCC clinical N as N0, N1, N2, N2b, N2c, N3, NX, and alcohol history as yes. Next, in the Genes section, we selected protein\_coding and chose "View Files in Repository". Finally, in the Files section, we selected RNA-Seq under Experimental Strategy.

The data repository yielded 74 cases that meet the criteria (including 36 cases with non-metastatic lymph node stage N0, NX: 3 cases, and 38 cases with metastatic lymph node stage (including N1: 13 cases, N2: 1 case, N2b: 16 cases, N2c: 8) among male patients with primary squamous cell carcinoma in the tongue, who have a history of alcohol consumption). We selected patient data for research based on additional criteria and filtered out irrelevant data. We then clicked on EXP (expression-genomic expression) and selected htseq count, which will provide 2 files, with the Primary Tumor file being the one to download. Furthermore, for each case, click on the case ID and download the clinical information of that case in TSV format. A metadata file was generated including basic sample information (case ID, age at diagnosis, gender, race, age, year of birth, year of death, cancer location, MNT staging, clinical stage, MNT classification, year of diagnosis, treatment regimen, smoking information, alcohol consumption). Raw\_count files included gene expression data (number of transcripts detected for each gene).

### *mRNA Sequencing Data Screening and Differential Expression Analysis*

mRNAseq data has been processed from raw data and transformed into processed data using the gencode.gene.info.v22 gene set. For this study, we only focused on protein-coding genes, removed duplicate genes, irrelevant columns, and genes marked as "NA". The selected classification for the study includes N0, NX as non-metastatic and N1, N2, N2a, N2b, N3 as metastatic stages. We used R package DESeq2 (version 1.40.0) (Love *et al.*, 2014) to remove genes with a total count <1 and is used in subsequent steps of the DESeq2 analysis. DESeq2 calculated the total number of highly expressed genes in the metastatic and non-metastatic groups and then passed differential expression analysis. It then generated plots and provided a list of genes with their log<sub>2</sub> fold change, baseMean (mean expression), and adjusted *p* value (<0.05).

### *Enrichment Functional Analysis and Survival Analysis*

We applied Metascape (version 3.520230601) (Zhou *et al.*, 2019) which utilizes clustering methods such as Gene Ontology (GO) and KEGG pathway analysis to analyze gene or protein datasets. Firstly, we created a list called "clinical-n" with two columns: one column represents genes in the non-metastatic group (log<sub>2</sub>foldchange < 0), and another column represents genes in the metastatic group (log<sub>2</sub>foldchange > 0). Metascape provides information about the molecular biological signals of the genes and transcription factors associated with those genes.

Additionally, the gene expression in bulk RNA-seq data was processed for GSEA (v.4.3.3) (Subramanian *et al.*, 2005; Huynh, 2023). For every gene set, 1000 permutations of GSEA were performed. We took advantage of the 188 C6 collection oncogenic signature gene set that is accessible in the Human MSigDB (v2023.2.Hs) of the Molecular Signatures Database.

From the top genes upregulated in 2 groups by differential expression analysis, we selected cancer-related genes that significantly patient outcomes by TCGA database. Survival analysis was performed based on gene expression levels of these genes using head–neck squamous carcinoma in TCGA database by GEPIA (v2). The method applied a log-rank test for the hypothesis evaluation. We included the Cox proportional hazard ratio and the 95% confidence interval in the survival plot (Tang *et al.*, 2017; Huynh *et al.*, 2022). These candidate genes were then investigated for survival probability and protein expression by immunohistochemistry staining in different cancer tissues from <https://www.proteinatlas.org> database (Uhlen *et al.*, 2017).

### ***Statistical analysis***

Different expression analysis was performed with adjusted  $p$  value  $<0.05$  using the *apecglm* method for effect size shrinkage. Metascape analysis,  $\log_{10}$   $p$  value was used to estimate the chance of the observed enrichment due to randomness (with default  $p$  value threshold  $10^{-2}$ ) (Timmons *et al.*, 2015). For GSEA analysis, the normalized enrichment score (NES) in Signal2Noise, which uses the standard deviation-scaled difference of means, was chosen based on an FDR-adjusted  $p$  value  $<0.05$ . Survival analysis was based on the expression status of one gene or a multi-gene signature and plotted by Kaplan-Meier curve with log-rank  $p$  value  $<0.05$ .

## RESULTS

### *Epidemiology and Clinical Status*

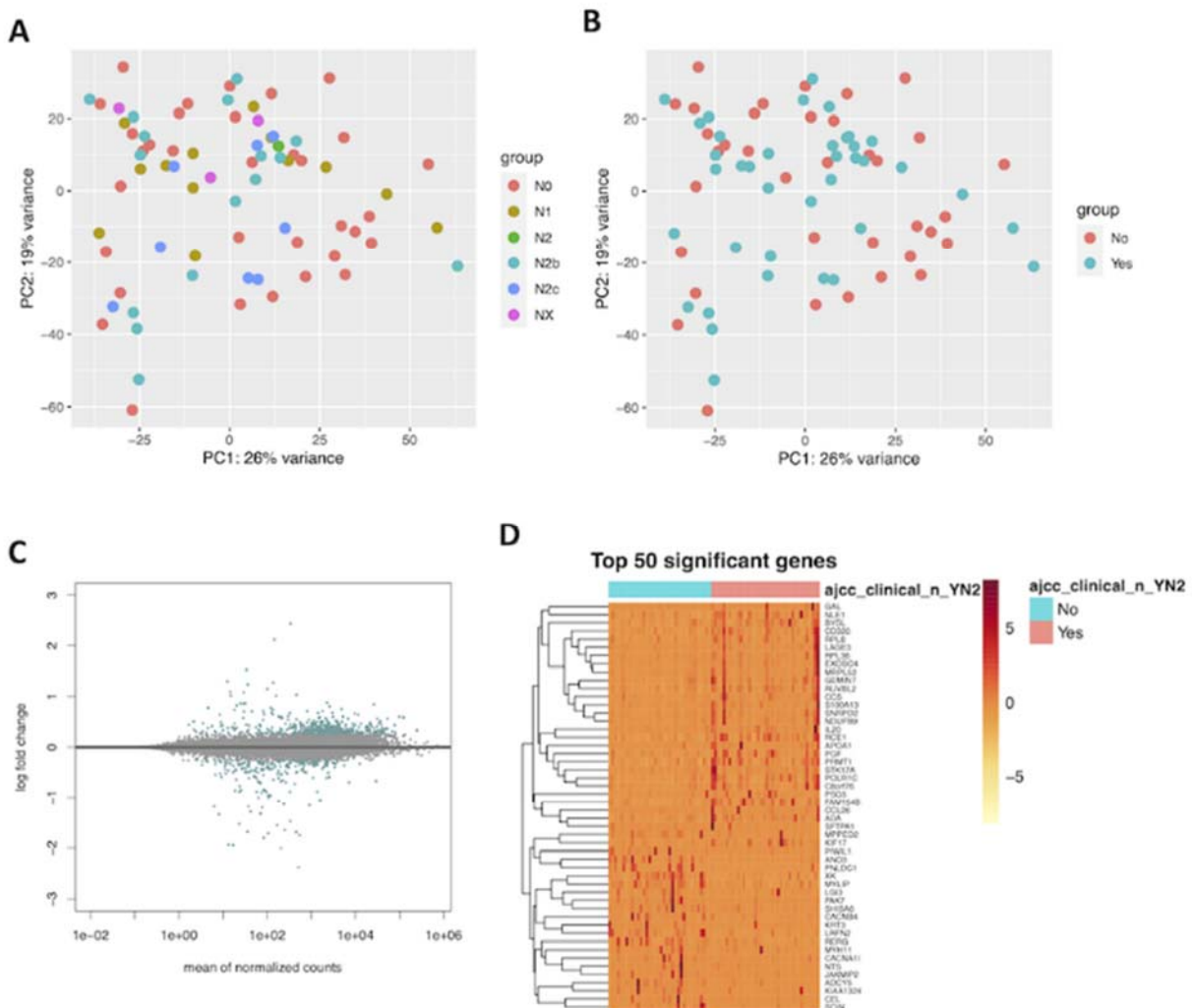
The study sample consisted of data from 74 male patients (Table 1). The oldest patient was 79 years old, and the youngest was 26. The study population was split into 2 age groups: under 40 and over 40. The majority of patients diagnosed with the disease were 40 years old or older, accounting for 93.2% of the cases. Most of the patients diagnosed with the disease were white people, comprising 87.8% of the sample. The primary location of tongue cancer was found to be 20.3% at the base of the tongue, compared to 79.7% in other and unspecified parts of the tongue. Regarding the metastatic staging according to AJCC, we observed that both N0 and N2b stages had relatively high proportions, accounting for 27% compared to the other groups (Table 1).

**Table 1** Summary of epidemiological information

Characteristic		Cases (n)	Percent (%)
Age at Diagnosis	>40	69	93.2
	<40	4	5.4
	Not reported	1	1.4
Race	White	65	87.8
	Asian	2	2.7
	American Indian or Alaska native	1	1.4
	Black or African American	4	5.4
	Not reported	2	2.7
Vital Status	Alive	47	63.5
	Dead	27	36.5
Primary Site	Base of tongue	15	20.3
	Other and unspecified parts of tongue	59	79.7
AJCC Clinical N	N0	33	44.6
	Nx	3	4.1
	N1	13	17.6
	N2	1	1.3
	N2a	0	0.0
	N2b	16	21.6
	N2c	8	10.8
	N3	0	0.0

### Differentially Expressed Genes in Metastatic and Non-Metastatic Patients

The results of Principal Component Analysis (PCA) of the sequenced samples indicated that the samples were grouped into different clusters depending on the differences in the two most important principal components, PC1 and PC2 (Fig. 1A, B). The analysis of data distances revealed that the samples formed distinct clusters based on the similarity of their data. From the two groups (non-metastatic and metastatic), we analyzed the differential gene expression between the two clusters. The MA plot and heatmap demonstrate a significant number of genes with differential expression between Group 1 and Group 2 (Fig. 1C, D). The list of genes with, baseMean, log<sub>2</sub> fold change and adjusted *p* value consisted of 384 genes, including 269 genes with log<sub>2</sub> fold change > 0 representing upregulated genes in metastatic group), and 115 genes with log<sub>2</sub> fold change < 0 representing upregulated genes in non-metastatic group.

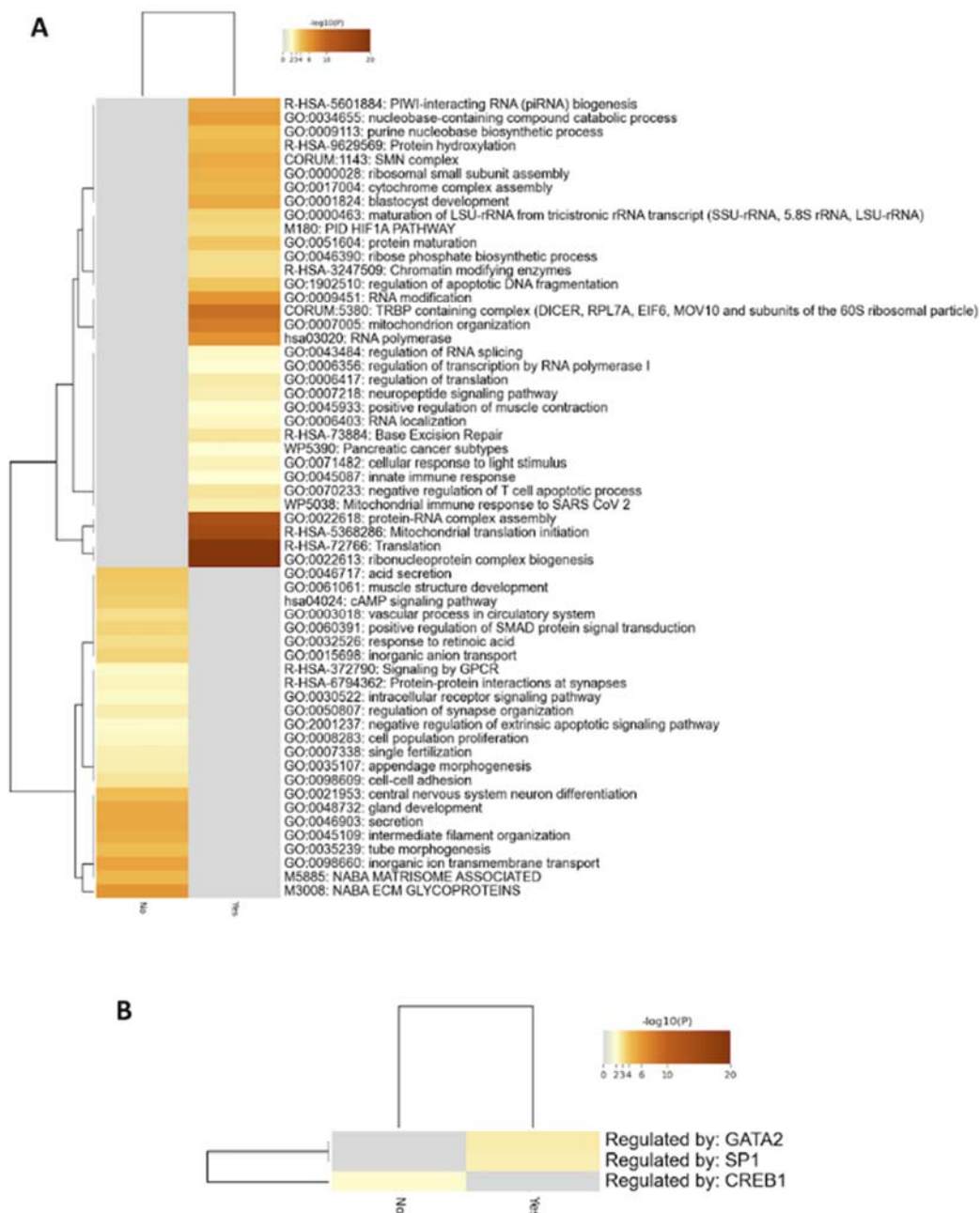


**Fig. 1** Distribution of samples according to principal component analysis (PCA): **A** classified by N; **B** classified by metastasis (No: non-metastasis, yes: metastasis). **C** MA plot shows the expressed genes with significant differences between group 1 without metastasis (below) and group 2 with lymph node metastasis (above). Gray dots: remaining genes, blue dots: genes with increased/decreased expression with statistical significance between group 2 vs cluster 1. **D** Heatmap graph showing the top 50 expressed genes with significant differences (lowest adjusted *p* value) between cluster 1 and cluster 2.



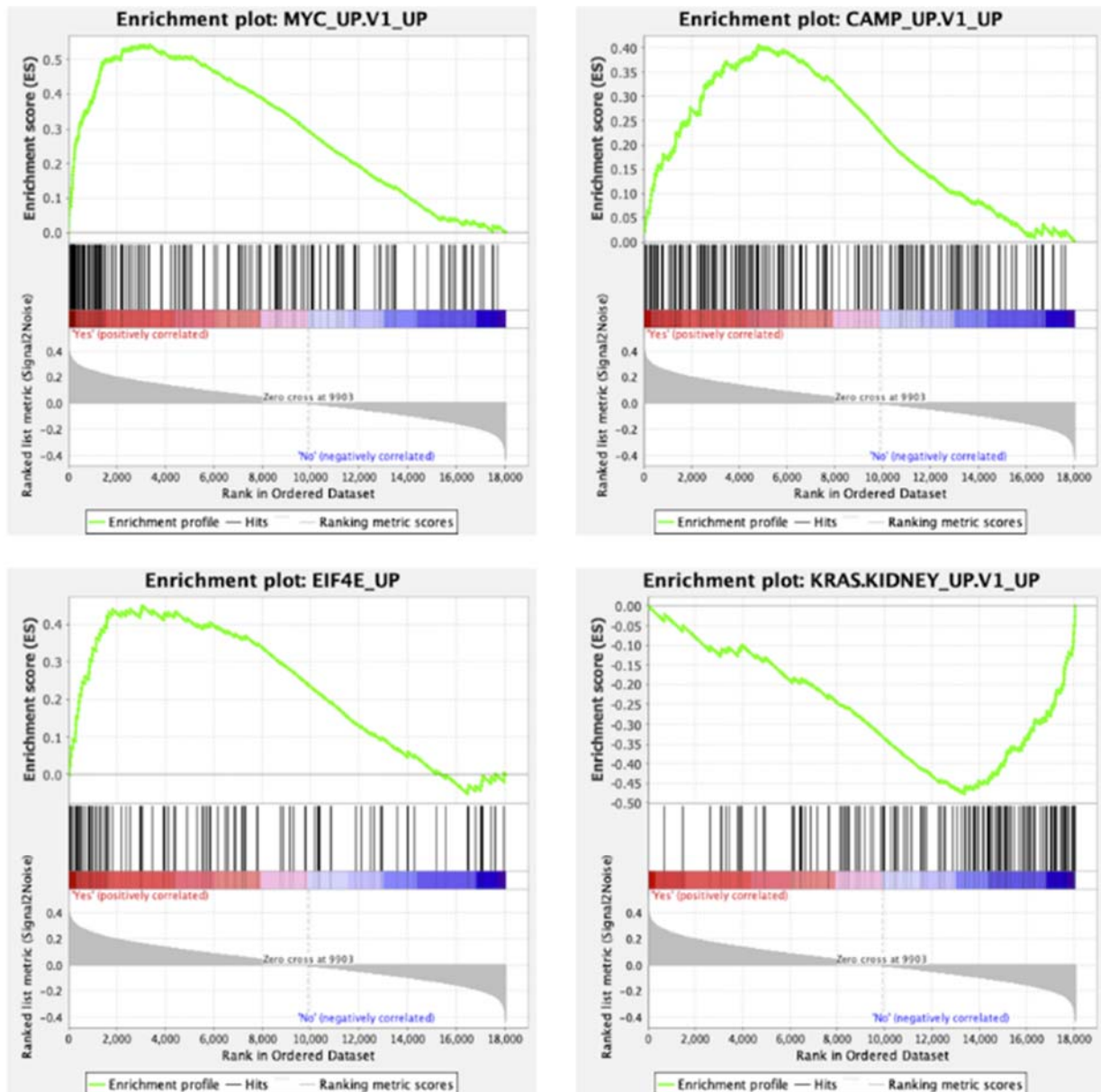
### Functions and Signaling Pathways of Highly Expressed Genes in Metastatic and Non-Metastatic Groups

The results of Enrichment functional analysis of dominantly expressed genes in the two groups revealed statistically significant differences, with the lowest  $p$  value (Fig. 2A). In general, the metastatic group increased transcription, translation, immune response, and cancer terms while non-metastasis increased morphogenesis, cellular organization, and cell adhesion terms. The analysis of transcription factors also showed a significant increase in transcription factors related to the metastatic group, such as GATA2 and SP1. In contrast, CREB1 was associated with the non-metastatic group (Fig. 2B).



**Fig. 2** Results of functional analysis of genes expressed in 2 clusters: **A** top 100 functions have statistically significant differences with the lowest  $p$  value; **B** results of analysis of differential transcription factors between 2 clusters.

In GSEA analysis, there were 76/188 gene sets upregulated in the metastatic group, including 9 gene sets that were significant at  $FDR < 25\%$ , 4 gene sets that were significantly enriched at nominal  $p$  value  $< 1\%$  and 12 gene sets that were significantly enriched at nominal  $p$  value  $< 5\%$ . However, in the non-metastatic group, 112 / 188 gene sets were upregulated with 0 gene set being significantly enriched at  $FDR < 25\%$ , 1 gene set significantly enriched at nominal  $p$  value  $< 1\%$  and 8 gene sets significantly enriched at nominal  $p$  value  $< 5\%$ . Fig. 3 presented the top NES of enhanced oncogenic signatures via MYC, cAMP, and EIF4E cancer genes in the metastatic group and KRAS cancer gene in the non-metastatic group.



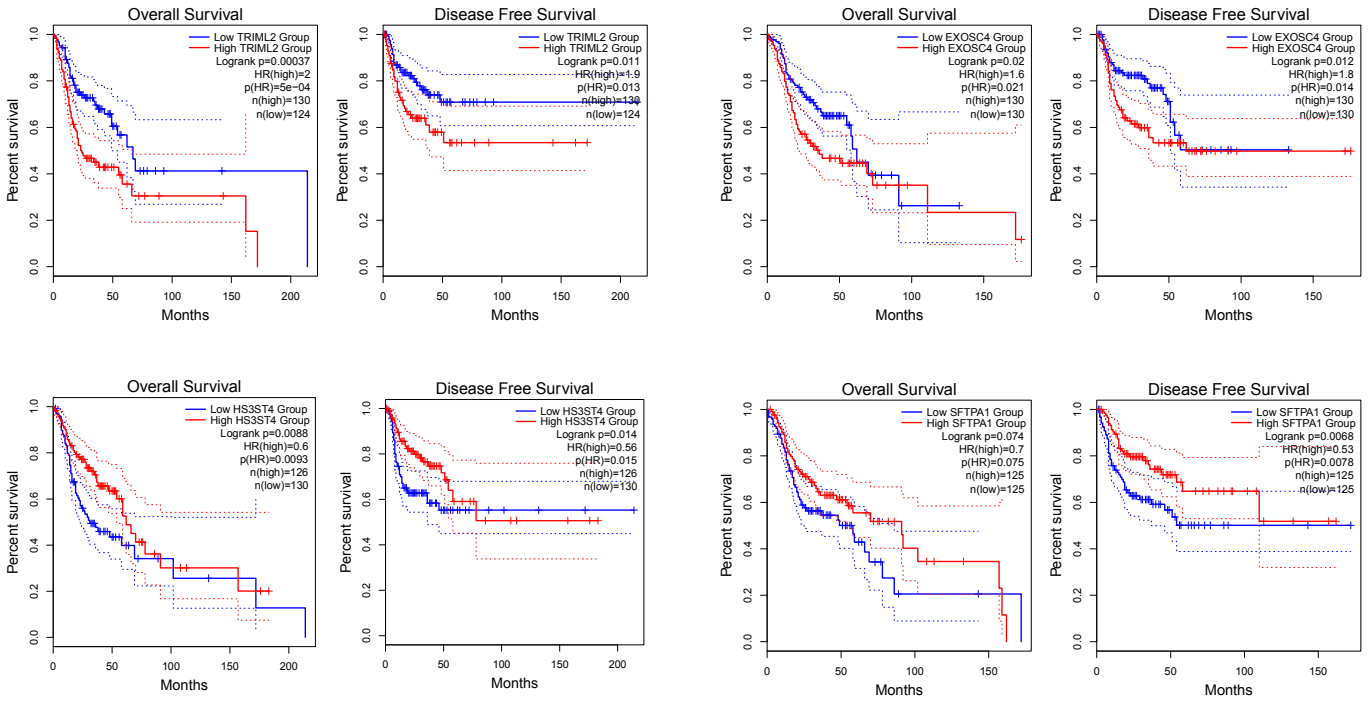
**Fig. 3** Selected oncogenic signature gene set that significantly upregulated in metastatic (Yes) and non-metastatic (No) groups.



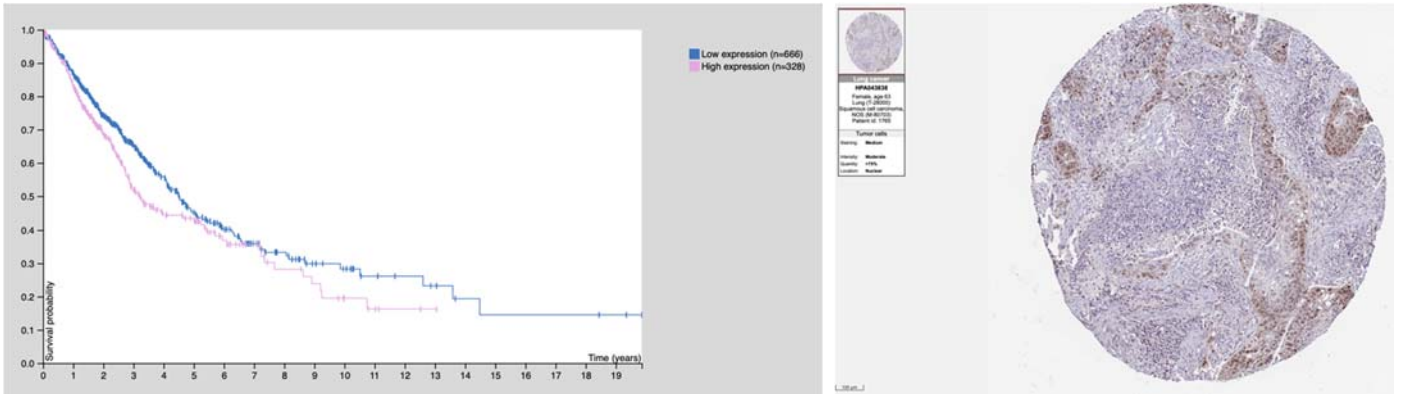
***Identification of Cancer-Related Genes in Metastatic and Non-Metastatic Groups***

We found that in the top 20 upregulated genes in the metastatic group, high expression of TRIML2 and EXOSC4 reduced percentages of both overall survival and disease-free survival in HNSC patients in general until 50-150 months. On the other hand, in the top 20 upregulated genes in the non-metastatic group, high expression of HS3ST4 and SFTPA1 increased percentages of both overall survival and disease-free survival in HNSC patients in general until 70-150 months (Fig. 4A). Further investigation in <https://www.proteinatlas.org> database showed that TRIML2 and EXOSC4 were unfavorable in lung and liver cancer via the high protein expression in lung and liver cancer samples with a low rate of overall survival (Fig. 4B, C).

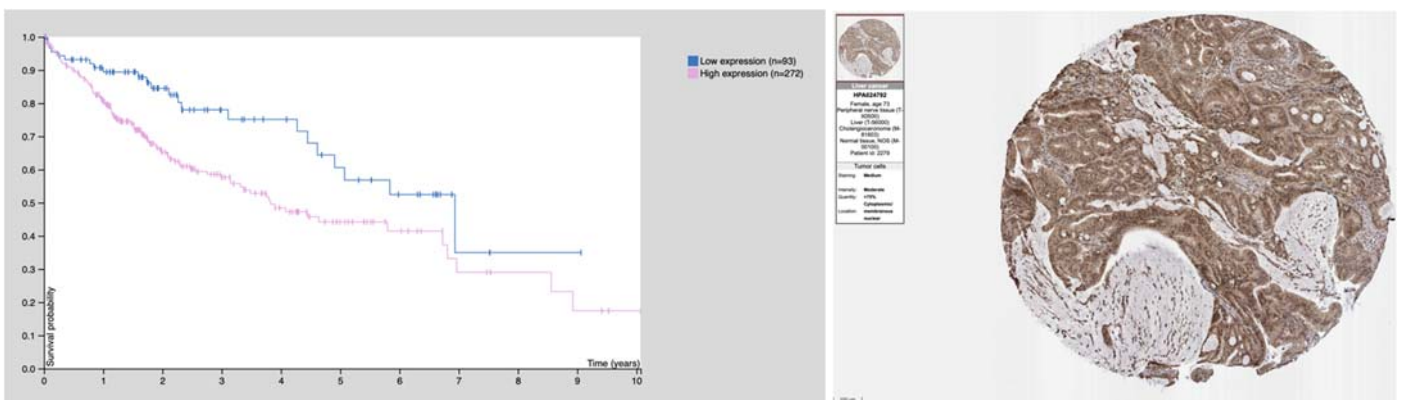
**A**



**B**



**C**



**Fig. 4** Survival analysis. **A** Overall survival and disease-free survival analysis of TRIML2, EXOSC4, HS3ST4 and SFTPA1. **B, C** Survival analysis (left panels) and protein expression by immunohistochemistry staining (right panels) of TRIML2 in lung cancer (B) and EXOSC4 in liver cancer (C) from <https://www.proteinatlas.org>.

## DISCUSSION

We performed the data analysis of 74 cases of male tongue cancer patients who consumed alcohol from TCGA regarding metastatic lymph node involvement in tongue cancer among male patients who consume alcohol. Non-metastasis (N0, NX) was observed in 36 out of 74 patients and metastasis (N1, N2, N2a, N2b, N3) was observed in 38 patients with 20.3% primary tumor located at the base of the tongue and 79.7% at other parts. In all cases, aging is thought to be linked to the risk of cancer in general and oral cancer in particular. From the age of 40, the risk of cancer begins to rise. The sample in this study had an average age of 64. The relationship between alcohol consumption and tongue cancer, especially in males, is significant. The exact mechanism through which alcohol increases cancer risk is complex. It may involve the conversion of alcohol into acetaldehyde, a toxic chemical and probable carcinogen, by enzymes in the oral cavity. Acetaldehyde can damage DNA and prevent the body from repairing the damage (Mehrotra *et al.*, 2022; Chou *et al.*, 2023). Hence, it is important to discover the overall gene expression profile of these objects to understand the microenvironment behavior of the cancer tissue regarding metastasis. Computational analysis revealed a significant difference in gene profiles between 2 groups. From the upregulated gene lists, function and signal pathways of highly expressed genes in metastatic and non-metastatic groups were performed. Notable molecular biological differences between the two groups can be described as follows: In the group with metastasis: transcription, translation regulation, and immune response indicated the cellular metabolism during division. In the group of non - metastasis: secretion activities, transmembrane transport, cell-cell adhesion as well as organization and morphogenesis for normal cell functions. The findings confirmed the exclusive metabolism of metastasis vs. non-metastasis via cell division (Lee *et al.*, 2021).

We determined the significant different expression genes between patients with and without lymph node and then performed the functional analysis. In the metastatic group, GATA2, SP1, and MYC are well-known transcription factors for many types of cancer progression and invasion from previous studies. Different genes regulated by transcription factors were identified in each group: CREB1 in non-metastasis and GATA2 and SP1 in metastasis. CREB1 is a protein that binds the CAMP 1 responsive factor which presents in a variety of viruses and cellular stimulants. There is no link between CREB1 and tongue cancer so far (Hoeffler *et al.*, 1988; Taylor *et al.*, 1990). GATA2 is a transcription factor that regulates the expression of genes for the embryonic development, self-renewal, and functionality of lymphatic system-forming, tissue-forming stem cells (Lee *et al.*, 1991). It plays important roles in many types of cancer including prostate, lung, breast, gastric tract cancers and is linked to poor prognosis (Aktar & Heit, 2023). Additionally, SP1 is a transcription factor regulating cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling. Sp1 promotes tumor progression, invasion and contributes to the hallmarks of cancer (Beishline & Azizkhan-Clifford, 2015; Xu *et al.*, 2023). These transcription factors may contribute to the cancer invasion in head-neck carcinoma that requires further investigation (Abu-Ghanem *et al.*, 2016).

Additionally, enrichment analysis by GSEA with oncogenic signatures also elucidated the upregulation of cancer gene sets in metastasis. GSEA revealed the significant upregulation of cancer-related genes in metastasis including MYC target genes, a proto-oncogene and transcription factor or EIF4E target genes, a eukaryotic translation initiation factor involved in directing ribosomes to the cap structure of mRNAs and other steps in RNA metabolism (Sonenberg *et al.*, 1979; Gardner *et al.*, 2002; Ramaswamy *et al.*, 2003). Non-metastasis enhanced KRAS target genes, a molecular on/off switch. These genes are very important in many types of cancer for prognosis, and diagnosis treatment targets (Yun *et al.*, 2009).

Interestingly, from the different expression gene profiles of 2 groups, overall survival and disease-free survival analysis of TRIML2 (a target of tumor-suppressor protein p53) and EXOSC4 (Exosome component 4, requiring for multiple cancer type progression) genes indicated the promising markers of metastasis HNSC (Kung *et al.*, 2015; Taniue *et al.*, 2022). The investigation in <https://www.proteinatlas.org> database showed that TRIML2 and EXOSC4 were highly expressed in lung and liver cancer samples. The markers were also significantly unfavorable in lung and liver cancer and could be prognostic markers. These findings strongly supported our finding and suggested TRIML2 and EXOSC4 as biomarkers in the next metastatic tongue cancer studies. In non-metastasis, HS3ST4 (enzyme heparan sulfate thought to play a role in HSV-1 pathogenesis) and SFTPA1 (Surfactant protein A1, roles in phagocytosis and chemotaxis of alveolar macrophages and induction of proliferation of immune cells) could be favorable in good prognosis HNSC patients (Ohka *et al.*, 2021; Yuan *et al.*, 2022).

Our present study focuses on computational analysis using the TCGA database. We used the same data processing methods to eliminate the potential batch effects. Although this is the largest data up to now, further studies need to contribute more samples from different ethics for better insight into the metastatic tongue cancer gene expression. Further validation experiments and clinical investigations including immunohistological-chemical staining, treatment response and survival analysis studies on the group of metastases to clarify the biological roles of suggested new markers in diagnosis and prognosis.

## CONCLUSION

Our study analyzed mRNA sequencing data from TCGA to investigate the differential gene expression of male patients with and without lymph node metastasis in tongue cancer. Our study suggested new prognostic biomarkers such as TRIML2 and EXOSC4 for unfavorable outcomes and HS3ST4 and SFTPA1 for favorable outcomes. These findings found that gene expression profiles may play a role in the progression and metastasis of tongue cancer. Further research is needed to validate these results and to develop targeted therapies for patients with tongue cancer.

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