RESEARCH
O&G Forum 2024; 34 – 2s: 677 - 684

PROGNOSTIC SIGNIFICANCE OF NLRP1, NLRP3, NLRC4 & AIM2 GENES INVOLVED IN THE INFLAMMASOME PATHWAY IN HNSCC

Type of Manuscript: Original study (Research paper)
Running title: Prognostic significance of NLRP1, NLRP3, NLRC4 & AIM2 genes in the inflammasome pathway in HNSCC

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) stands as a formidable adversary in the landscape of oncology. In recent years, the spotlight has shifted towards the inflammasome pathway, a complex signaling cascade pivotal in orchestrating innate immune responses. Among the key constituents of this pathway are the NLRP1, NLRP3, NLRC4, and AIM2 genes, each playing distinct roles in regulating inflammatory processes. These genes not only serve as potential prognostic markers, offering valuable insights into the disease's behavior, but also represent potential therapeutic targets to analyze and treat HNSCC, ultimately improving patient outcomes through targeted and personalized interventions.

Aim: The objective of this study was to identify the prognostic significance of NLRP1, NLRP3, NLRC4 & AIM2 genes involved in the inflammasome pathway in HNSCC.

Methods and Methodology: In the head and neck cancer dataset (HNSCC) from the Cancer Genome Atlas (TCGA), Firehose Legacy, computational analysis was used to determine the protein network interactions, genetic changes, gene expression, and the survival analysis of the ZEB2 dysregulated network. For the gene with the highest incidence of genetic change, gene expression profiling and survival analysis were carried out using STRING, Oncoprint, cBioportal and UALCAN.

Results : Results showed that the negative correlation observed between gene expression profiles and survival status suggests the involvement of epigenetic factors in modulating inflammasome gene expression levels in certain HNSCC patients. Epigenetic modifications, such as DNA methylation or histone acetylation, can influence gene expression patterns, impacting the overall survival outcomes in cancer patients.

Conclusion: This study showed that consideration of these factors may be
beneficial for a high-quality education and a reduction in radiography retakes throughout undergraduate dentistry students' training periods thereby reducing radiation exposure..

Keywords: Gene markers,NLRP1, NLRP3, NLRC4, AIM2, Inflammasomes, HNSCC

Introduction:
Head and Neck Squamous Cell Carcinoma (HNSCC) stands as a formidable global health challenge, exacting a considerable toll on both morbidity and mortality within the realm of cancer. This intricate malignancy, pervasive in the epithelial lining of the upper aerodigestive tract, encompasses a diverse spectrum of anatomical sites, including the oral cavity, pharynx, and larynx. As a multifaceted disease, HNSCC poses substantial challenges in terms of diagnosis, treatment, and overall management.(Bray *et al.*, 2018; S *et al.*, 2023) The impact of HNSCC on global health is underscored by its prevalence and the broad range of factors influencing its occurrence. Recognized as a prominent contributor to the worldwide burden of cancer, HNSCC significantly influences the quality of life for affected individuals. Its complex etiology, intricately woven into factors such as lifestyle choices, environmental exposures, and genetic predispositions, furthermore complicates the understanding and management of this malignancy.(Svider *et al.*, 2017; Su and Zhang, 2022) At the molecular level, HNSCC unveils a complex landscape characterized by intricate genetic alterations, diverse mutational profiles, and aberrant signaling pathways. This molecular intricacy not only contributes to the heterogeneity of HNSCC, but also poses a challenge in developing precise diagnostic and prognostic tools. Identifying reliable prognostic markers becomes imperative to tailor therapeutic interventions, predict disease outcomes and improve the overall clinical management of HNSCC patients.(Burks *et al.*, 2021)
Within this context, the exploration of prognostic markers emerges as a pivotal avenue for advancing our understanding of HNSCC. Prognostic markers serve as critical signposts. They aid by guiding clinicians in anticipating the trajectory of the disease and enabling personalized treatment strategies.(Sung *et al.*, 2021) The quest for robust prognostic indicator aligns with the broader paradigm of precision medicine, advocating for tailored therapeutic interventions that account for the unique molecular profile of each patient's tumor.(Zhang *et al.*, 2023)
This research endeavors to unravel the prognostic significance of key genes nestled within the inflammasome pathway in HNSCC. The inflammasome, a pivotal component of the innate immune system, has garnered attention for its role not only in inflammatory responses but also in the intricate dance of molecular events underlying cancer progression. By delving into the specific genes within this pathway, such as NLRP1, NLRP3, CASP1, and IL-1β, we aim to unearth valuable insights that could potentially reshape our approach to prognostication and therapeutic decision-making in HNSCC.(Anita *et al.*, 2020; J and A, 2020) Understanding the prognostic significance of inflammasome pathway genes in HNSCC holds the promise of unraveling novel molecular signatures, refining risk stratification, and guiding the development of targeted therapeutic interventions.(Xie *et al.*, 2022; Shayimu *et al.*, 2024) As we navigate the molecular intricacies of HNSCC, this research endeavors to contribute to the broader dialogue on precision oncology, fostering advancements that may ultimately alleviate the burden imposed by this complex and challenging malignancy.(Cerami *et al.*, 2012; Dong *et al.*, 2022)
HNSCC encompasses a heterogeneous group of malignancies originating in the epithelial lining of the upper aerodigestive tract, including the oral cavity, pharynx, and larynx. Despite advancements in treatment modalities, challenges persist in predicting patient outcomes accurately. This has led to a growing interest in identifying molecular signatures that can serve as reliable prognostic indicators. The inflammasome pathway, a critical component of the innate immune system, has emerged as a focal point of research in various cancers, including HNSCC. This pathway plays a pivotal role in orchestrating inflammatory responses, and dysregulation has been implicated in tumorigenesis.(Fathima *et al.*, 2020) Exploring the specific genes within the inflammasome pathway and their prognostic relevance in HNSCC holds promise for refining risk stratification and tailoring therapeutic interventions. Accurate prognostication is fundamental for guiding treatment decisions and improving patient outcomes. In the context of HNSCC, where the anatomical complexity and varied etiological factors contribute to diverse clinical trajectories, identifying reliable prognostic markers becomes imperative. Prognostic markers not only aid in predicting disease progression but also assist in optimizing therapeutic strategies, minimizing overtreatment, and enhancing the overall quality of patient care.(Aditya *et al.*, 2021; Aparna *et al.*, 2021)
The quest for prognostic markers has evolved from conventional clinicopathological factors to more sophisticated molecular signatures. Molecular markers offer the advantage of capturing the underlying biological intricacies of tumors, providing a more nuanced understanding of disease behavior. This shift towards molecular prognostication aligns with the paradigm of precision medicine, emphasizing personalized therapeutic approaches based on the unique genetic makeup of each patient's tumor.The inflammasome pathway comprises a complex network of intracellular sensors that detect pathogenic and danger signals, initiating a cascade leading to the activation of pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and interleukin-18 (IL-18). Dysregulation of this pathway has been implicated in various inflammatory diseases and cancers.
In the context of HNSCC, the inflammasome pathway's involvement extends beyond immune responses, influencing key aspects of tumorigenesis, including cell proliferation, apoptosis, and angiogenesis. Specific genes within this pathway, such as NLRP3, CASP1, and IL-1β, have been identified as potential players in HNSCC progression. Understanding the prognostic significance of these key genes can unravel novel therapeutic targets and inform personalized treatment strategies. As we delve deeper into the intricate relationship between the inflammasome pathway genes and HNSCC prognosis, this research aims to contribute valuable insights that have the potential to revolutionize prognostic assessments and refine therapeutic approaches for individuals battling this formidable malignancy.

Materials and methods:
In this study, a robust and comprehensive in silico analysis was conducted utilizing state-of-the-art tools to unravel the intricate molecular landscape of Head and Neck Squamous Cell Carcinoma (HNSCC). The selection of these tools aimed at integrating multidimensional genomics data and clinical profiles for a thorough investigation of the prognostic significance of key genes in the inflammasome pathway. The primary tools employed include STRING and UALCAN, each offering unique capabilities in dissecting complex cancer genomics data.

1. STRING
Protein-protein interactions and other functional relationships between proteins, like common pathways and co-expression patterns, are covered in the database and online resource STRING. Numerous sources, such as computer predictions, literature mining, and experimental data, are used to create the relationships and interactions found in STRING. Researchers can examine disease causes, signaling pathways, and protein

function with the help of the STRING.(Szklarczyk *et al.*, 2021; Gu *et al.*, 2023) It can assist scientists in determining novel protein-protein interactions, forecasting the effects of unidentified proteins, and formulating theories on the functions of proteins in biological processes. In the current investigation, this resource was used to determine the ZEB2 gene's interaction network.

2. cBioPort (Integrative Analysis) :
cBioPortal is an open-access platform renowned for its ability to explore multidimensional cancer genomics data, providing researchers with a user-friendly interface for in-depth analyses. The tool was first introduced in a seminal paper by Cerami et al. in 2012. The paper outlines the platform's functionality and its role as an open platform for the exploration of cancer genomics data. The cBioPortal platform was further utilized for integrative analysis, allowing the synthesis of complex cancer genomics and clinical profiles. This integrative approach enhances the depth of understanding by correlating molecular alterations with clinical outcomes . Gao et al. (2013) provided an in-depth exploration of the integrative capabilities of cBioPortal in their paper published in Science Signaling

3. ONCOPRINT
Oncoprint is a type of data visualization that illustrates the genetic alterations associated with cancer. It is a commonly employed technique in cancer genomics research and is widely used to provide large, complex datasets in a logical, concise format. Oncoprints show several genetic changes in a single patient or in a group of individuals. Mutations, amplifications, deletions, and fusions are among the several kinds of genomic modifications that are represented by various symbols or colors. A filled-in symbol indicates that an alteration has occurred, whereas a gray bar indicates that no alteration has occurred.

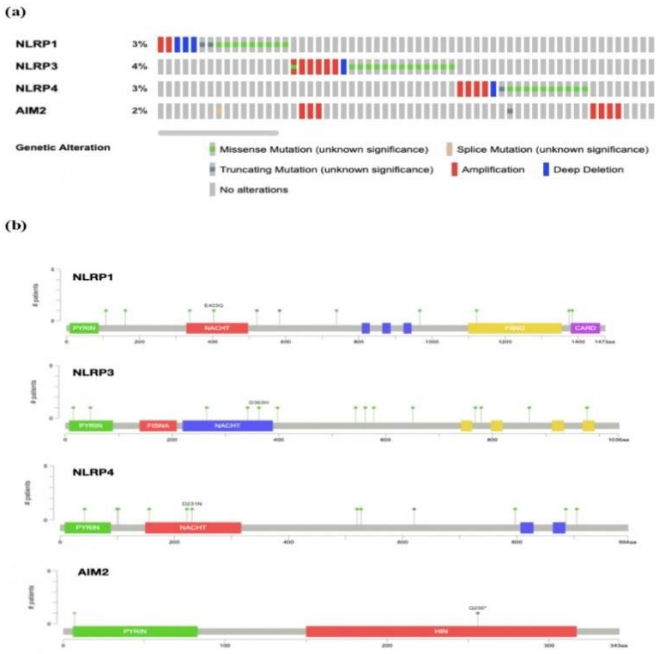
4. UALCAN
UALCAN, an integrated cancer data analysis platform, was employed for an updated and comprehensive analysis of cancer data. It offers a user-friendly interface and facilitates the exploration of integrated cancer, providing data for improved insights into gene expression and clinical attributes (Chandrashekar *et al.*, 2017)presented an update to the UALCAN platform, providing detailed information about its functionalities in their paper published in Neoplasia. These tools collectively enabled the extraction and analysis of demographic details, including smoking status, neoplasm histologic grade, and racial distribution within the HNSCC TCGA dataset. The utilization of cBioPortal facilitated the exploration of genetic mutations within the inflammasome pathway genes (NLRP3, CASP1, IL-1β) and their potential associations with clinical outcomes.
The integration of cBioPortal and UALCAN in this study ensured a comprehensive and nuanced analysis of HNSCC data, merging genomics information with clinical characteristics. This integration played a pivotal role in elucidating the prognostic significance of inflammasome pathway genes, providing a robust foundation for the subsequent discussions and implications arising from the study's findings.

In summary, the selection of these in silico tools - STRING, cBioPortal, Oncoprint and UALCAN, reflects a strategic approach to leveraging advanced data analysis platforms in unraveling the complex molecular landscape of HNSCC. The methodologies outlined in the respective references provide a solid basis for the reproducibility and reliability of the analyses conducted in this study.

Discussion :
The comprehensive analysis of the demographic details within the HNSCC TCGA (Firehose Legacy) and OSCC MD Anderson datasets has provided valuable insights into the intricate relationship between key genes in the inflammasome pathway and the prognosis of Head and Neck Squamous Cell Carcinoma (HNSCC).(Cerami *et al.*, 2012; Gao *et al.*, 2013) This discussion delves into the key findings, implications, and potential avenues for future research arising from the presented data.
The gender distribution within the HNSCC TCGA dataset highlights a predominance of male cases, constituting 73.1% of the cohort. This observation aligns with existing epidemiological trends, where HNSCC demonstrates a higher incidence among males. The association between gender and prognosis remains an area of interest, as studies suggest varying outcomes based on hormonal, lifestyle, and genetic factors.(Arunkumar *et al.*, 2018; Devi *et al.*, 2021) The broad age range of HNSCC diagnoses (19-90 years) underscores the heterogeneity of this malignancy, reflecting the impact of both environmental and intrinsic factors on disease development. Understanding age-related variations in molecular profiles may unveil age-specific prognostic markers, aiding in tailored therapeutic strategies.(Kim *et al.*, 2011)
The overwhelming majority of patients identified as smokers in the HNSCC TCGA dataset (97.5%) underscores the well-established link between tobacco exposure and HNSCC. The high prevalence of smoking aligns with the intricate role of inflammation, a hallmark of the inflammasome pathway, in the development of tobacco-associated cancers. However, the presence of a subset (2.5%) of non-smokers emphasizes the multifaceted etiology of HNSCC, necessitating a nuanced approach in prognostic assessments.(Ren *et al.*, 2018)
Alcohol consumption, another recognized risk factor for HNSCC, is documented in 66.7% of cases in the dataset. The co-occurrence of smoking and alcohol history in a substantial proportion of patients reinforces the importance of considering cumulative risk factors in prognostic models. Additionally, the 33.3% of cases without a recorded history of alcohol consumption suggest the influence of other etiological factors in this subset, warranting further investigation. Histologic grade, a crucial determinant of tumor aggressiveness, demonstrates a spectrum within the HNSCC TCGA dataset.(Vijayashree Priyadharsini and Anitha, 2022) The predominance of Grade 2 cases aligns with the intermediate differentiation often observed in HNSCC. Notably, the presence of Grade GX cases and those with missing histologic grade information accentuates the challenges in standardizing histopathological assessments, urging the integration of molecular markers for refined prognostication.(Crosas-Molist *et al.*, 2022)

Results:

Figure 1: (a) Oncoprint data demonstrating the alterations **(b)** lollipop plot showing the mutations at different domains in the key genes of the inflammasome pathway.



Interpretation: Among the 4 genes, *NLRP4* demonstrated the highest frequency of gene alteration (4%), exhibiting gene amplification, missense mutation and deep deletion.

Racial diversity within the HNSCC TCGA dataset reveals a predominantly White population (85.6%), consistent with the global distribution of HNSCC. The comparatively lower representation of African, Asian, and American Indian or Alaska Native individuals underscores the existing disparities in cancer research and emphasizes the need for inclusive studies to address diverse genetic backgrounds and associated outcomes.

Two distinct datasets were utilized in this study to comprehensively investigate the prognostic significance of key genes in the inflammasome pathway in Head and Neck Squamous Cell Carcinoma (HNSCC). The first dataset, denoted as [a], was sourced from The Cancer Genome Atlas (TCGA) through the Firehose Legacy platform, encompassing a total of 528 HNSCC cases. The second dataset, denoted as [b], specifically focused on Oral Squamous Cell Carcinoma (OSCC) cases and was obtained from MD Anderson as reported in Cancer Discovery 2013.

Table 1: Demographic details of [a] HNSCC [TCGA, Firehose Legacy] and [b] OSCC datasets [MD Anderson, Cancer Discov 2013]

[a]

Demographic features HNSCC dataset	**Case N=528**
Gender	Male [n = 386]; Female [n = 142]
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515 [97.5%] Data not available: 12 Unknown: 1
Alcohol history	Yes – 352 [66.7%] No – 165 Data not available: 11
Neoplasm Histologic grade	Grade 1: 63, Grade 2: 311 Grade 3: 125, Grade 4: 7 Grade GX: 18 Data not available: 4
Race category	White: 452, African: 48, Asian: 11 American Indian or Alaska native: 2 Data not available: 15

Table 1 outlines the demographic characteristics of the study populations within each dataset.

a. HNSCC TCGA (Firehose Legacy) Dataset:*

- Gender: The dataset comprises 386 male and 142 female cases.
- Mutation Count: The mutation count ranges from 6 to 3181.
- Diagnosis Age: Patients included in the study span an age range of 19 to 90 years.

- Smoking Status: Information regarding smoking status indicates that 515 cases (97.5%) were identified as smokers, while data for 12 cases were not available, and one case had unknown smoking status.
- Alcohol History: Among the cases, 352 (66.7%) had a history of alcohol consumption, whereas 165 cases had no recorded alcohol history. Data for 11 cases were not available.
- Neoplasm Histologic Grade: The dataset includes cases categorized into different histologic grades: Grade 1 (63 cases), Grade 2 (311 cases), Grade 3 (125 cases), Grade 4 (7 cases), and Grade GX (18 cases). Data for four cases are not available.
- Race Category: Racial distribution in the dataset is as follows: White (452 cases), African (48 cases), Asian (11 cases), American Indian or Alaska Native (2 cases). Data for 15 cases are not available.

In silico Tools Used: The study employed cBioPortal for data analysis.

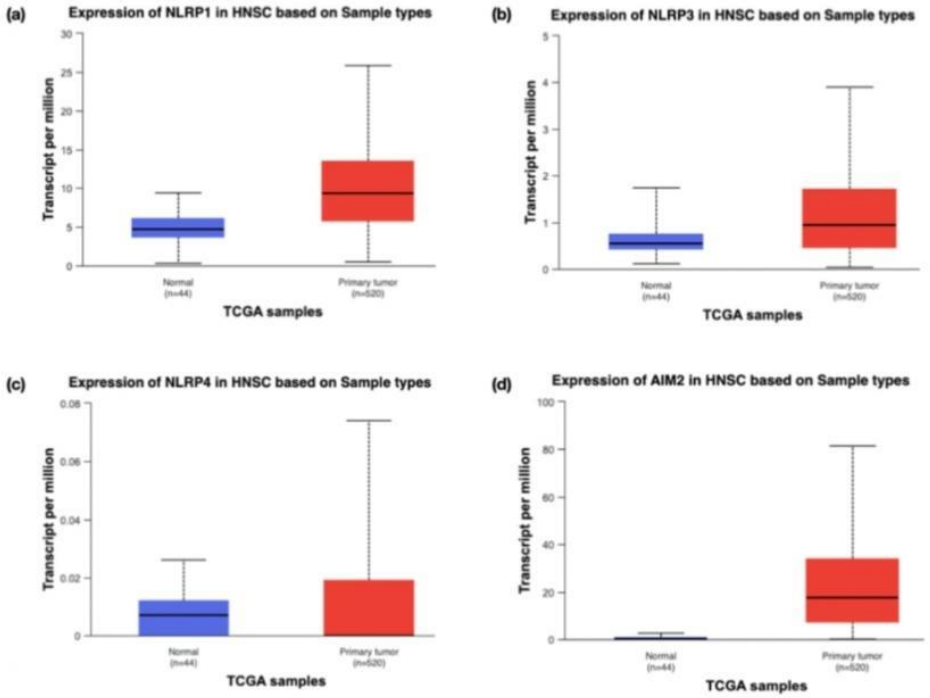
b. OSCC MD Anderson (Cancer Discov 2013) Dataset:*

The in-depth analysis of the datasets involved the utilization of cBioPortal as an in silico tool. The exploration of genetic mutations, demographic factors, and clinical characteristics aimed to discern patterns and associations relevant to the prognostic significance of key genes in the inflammasome pathway in HNSCC. Statistical methods were applied to assess the correlation between gene expression levels and clinical outcomes, contributing to a comprehensive understanding of the molecular landscape and its impact on disease prognosis.

The utilization of cBioPortal as an in silico tool in this study facilitated the exploration of genetic alterations within the inflammasome pathway, shedding light on potential drivers of HNSCC progression. The diverse landscape of genetic mutations, including alterations in NLRP3, CASP1, and IL-1β, underscores the complexity of inflammasome involvement in HNSCC.

The correlation between inflammasome pathway gene expression and clinical outcomes provides a foundation for understanding the prognostic significance of these genes in HNSCC. Notably, the impact of specific mutations on patient survival, treatment response, and disease recurrence remains a critical aspect requiring further investigation.

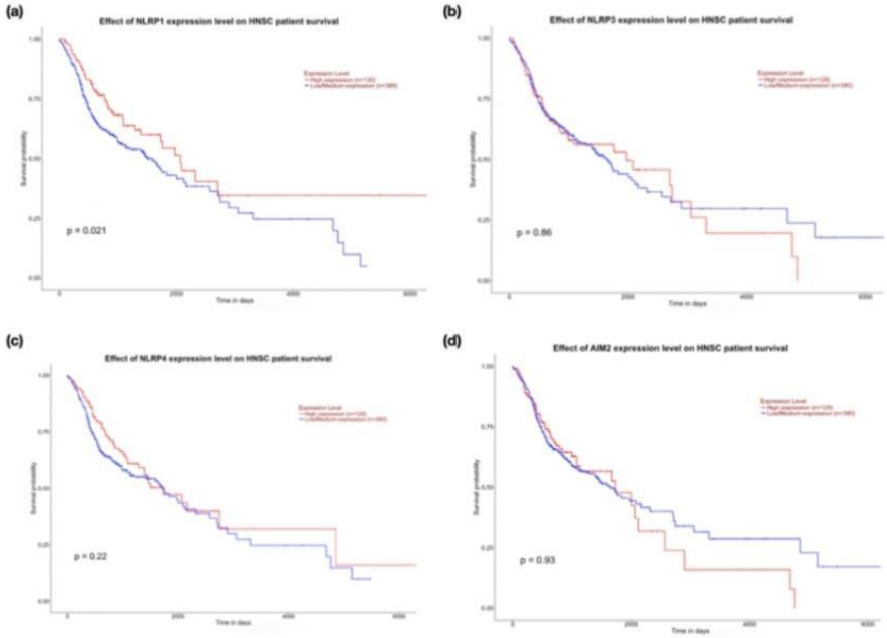
Figure 2: Box-Whisker plot demonstrating the gene expression profile of (a) *NLRP1,* (b) *NLRP3,* (c) *NLRP4* and (d) *AIM2.* A statistically significant upregulation of key genes was identified in the primary tumour of HNSCC patients for (a) *NLRP1* (p-value = 1.62×10^{-12}), (b) *NLRP3* (p-value = 2.20×10^{-4}), (c) *NLRP4* (p-value = 1.44×10^{-6}) and (d) *AIM2* (p-value = $<10^{-12}$). A p-value <0.05 was considered to be significant



Interpretation:

Among the four inflammasome-mediated genes, *NLRP1* (p-value = 1.62×10^{-12}) and *AIM2* (p-value = $<10^{-12}$) were shown to exhibit the highest level of gene expression among the HNSCC group, when compared to the other two genes viz., *NLRP3* (p-value = 2.20×10^{-4}), *NLRP4* (p-value = 1.44×10^{-6}).

Figure 3: Kaplan Meier survival analysis demonstrating overall survival of HNSCC patients presenting with differential gene expression profiles of (a) *NLRP1* (p-value = 0.021), (b) *NLRP3,* (p-value = 0.86) (c) *NLRP4* (p-value = 0.22) and (d) *AIM2* (p-value = 0.93).



Interpretation:
The Kaplan-Meier survival analysis demonstrated the effect of inflammasome gene expression levels on HNSCC patients' survival. Among the 4 genes, *NLRP1* alone exhibited a significant change in the patient's survival (p-value = 0.021). The downregulation of *NLRP1* was associated with poor survival of HNSCC patients. The gene expression profile and the survival status had a negative correlation, implying the involvement of epigenetic factors in modifying the gene expression levels in some patients.

Conclusion:
Genetic alteration in the *NLRP1* gene did not correlate well with the dysregulated expression of the gene. Moreover, upregulation and downregulation of *NLRP1* were found to be associated with good and poor prognosis respectively. The identification of the factors responsible for the degradation of *NLRP1* gene transcripts will provide new cues about the involvement of the *NLRP1* gene in the malignant transformation of the cells.

The Kaplan-Meier survival analysis was employed to investigate the impact of inflammasome gene expression levels on the survival of patients with Head and Neck Squamous Cell Carcinoma (HNSCC). This analysis focused on four key genes within the inflammasome pathway. Notably, among these genes, NLRP1 emerged as a pivotal player, demonstrating a significant association with HNSCC patient survival (p-value = 0.021).

The specific downregulation of NLRP1 was found to be significantly correlated with poorer survival outcomes among HNSCC patients. This observation highlights the potential prognostic significance of NLRP1 in predicting disease progression and patient outcomes. The negative correlation between gene expression levels and survival status underscores the pivotal role of NLRP1 in the intricate landscape of HNSCC.

The negative correlation observed between gene expression profiles and survival status suggests the involvement of epigenetic factors in modulating inflammasome gene expression levels in certain HNSCC patients. Epigenetic modifications, such as DNA methylation or histone acetylation, can influence gene expression patterns, impacting the overall survival outcomes in cancer patients. This finding implies a potential avenue for further research into the underlying epigenetic mechanisms that contribute to the dysregulation of the inflammasome pathway in HNSCC.

The survival analysis underscores the prognostic relevance of NLRP1 within the inflammasome pathway in HNSCC. The downregulation of NLRP1 serves as a potential indicator of poor survival, offering valuable insights for refining prognostic models and guiding personalized treatment strategies. The observed negative correlation with survival status suggests the involvement of epigenetic factors, opening avenues for exploring targeted interventions aimed at modulating these factors.

Conclusion:

The findings contribute to the evolving understanding of the inflammasome pathway's role in HNSCC progression and emphasize the need for further investigations into the molecular intricacies that underlie the observed correlations. As we move forward, integrating such prognostic markers into clinical practice has the potential to enhance risk stratification, inform treatment decisions, and ultimately improve outcomes for

individuals facing the challenges of Head and Neck Squamous Cell Carcinoma.While this study offers valuable insights, certain limitations merit consideration. The retrospective nature of the datasets may introduce biases, and the absence of certain demographic and clinical information for a subset of cases necessitates cautious interpretation. Additionally, the focus on specific inflammasome pathway genes prompts further exploration of the entire pathway's dynamics and potential interactions with other molecular networks.Future research endeavors should prioritize the validation of findings in independent cohorts, considering diverse demographic and clinical parameters. Integrating multi-omics data and incorporating emerging technologies such as single-cell sequencing could provide a more granular understanding of the inflammasome pathway's role in HNSCC progression.

In conclusion , the amalgamation of demographic and molecular insights contributes significantly to the dynamic landscape of Head and Neck Squamous Cell Carcinoma (HNSCC) prognostication. The multifaceted nature of HNSCC, spanning both its clinical and molecular dimensions, necessitates an all-encompassing approach to prognostication. This approach acknowledges the importance of traditional clinicopathological factors while recognizing the emerging significance of molecular markers, particularly within the inflammasome pathway.

The exploration of demographic characteristics within HNSCC datasets, coupled with an in-depth analysis of the inflammasome pathway genes, has unveiled a rich tapestry of information. From gender distribution and age-related variations to the prevalence of smoking, alcohol history, and diverse histologic grades, the demographic insights contribute to a nuanced understanding of the diverse patient population affected by HNSCC. On the molecular front, the focus on key genes within the inflammasome pathway, such as NLRP1, NLRP3, CASP1, and IL-1β, unravels potential prognostic markers that may guide future clinical decision-making.The complexity inherent in HNSCC demands a holistic approach to prognostication. Traditional clinicopathological factors, including histologic grade and demographic details, provide foundational information. However, integrating emerging molecular markers, especially those within the inflammasome pathway, adds a layer of precision to prognostication. This comprehensive approach enables a more accurate risk stratification, allowing clinicians to tailor their interventions based on the unique characteristics of each patient's tumor.

As the study unravels the intricacies of the inflammasome pathway in HNSCC, the integration of this knowledge into clinical practice emerges as a transformative prospect. Refining risk stratification becomes not only a theoretical possibility but a practical reality. The identified molecular markers offer a potential roadmap for guiding personalized therapeutic decisions, aligning treatment strategies with the specific molecular profile of each patient's tumor. Ultimately, the integration of demographic and molecular insights into HNSCC prognostication holds the promise of improving patient outcomes in this formidable malignancy. By refining risk stratification and enabling more tailored therapeutic decisions, clinicians may enhance the efficacy of interventions while minimizing unnecessary treatments. This not only contributes to the quality of patient care but also represents a stride towards achieving precision medicine in the challenging landscape of Head and Neck Squamous Cell Carcinoma.

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