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GENETIC ALTERATIONS IN CELL CYCLE CONTROL GENES AND ITS PUTATIVE ASSOCIATION WITH HNSCC

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ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is the most common form of cancer of the hypopharynx, oropharynx, lip, oral depression, nasopharynx, or larynx. The transformation of a normal cell to a cancerous one involves complex interplay of genes involved in several cellular processes such as cell cycle arrest, DNA repair process, activation of tumor suppressors etc., The cell cycle is a strictly controlled and integrated set of events that enables the cell to grow and proliferate through phase G1, S, G2 and M phases. The main goal of this controlled occurrence is to ensure that DNA is duplicated in the S phase and distributed evenly to the daughter cells. Cycline dependent kinases (CDKs), which provide a regulated transition from one phase to the next, regulate the completion of various phases. The present study follows a retrospective observational study design. The source of patient's data was procured from the cBioportal database. This database hosts an exhaustive collection of HNSC patient's details from different cohorts. Gene alterations identified in the genes controlling the cell cycle process was recorded. About eight genes viz., *CDKN2A* (54%),

CDKN2B (28%), *CCND1* (24%), *MYC* (13%), *JAK2* (7%), *RB1* (6%), *CDK6* (6%) and *E2F5* (6%) with the highest frequency of alterations (>5%) were further analyzed for their gene expression profile. Except for the *MYC* gene all other genes showed a differential expression pattern which was statistically significant. Furthermore, survival curve analysis was carried out for the seven genes displaying an increase in gene expression relative to that of the normal tissue. Out of the seven genes only two genes *CCND1* and *CDKN2A* significantly influenced the survival probability of HNSCC patients. Thus the present study identifies *CCND1* and *CDKN2A* as the candidate genes showing a strong association with HNSCC. However, further experimental validation of the results are warranted to confirm the role of these genetic alterations with the development and progression of HNSCC.

Keywords: Cell cycle control genes, head and neck squamous cell carcinoma, cyclin dependent kinases, amplification, deletions, variants

INTRODUCTION

Head and neck cancer represents the sixth most common form of cancer afflicting a larger population of males belonging to the south Asian region **(Bray *et al.*, 2018)**. Oral squamous cell carcinoma is the predominant form of head and neck cancer which accounts to about 95% of all cancers occurring in the head and neck region **(Mandal *et al.*, 2016)**. Despite advancements in treatment modalities and identification of candidate genes associated with the cancer type, the mortality and five year survival rate of patients has not improved significantly **(Perez-Sayans *et al.*, 2020)**. Since HNSC is a complex multifactorial disorder involving environmental, lifestyle and more importantly the genetic factors **(Abusail *et al.*, 2013; Maasland *et al.*, 2014)**. The investigations based on computational tools have identified several novel mutations to be associated with HNSCC.

Cell cycle control proteins serve as checkpoints so as to correct the errors and lesions in the DNA molecule. Any aberrations in the genes encoding these proteins would affect the process of cell cycle regulation resulting in adverse consequences. The lesions in the DNA might go unrepaired, DNA damage response may not be elicited and the functioning of tumor suppressors may be severely hampered. Several gene alterations have been identified in the genes encoding cell cycle proteins. Around six SNPs were found to confer the highest risk of oral cancer. Polymorphisms of p27 *(rs34329)*, cyclin E *(rs1406)*, Rb1-2 *(rs3092904)*, cyclin H *(rs3093816),* cyclin D1-1 *(rs647451)* and cyclin D2 *(rs3217901)* genes have been shown to be associated

with the oral cancer risk. The cumulative effect of all the polymorphisms are known to severely affect development, progression and invasion of tumors **(Murali *et al.*, 2014; Multani and Saranath, 2016)**. Previously our team has a rich experience in working on various research projects across multiple disciplines **[1–15]**. Hence, the present study was aimed at identifying alterations in the cell cycle control genes, followed by the assessment of gene expression profiles relative to normal tissues. In addition, survival curve analysis was also carried out to demonstrate the survival probability in HNSCC patients showing differential expression patterns of crucial gene products.

METHODOLOGY

Data retrieval

The present study follows a retrospective observational study design. The source of patient's data was procured from the cBioportal database. This database hosts an exhaustive collection of patient's details from different cohorts **(Table 1)**. The TCGA, Firehose legacy data set consisted of 528 head and neck squamous cell carcinoma cases of which sequencing and copy number alteration data were available for 512 tumor samples. A complete profile of mutated, amplified, deleted genes was available for each of the cases in the dataset. The demographic details of the cases in the dataset have been provided in **Table 1**. A list of vital genes related to family was obtained from the "HUGO Gene Nomenclature Committee at the European Bioinformatics Institute" (www.genenames.org/data/) database. User defined queries based on these genes were submitted in the cBioportal database and the resultant Oncoprint data was used for further analysis **(Cerami *et al.*, 2012; Gao, *et al.*, 2013)**.

Oncoprint data analysis

The Oncoprint data provides information on the frequency distribution of variations in each of the genes selected, type of variation, changes in the protein coding amino acids, gene amplification, deletions, insertions, frameshifts, splice site mutations etc. These details can be used to (a) derive a putative association between the disease phenotype and genotype, (b) identify the variations in less understood pathways or genes, and (c) identify any novel variations which can be associated with the disease phenotype **(Cerami *et al.*, 2012; Gao, *et al.*, 2013)**.

Gene expression and survival curve analysis

The expression of the gene in HNSCC was analysed using the UALCAN (http://ualcan.path.uab.edu/cgi-bin/TCGA-survival1.pl?) database. Survival curve analysis based on the tumor grade and

expression profile was performed to demonstrate the putative role of Rho family g genes with HNSC. Gene expression data is expressed as transcripts per million (TPM) which is a normalization method for RNA-seq data. The TPM values used for the generation of box-whisker plots were also used to determine the significant difference between the groups. The t test was performed using PERL script with the comprehensive perl archive network (CPAN) module. Combined survival effect analysis of gene expression and other clinical parameters such as race, gender, tumor grade, cancer subtypes were assessed using multivariate Kaplan-Meier survival analysis **(Chandrashekar *et al.*, 2017)**.

RESULT AND DISCUSSION

Computational approach is considered to be the most widely used method by researchers because of its ease of use. An exhaustive screening could be performed using these platforms. A specific pathway or a candidate gene can be analyzed for its association with disease phenotype. The preliminary results obtained from such studies can be employed for screening of the population which could result in identification of lead molecules for the purpose of diagnosis or therapy (Vijayashree and Paramasivam, 2020). Hence in the present study the cBioportal and UALCAN database were used to assess the genetic alterations and gene expression in the HNSCC dataset. The primary database, cBioportal which hosts several datasets of which the TCGA dataset (TCGA, Firehose Legacy) was selected for the present study. The TCGA dataset consisted of 528 HNSCC patients (530 samples).

The present study identified several alterations in about 34 cell cycle controls genes. About eight genes viz., *CDKN2A* (54%), *CDKN2B* (28%), *CCND1* (24%), *MYC* (13%), *JAK2* (7%), *RB1* (6%), *CDK6* (6%) and *E2F5* (6%) showed highest frequency of alterations (>5%) **(Figure 1)**. Out of the seven genes only two genes *CCND1* and *CDKN2A* were shown to significantly influence the survival probability of HNSCC patients **(Figures 2a-2g)**. The HNSC patients presenting with a high level expression of *CCND1* gene showed a low survival probability when compared to patients with low/medium level expression. On the other hand patients with low/medium level expression of *CDKN2A* gene presented with a low survival probability when compared to patients with high level expression **(Figures 3a, 3b)**.

These genes were further analyzed for their gene expression profile using the UALCAN database. All the genes showed relatively high expression levels when

compared to the normal tissue. Interestingly, the genes *CDKN2A* and *CDKN2B* showed the highest frequency of deep deletions and truncating mutations. Also, the *CCND1* gene showed a greater frequency of gene amplification among all the 34 genes investigated. Except for the *MYC* gene all other genes showed a differential expression pattern which was statistically significant (p value <0.05). Furthermore, survival curve analysis based on the Kaplan Meier method was carried out for the seven genes displaying an increase in gene expression relative to that of the normal tissue.

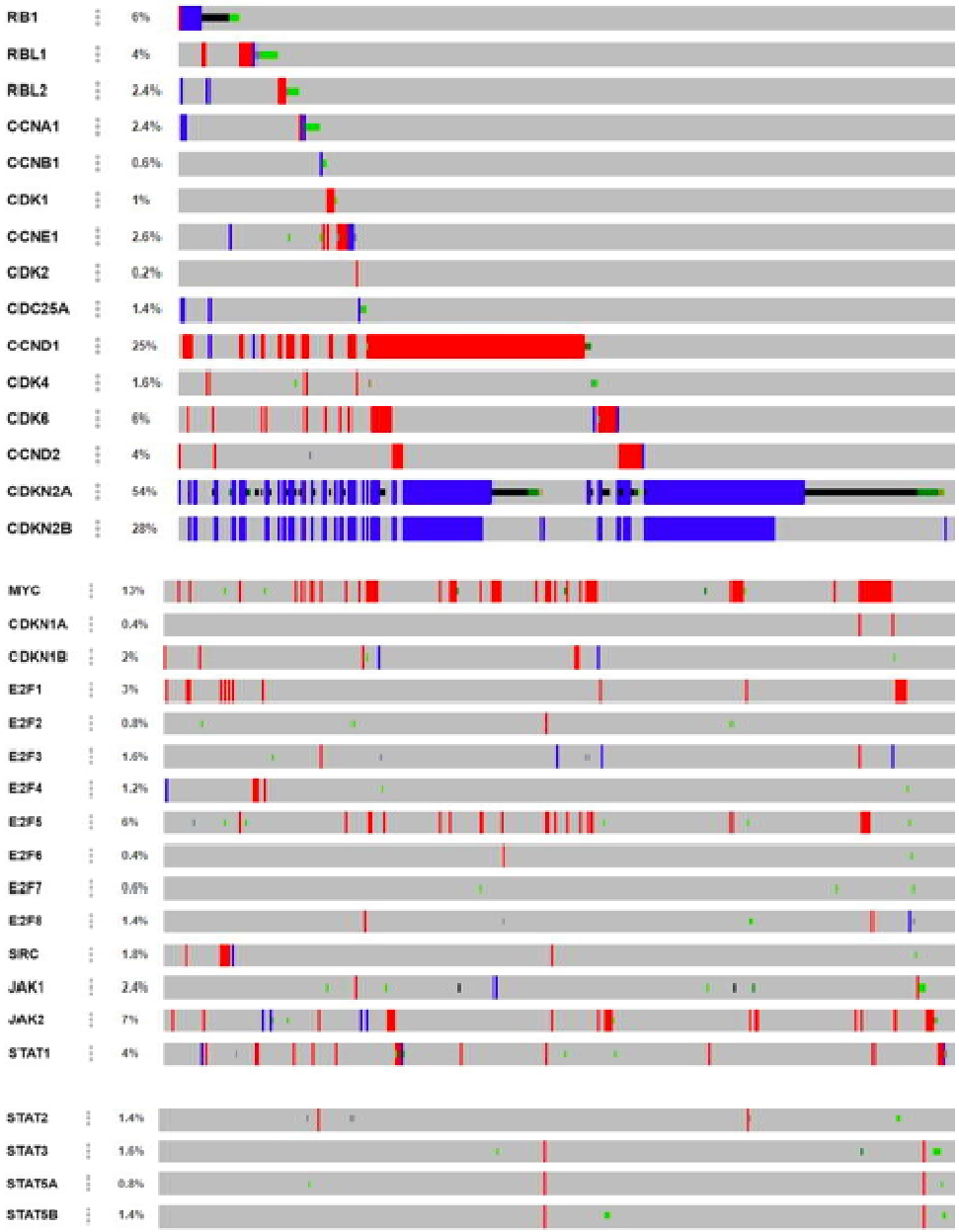
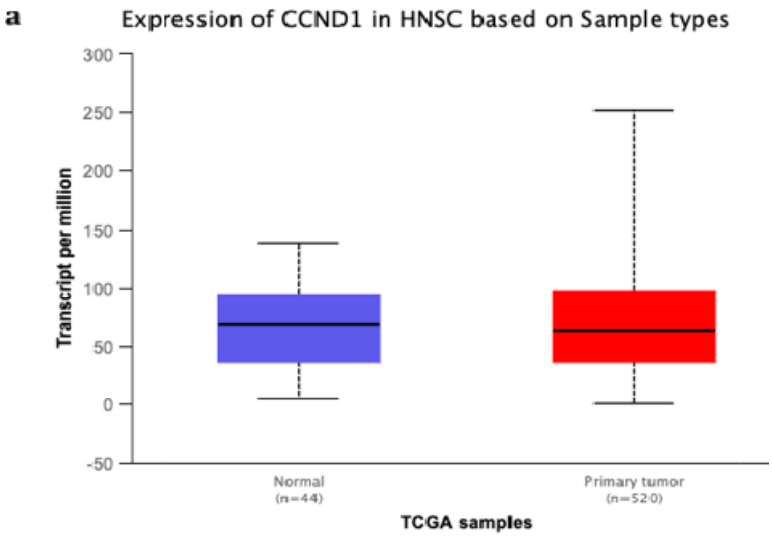
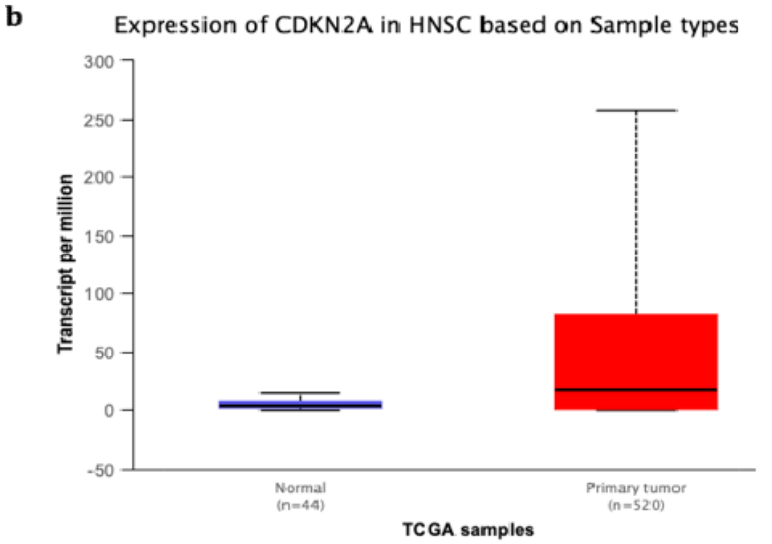
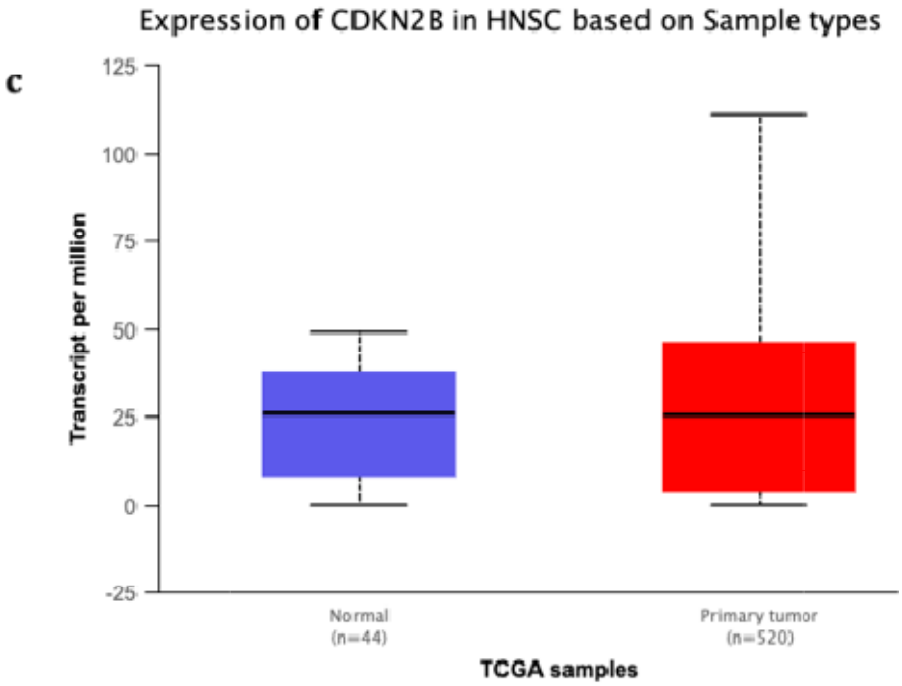


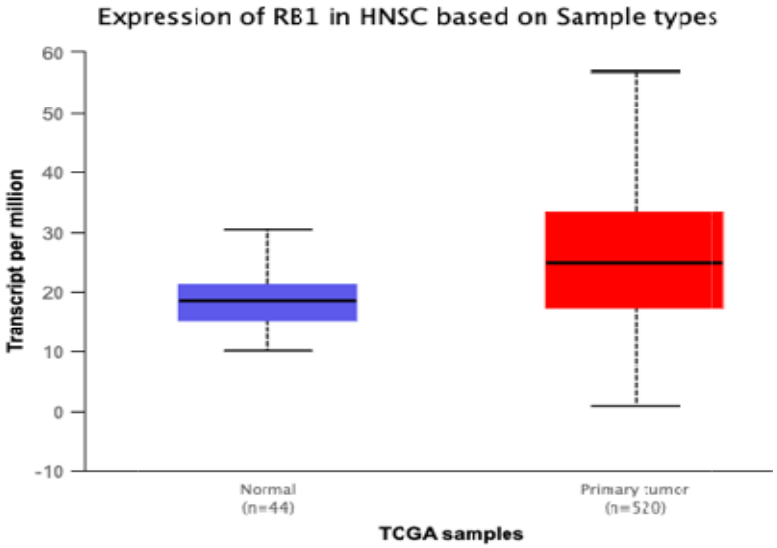
Figure 1: Oncoprint data depicting the alterations in cell cycle control genes. Each of the grey bars represents HNSC patients included in the TCGA dataset


a
Expression of CCND1 in HNSC based on Sample types
Transcript per million
300
250
200
150
100
50
0
-50
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples

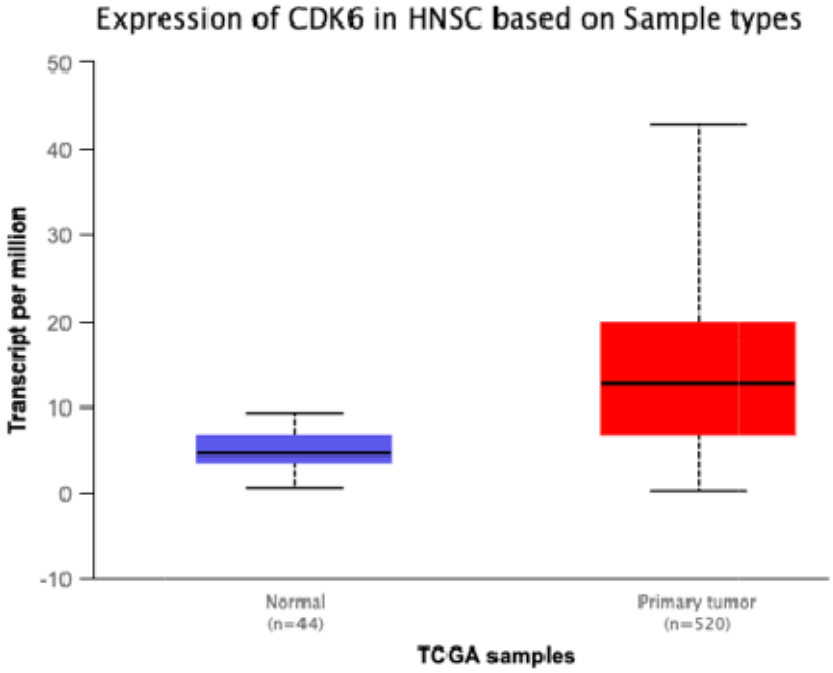

b
Expression of CDKN2A in HNSC based on Sample types
Transcript per million
300
250
200
150
100
50
0
-50
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples


c
Expression of CDKN2B in HNSC based on Sample types
Transcript per million
125
100
75
50
25
0
-25
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples

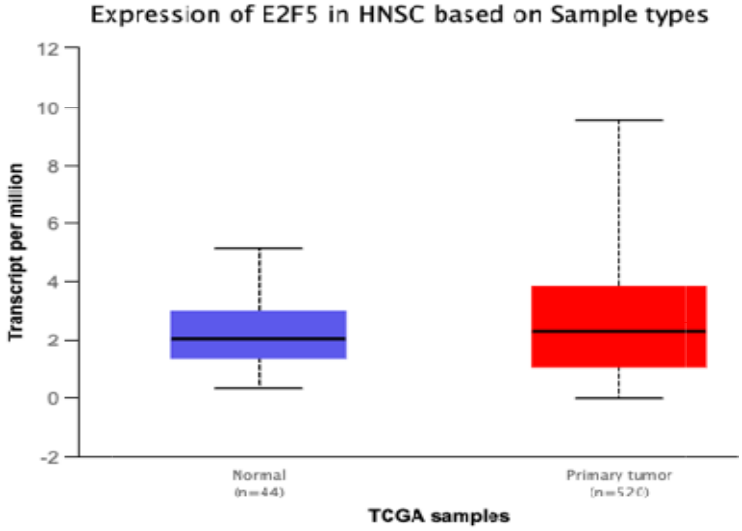
d

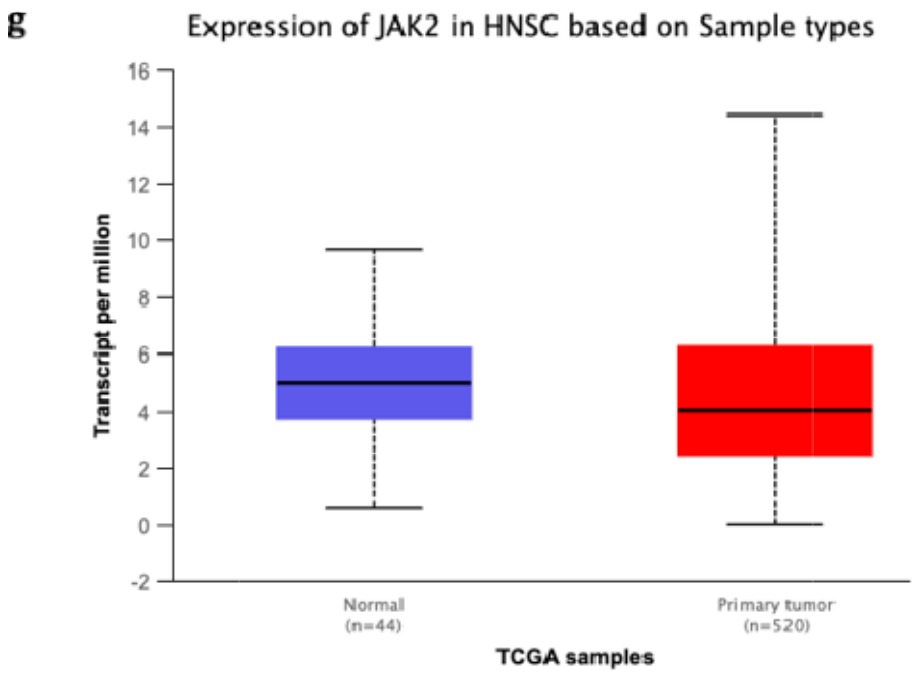

Expression of RB1 in HNSC based on Sample types
Transcript per million
60
50
40
30
20
10
0
-10
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples

e

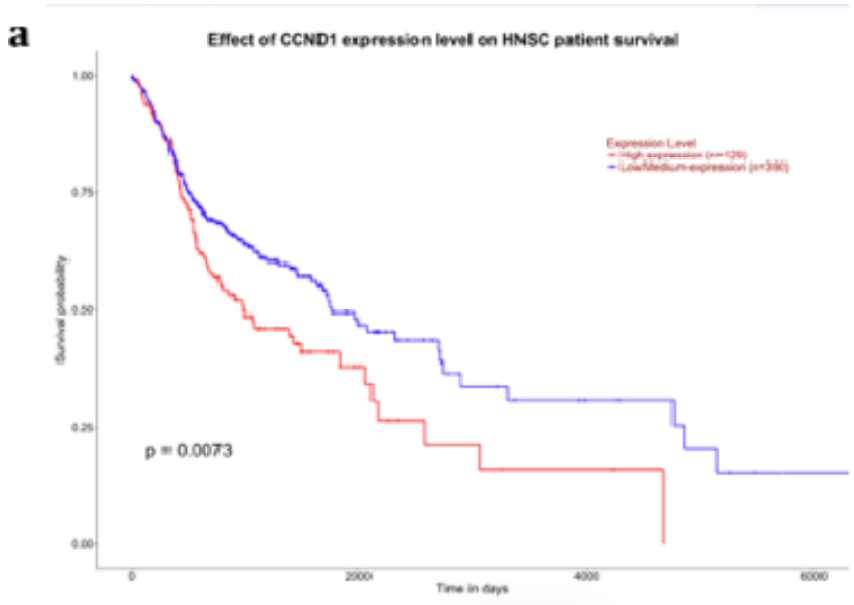

Expression of CDK6 in HNSC based on Sample types
Transcript per million
50
40
30
20
10
0
-10
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples

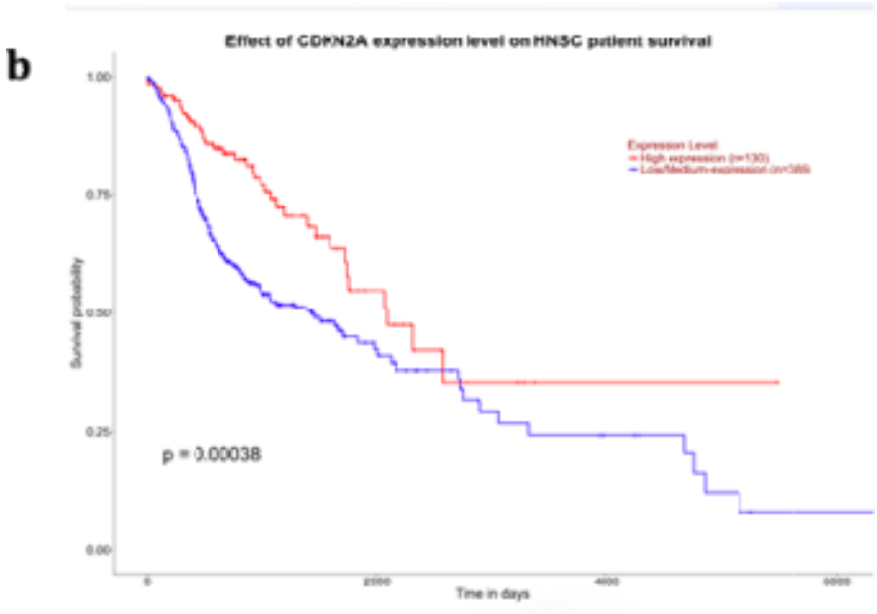
f


Expression of E2F5 in HNSC based on Sample types
Transcript per million
12
10
8
6
4
2
0
-2
Normal
(n=44)
Primary tumor
(n=520)
TCGA samples



Figures 2: Box-Whisker plot showing relative expression of (a) *CCND1* in HNSC and normal tissues (p = 4.312 x 10^{-9}), (b) *CDKN2A* (p = <10-^{12}), (c) *CDKN2B* (p = 5.02 x10-^{4}), (d) *RB1* (p = 2.26 x 10^{-9}), (e) *CDK6* (p = 1.62 x 10^{-12}), (f) *E2F5* (p = 4.03 x 10^{-5}) and (g) *JAK2* (p = 4.06 x 10^{-2}). A p value less than <0.05 is considered to be significant.





Figures 3: Kaplan–Meier plots showing the effect of (a) *CCND1* gene expression on HNSC patient's survival. The x-axis represents time in days and y-axis shows the survival probability. The blue line indicates low/medium level expression and the red line indicates high level expression of the *CCND1* gene which returned a significant p value = 0.0073. The HNSC patients presenting with a high level expression showed a low survival probability when compared to patients with low/medium level expression. (b) The differential gene expression pattern of *CDKN2A* gene with HNSC patient's survival returned a significant p value = 0.00038. The red line indicates high level expression and the blue line indicates low/medium level expression of the *CDKN2A* gene in HNSC patients. Here, patients with low/medium level expression presented with a low survival probability when compared to patients with high level expression. A p value less than 0.05 is considered to be significant.

Zhang *et al.*, identified 28 differentially expressed genes while comparing the expression profiles of primary tumor of oral cancer exhibiting lymph node metastasis and tumors without lymph node metastasis. Among the genes identified *CCND1, JUN* and *SPP1* were found to be associated with the focal adhesion pathway. Silencing of the three genes in the oral cancer cell line OECM-1also proved that these genes play essential roles in cancer cell invasion. The replication of the results in clinical samples showed that the expression of these genes were associated with short survival and metastasis. A meta-analysis conducted by **Ramos-García *et al*,** indicated a significant association between *CCND1* amplification or overexpression in the progression of potentially malignant tumor of head and neck to HNSCC.

The *CCND1* or the cyclin D1 protein is known to play a critical role in G1 to S transition. A meta-analysis was conducted to assess genotype phenotype association between A870G with the risk of developing oral cancer. It was found that the G allele of *CCND1* gene was associated with a decreased risk of developing oral cancer in Asian population **(Wang *et al.*, 2013)**. **Lin *et al.*,** demonstrated that the harzard ratio for *CCND1* +870 A>G polymorphism with an increased tumor size and a poor survival

rate as assessed by log rank test and Cox proportional hazards model analysis.

Thus the present study identifies *CCND1* and *CDKN2A* as the candidate genes showing a strong association with HNSCC. However, further experimental validation of the results are warranted to confirm the role of these genetic alterations with the development and progression of HNSCC. Our institution is passionate about high quality evidence based research and has excelled in various fields **[16–37]**. We hope this study adds to this rich legacy.

CONCLUSION

The present study investigates the association of members of the cell cycle control gene family with HNSC. The results provided preliminary evidence on the fact that the genes *CCND1* and *CDKN2A* could play a vital role in the progression of HNSCC. Further experimental validation employing genotyping assays, functional analysis using animal models and population-wide screening of genetic variants could reveal the actual association of these genes with the development and progression of OSCC.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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