Genetic alterations in *Cullin* family of genes and their putative association with HNSCC.

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Abstract

The present study was conducted to analyse the mutations in *Cullin* genes and to determine their possible association with HNSCC. HNSCC comprises a heterogeneous group of malignancies predominantly affecting the squamous epithelium of the head and neck due to factors such as tobacco use and genetic mutations. Alterations in specific genes affect the encoded protein resulting in malignant transformation of normal cells. Identifying such alterations in genes could aid in early detection and diagnosis of HNSCCs. The *Cullin* family consists of eight genes which work in a cascade mechanism. The oncoprint data obtained after producing the query showed frequency distribution of gene mutations. These mutations were further analyzed using the gnomAD database, to identify mutations which are already reported and those which are novel.

Keywords: HNSCC, Mutation, Inhibition, Molecular marker, Novel, Variants, Apoptosis.

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Introduction

HNSCC comprises a heterogeneous group of malignancies predominantly affecting the squamous epithelium of the oral cavity, oropharynx, larynx and hypopharynx incident in more than one million individuals per year [1]. The primary cause of HNSCC is the uncontrolled use of tobacco and smoking which may result in genetic alterations thus paving way for tumor growth [2]. The Human Papilloma Virus is also considered as a significant risk factor in developing HNSCC [3]. Most HNSCCs in addition to other cancers can be identified based on the presence of certain tumour marker genes. Overexpression of these genes or an abnormality can help in early detection, diagnosis and treatment of cancers [4]. One such gene family is the *Cullin* gene. Increased expression of *Cullin* genes, particularly CUL4A, is the most common gene associated with breast cancer, hepatocellular carcinomas, adrenocortical carcinomas, medullary blastomas and other Head and Neck Squamous Cell Carcinomas (HNSCC) [5]. However, the mutations in certain genes are visible only at a later stage which reduces the prognosis of the disease [6].

The *Cullin* family of genes are molecular proteins whose actions are particularly useful in the post translational modification of various proteins. These hydrophobic *Cullin* genes are predominantly found in bacteria, mammals and plants [7]. The *Cullin* family consists of eight genes namely, CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 and CUL9 which on the whole provide reinforcement for ubiquitin ligases [8]. These genes also function in organ regeneration and blastema formation. The *Cullin* organised *Cullin* Ring Ligases (CRLs) help in ubiquitination of substrates proximal to the E2 ubiquitin ligase enzyme [9]. The activation of these *Cullin* genes and CRLs occurs by a cascade mechanism consisting of E1, E2 and E3 enzymes respectively. Further activation of CRL complexes parallels the conformational changes between carboxy terminal end and ring substitutes of *Cullin genes* and CRLs [10,11]. The overexpression of CUL4B gene is known to increase the growth of tumor cells and promote metastasis. Thus, overexpressed genes can be detected with ease at an early stage of cancer and hence act as potent molecular markers [12]. However, certain mutations or modifications in these genes show multi drug resistance character which potentiates the need for antimicrobials [13-16].

To comprehend the effect of overexpression of *Cullin* genes and its relation to the detection and diagnosis of head and neck squamous cell carcinomas, the present study was adopted. Further, the mutations in these genes can be used to develop therapeutic drugs against specific targets and microorganisms [14-17]. Our team has extensive knowledge and research experience that has translated into high quality publications [18-27]. The present study aims at understanding the mutations in the *Cullin* family of genes and how its overexpression has the potential to aid in early treatment of HNSCC.

Materials and Methods

Data source

The present study follows a retrospective study design. The cBioportal database was employed to obtain the patient data from various cohort studies [28]. The TCGA Firehose legacy consisted of 528 HNSCC cases of which 496 samples provided the copy number alteration data. Independent of this, the different mutations, alterations, amplifications and deletions of genes were available with respect to the genes present. The TCGA data set also provided information on the demographic details of the sample collected (Table 1).

Gender	Male (n=386)
	Female (n=142)
Mutation count	6-3181
Diagnosis age	19-90 years
Smoking status	Smokers: 515
	Data not available: 12
	Unknown: 1
Alcohol history	Yes-352
	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. *Demographic details of patients obtained from cBioportal database for the present study.*

The most significant genes part of the *Cullin* family which play a role in apoptosis were obtained by literature search- CUL1, CUL2, CUL3, CUL4A, CUL4B, CUL5, CUL7 and CUL9.

The final oncoprint data was obtained preceding the submission of user based queries to the cBioportal database and further analysis was conducted [29].

Oncoprint data analysis

The oncoprint data provides precise information of the frequency distribution of variations and mutations visible in each of the chosen genes for the study, the type of variations, changes in the proteins coding for amino acids and gene amplification.

The oncoprint data aids in linking the diseased genotype and phenotype **[30].**

gnomAD data analysis

This data represents the aggregation of different exome sequences which provides easy widespread access to the entire community. The newer mutations were identified while comparing already existing mutations recorded in the database [31].

Gene expression and survival analysis

The expression of the gene presenting with highest frequency of gene alteration in HNSCC was analysed using the UALCAN database. Survival curve analysis based on the tumor grade and expression profile was performed to demonstrate the putative role of *Cullin* family of genes with HNSCC. Gene expression data is expressed as Transcripts Per Million (TPM) which is a normalization method for RNA-seq data. Combined survival effect analysis of gene expression and other clinical parameters such as race, gender, tumor grade, cancer subtypes were assessed using log-rank test that generated a p value which was further used to indicate statistical significance of survival correlation between groups. The t tests were performed using PERL script with the Comprehensive Perl Archive Network (CPAN) module [32].

Results

The cBioportal database was employed as the primary database for the collection of samples from various cohorts and the TCGA among these was chosen for the present study. The data set consisted of 528 head and neck squamous cell carcinomas whose male to female ratio stood at 2.7:1. The data was collected from individuals who varied in age as 19-90 years. Based on origin and identity, 67% of the samples were American derived, 85.6% African origin, 9.1% Asian derived

and 2.1% American Indian derived. The samples also showed the presence of grade 2 neoplasms in 59% of the individuals (Figure 1).

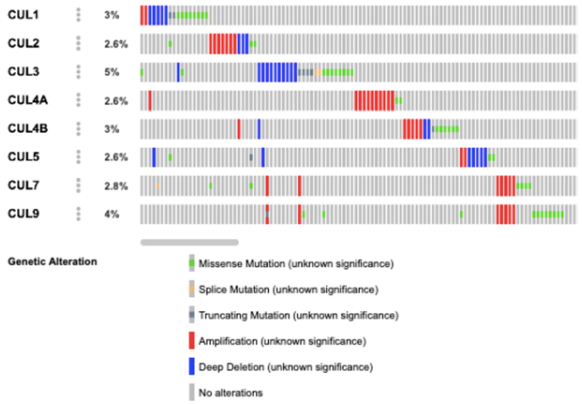

Figure 1. *Oncoprint data depicting the genetic alterations in Cullin family of genes in HNSCC patients.*

In order to comprehend the genetic alterations obtained, the oncoprint data analysis was suggested. From the table drafted for these variations, it was observed that the CUL3 gene showed the maximum amount of variations among all the 8 genes tested, which accounts for nearly 5% of the variations. The genes CUL1, CUL2, CUL4A, CUL4B, CUL5, CUL7 and CUL9 showed amplification mutation while deep deletion mutations were visible in CUL1, CUL2, CUL3, CUL4B and CUL5 genes. The highest number of genetic alterations *i.e.* 17 alterations was depicted by the CUL3 gene (Figure 1). Genetic variations expressed in E695*, Q584*, K521*, Q113*, Q163* were of the stop codon type of mutation. At particular loci, the expression of these genes prohibited the synthesis of various other proteins and hence prevented further divisions. L436FFS*9, N565IfS*18, R33SPfs*34 expressed frameshift mutations at their respective loci and altered the reading of the genes. Significant splicing mutations were demonstrated by the CUL3 gene. Gene alterations of R709Q and E56K correspond to contemporary mutations or pre-existing mutations (Table 2).

Gene	Protein coded	Cytogenetic loci	% of genetic alterations	Gene alterations		
CUL1	Cullin 1	7q36.1	3	Amplification		
				Deep deletion		
				E485K		
				E499D		
				E695*		
				E292K		
				E439K		
				Q584*		
				G79E		
				D239N		
				E215K		
				E194K		
				T501I		
CUL2	Cullin 2	10p11.21	2.6	Amplification		
				Deep deletion		
				D245H		
				I42V		
				D11H		
CUL3	Cullin 3	2q36.2	5	Deep deletion		
				E702D		
				D426H		
				R709Q		
				L321F		

				A115T		
				T509M		
				K521*		
				E206Q		
				M287R		
				Q113*		
				L418R		
				R709W		
				Q163*		
				L436FFS*9		
				X496_splice		
				X127_splice		
CUL4A	Cullin 4A	13q34	2.6	Amplification		
				Y227C		
				Q690P		
CUL4B	Cullin 4B	Xq24	3	Amplification		
				Deep deletion		
				E900K		
				D812H		
				Y431S		
				Q36*		
				T726S		
				F606L		
				P688Q		
CUL5	Cullin 5	11q22.3	2.6	Amplification		
				Deep deletion		
				K232N		
				N565Ifs*18		
				S771C		
				S503Y		
CUL7	Cullin 7	6p21.1	2.8	Amplification		
				K1431N		
				E1334K		
				S149N		
				E1533K		
				Q549R		
				A1481=		
				R1094H		
				S1531N		

				P409L		
CUL9	Cullin 9	6p21.1	4	Amplification		
				R335Pfs*34		
				N1268D		
				M189V		
				D1554N		
				E56K		
				R1409W		
				R1519W		
				R1945Q		
				L481F		
				T159S		
				H1599Y		
				E292K		

Table 2. *Gene alterations in Cullin family of genes as obtained from the oncoprint data.*

Changes in the DNA which led to shortening of proteins or in other words truncating mutations were expressed by the CUL3 gene only. Significant missense mutations were visible in CUL1, CUL3 and CUL9 genes.

From the oncoprint data it was observed that amplification of CUL9 gene was accompanied by amplification of CUL7 gene in the same HNSCC patients. Gene expression profile analysis for the CUL3 gene in normal individuals and in patients suffering from HNSCC, it was noted that the expression of this gene was significantly down regulated in individuals' wth HNSCC.

The 'p' value was found to be 2.199 x10-4 suggesting that the decline in expression of CUL3 gene was significant (Figure 2).

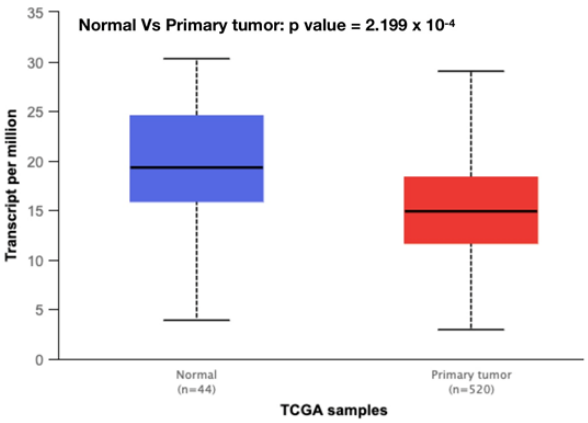

Figure 2. *Box whisker plot showing the gene expression profile of CUL3 in HNSCC based on sample types (blue bars represent normal; red bars represent primary tumor). The gene expression was found to be statistically significant with a p value of 2.199 × 10^{-4}.*

The Kaplan-Meier plot for the CUL3 gene expression among different groups was assessed further. When low/medium expression males and high expression males were compared, it was found that the survival rates between the two groups had a significant difference suggesting that high expression males had a higher chance of survival. Similarly, on comparing high expression males and low expression females, it was predicted that a higher survival rate for high expression males (Figure 3 and Figure 4).

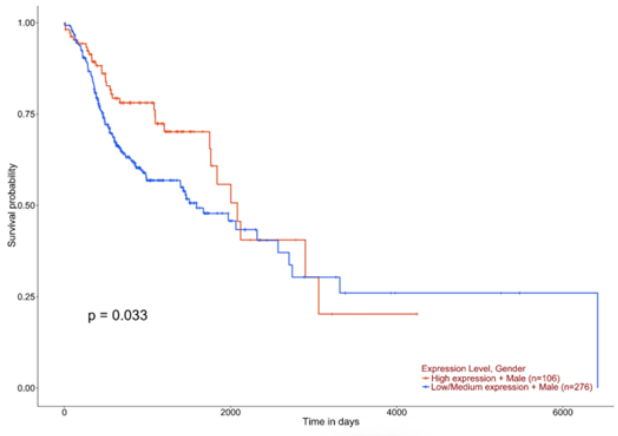

Figure 3. *Kaplan Meier plot showing the effect of CUL3 expression level and gender on HNSCC patients. The x-axis represents the time in days and the y-axis represents the survival probability. The blue line indicates low expression of CUL3 in males and the red line indicates high expression in males. A significant difference in the level of gene expression between the two groups were observed (p=0.033); p<0.05-significant.*

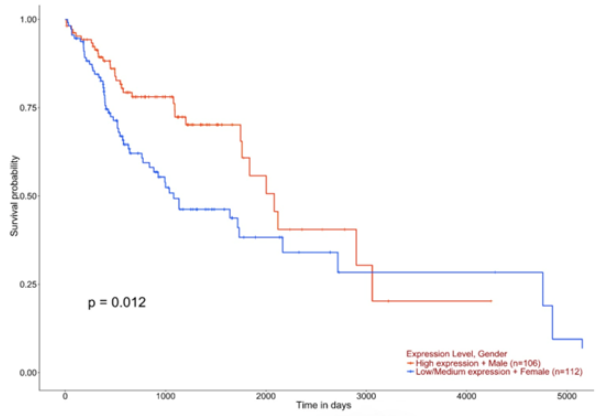

Figure 4. *Kaplan Meier plot showing the effect of CUL3 expression level and gender on HNSCC patients. The x-axis represents the time in days and the y-axis represents the survival probability. The blue line indicates low/medium expression of CUL3 in females and the red line indicates high expression in males. A significant difference in the level of gene expression between the two groups was observed (p=0.012); p<0.05-significant.*

Discussion

Head and neck squamous cell carcinoma arises either due to genetic factors or acquired factors such as smoking and chewing of tobacco and accumulation of reactive oxygen species and free radicals occur in the body which lead to various pathological changes and destruction of normal tissues [33]. The severity of these diseases is attributed to the inflammatory factors caused by microbes, genetic, epigenetic factors and the quality and quantity of the genetic variations induced [34,35]. Various molecular markers are used for the detection and diagnosis of HNSCC. The overexpression of the CUL3 gene in particular helps in metastasis and progression of cancer cells. These genes also alter the enzymes involved in the cellular processes such as matrix metalloprotease which can be detected by various screening methods [36]. Even a slight increase in the levels of CUL3 gene can prove to be useful in the detection of HNSCC. The functioning of these *Cullin* genes, their apoptotic pathway inhibition and overexpression have been discussed. The *Cullin* genes were also capable of inhibiting the replication process and acting as stop codons [37].

Based on previous researches conducted, it was found that over 70% of the mutations in the *Cullin* family corresponded to missense mutations while 20% were nonsense mutations and 10% were splice mutations [38]. The present study showed that maximum mutations were a resultant of amplification of *Cullin* genes while splice mutations were expressed in significantly lower concentrations in the CUL3 gene. The survival rates of different groups were compared with the existing studies. The survival rates in high risk individuals *i.e.* individuals more prone to HNSCC have significantly lower survival rates when compared with low risk individuals. In addition, patients with low risk of HNSCC expressed histological gradings of G2 and G3 neoplasms. When correlated with the present study, the individuals expressed G2 levels of neoplasm. The results are contraindicated to the present findings [39].

The combined effects of CUL3 gene and other molecular markers prove to be effective in inhibiting certain pathways responsible for the growth and metastasis of cancer cells in the body. In comparison with existing research, the *Cullin* genes overexpression and mutations and contraindicated but the survival analysis of the present study are in accordance with the survival rates obtained for various groups performed in the previous researches [40].

In addition to the effects against HNSCC, the *Cullin* family of genes are also well known for inhibiting the growth of tric cancer cells and oral cancer. The *Cullin* genes along with other markers such as EGFR can inhibit regulatory pathways that aid in the proliferation of these cancers [41]. This inhibition occurs by interfering with various cellular processes such as proliferation, differentiation and apoptosis. The post translational modification helps in activation of the CRL molecules [42]. Moreover, these genes can improve the efficiency of the DNA repair mechanisms after genetic insults and in turn protect the genome from further damage [43].

The results of the present study were enhanced by the facts and results of existing research [44-47]. Other types of studies involving microbial targets have been studied by us employing in silico tools [48-50]. The present study serves certain limitations due to the limited sample size and restriction to the effects in a particular type of population. Despite these limitations, the present study proves to be useful as a base for advanced researches and development of novel drugs against these diseases which are influenced by properties such as membrane permeability, bioavailability and various genetic components which could be significant in the early detection and diagnosis of head and neck squamous cell carcinoma.

Conclusion

In conclusion, the present study highlights the importance of molecular markers and the usefulness of the *Cullin* family of genes in the detection of head and neck squamous cell carcinomas. Furthermore, the expression and mutations of these genes prove to be useful in predicting the prognosis and survival rates of HNSCC individuals.

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Conflict of Interest

The authors declare no conflict of interest.

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