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Computational Approach to Identify Mutations in Genes of Notch Signaling Pathway and Its Association with OSCC

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Authors' contributions

This work was carried out in collaboration among all authors. Author JVP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ASSG and AP managed the analyses of the study. Author MS managed the literature searches and certain computational analysis. All authors read and approved the final manuscript.

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ABSTRACT

Derailments in signal transduction pathways are associated with the development of tumors. One such vital pathway is the *Notch* signaling pathway which is associated with various processes of carcinogenesis such as proliferation of cells, cell renewal, angiogenesis and oncogenic microenvironment preservation. Interestingly, Notch also plays a pivotal role in tumor development by acting as an oncogene as well as tumor suppressor gene. In view of this fact, the present study was designed to analyze mutations in Notch signalling pathway which might have a crucial role in

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the etiology of oral squamous cell carcinoma (OSCC) using computational approach. The Cancer Gene Atlas data set hosted in the cBioportal was used in the present study. These samples were queried for the presence of mutations in Notch signalling genes which included a predefined list of 55 genes. Further, the Oncoprint data obtained was compared to that of gnomAD database which identified novel and reported mutations in the genes analyzed. Additionally, I-Mutant and MutPred analysis was carried out to determine the stability and pathogenicity of the variations recorded. Among 55 genes analysed, *SPEN* gene was shown to possess the highest frequency of mutation (5%) followed by *FBXW7, Notch1, EP300, NUMB,* and *RBPJL* genes. Most of the mutations identified were novel as assessed using the control dataset from the gnomAD database. The stability of the protein was found to decrease upon nucleotide substitution. Finally, the MutPred score revealed that most of the mutant proteins were pathogenic. Several novel mutations have been identified in the pathway analyzed. Functional analysis of these variants using experimental approaches would aid in dissecting their association with OSCC.

Keywords: Oral cancer; Notch; SPEN; in silico; mutations.

1. INTRODUCTION

Asia ranks top most in the incidence of cancer with the highest incidence rate of 48.4%, followed by Europe (23.4%), America (21%), Africa (5.8%) and Oceania (1.4%). Cancer of lip and oral cavity occupies the 16th position among all cancer types, with 11th and 19th position among male and female, respectively, worldwide [1]. Oral cancer is considered to be the major public health problem in the Indian subcontinent [2]. According to Globocan, 2018, the incidence of oral cancer in India ranks the second among all cancers in both the sexes and first and fourth among major cancer types in male and female, respectively [1]. The major difference in incidence of oral cancer might be attributed to ageing of population and prevalence of specific risk factors [3]. Individuals in the low-income groups are mostly affected due to exposure to risk factors such as tobacco chewing, smoking, alcoholism etc., which exerts an adverse effect on the DNA.

Although oral cancer presents as a multifactorial trait the underlying genetic mechanism needs to be dissected so as to gain knowledge about the disease pathogenesis. Alterations in the signalling pathways have been linked to the development of tumours in the oral cavity. Some of the signal transduction pathways which have been studied extensively in head and neck squamous cell carcinoma are the Phosphoinositide 3-kinase (PI3K), Ras homologue (Rho) and TGFβ/SMAD pathways [4,5]. These pathways have been implicated in neoplastic transformation, tissue invasion and metastasis [6].

The Notch signalling pathway is an evolutionarily conserved pathway mainly involved in cell-cell communication [7]. The fate of the cells at each stage of embryonic development is decided by the genes of this cascade. Any dysregulation of this pathway is associated with genetic disorders including cancer. Several reports have demonstrated the pivotal role of *Notch1* in the development of cancer. The expression of Notch1 was found to be downregulated in epithelial dysplasia [8]. Tumor inducing effect is promoted by the loss of Notch1, whereby the integrity of the barrier is lost creating a wound like environment in the underlying stroma [9]. Computational evaluations on OSCC data showed that Notch1 was the fourth highest protein of interest related to oral cancer. Loss of function mutations of the Notch1 gene have been found in approximately 10% of OSCC cases [10]. Inhibition of Notch signalling has been proposed as an alternative adjuvant therapy for radio and chemotherapy [11,12].

2. MATERIALS AND METHODS

2.1 Sample Data Set

The cBioPortal for Cancer Genomics (http://cbioportal.org) integrates an exhaustive collection of molecular profiling information from cancer tissues and cell lines [13,14]. The database is user friendly and hosts genetic, epigenetic and proteomic information of the cases registered. The sample data set includes sequence information of forty oral squamous cell carcinoma cases (OSCC) which is used for the study. Demographic details of cases in the Oral Squamous Cell Carcinoma (MD Anderson, Cancer Discov 2013) dataset were recorded.

2.2 Mutation Analysis

A single cancer query for mutation analysis was initiated by selecting the oral squamous cell

carcinoma cases from the cBioPortal database. The case set included forty sequenced tumors which were analyzed for mutations in genes associated with Notch signalling pathway. The gene cluster includes *ADAM10, ADAM17, APH1A, APH1B, ARRDC1, CIR1, CTBP1, CTBP2, CUL1, DLL1, DLL3, DLL4, DTX1, DTX2, DTX3, DTX3L, DTX4, EP300, FBXW7, HDAC1, HDAC2, HES1, HES5, HEYL, ITCH, JAG1, JAG2, KDM5A, LFNG, MAML1, MAML2, MAML3, MFNG, NCOR2, NCSTN, NOTCH1, NOTCH2, NOTCH3, NOTCH4, NRARP, NUMB, NUMBL, PSEN1, PSEN2, PSENEN, RBPJ, RBPJL, RFNG, SNW1, SPEN, HES2, HES4, HES7, HEY1, HEY2.*

2.3 OncoPrint Data Analysis

Submission of query returned a window with OncoPrint data which demonstrates the presence of mutations in crucial genes associated with the Notch signaling pathway. The somatic mutation frequency and the site of mutation in the candidate genes were documented.

2.4 Protein Network Interactions

The network of proteins interacting with Notch1 signalling pathway was assessed by submitting the query gene in the STRING database [15].

2.5 gnomAD Analysis

The genome aggregation database (gnomAD) is an exhaustive collection of data spanning 125, 748 exome sequences and 15,708 whole genome sequences from unrelated individuals sequenced and deposited as part of various disease-specific or population genetic studies. This data source was used to verify whether the variants identified in the present study are reported elsewhere in the other populations. The search could also provide an insight about the minor allele frequency of the variants in the population by which nature of the variants can be ascertained [16] (Table 2).

2.6 Protein Stability Analysis

I-Mutant v3.0 is a support vector machine (SVM)-based tool for the automatic prediction of protein stability changes upon single point mutations. The software's predictions are based on the protein sequence. The predictions were classified into three classes: neutral mutation ($-0.5 \leq DDG \geq 0.5$ kcal/mol), large decrease (<-0.5 kcal/mol), and a large increase (>0.5 kcal/mol). The free energy change (DDG) predicted by I-Mutant 3.0 is based on the difference between unfolding Gibbs free energy change of mutant and native protein (kcal/mol) [17].

2.7 MutPred Analysis

MutPred v2 is a standalone and web application developed to classify amino acid substitutions as pathogenic or benign in humans. The wild-type protein sequence in FASTA format is used for the purpose and the substitution sites identified. The probability of the mutation being deleterious is reported (http://mutpred.mutdb.org/).

Table 1. Demographic details of cases in the oral squamous cell Carcinoma (MD Anderson, cancer discov 2013) dataset

Demographic features	Cases (N=40)
Gender distribution (Male: Female ratio)	Male = 28
	Female = 12
Diagnosis age	26 - 85 years
HPV status	Positive: 1
	Negative: 11
	Not detected: 28
Smoking status	Smoker: 29
	Non-Smoker: 11
Daily alcohol	Alcoholic: 9
	Non-alcoholic: 31
Mutation count	10 - 173

Table 2. List of genes carrying mutations involved in NOTCH signaling pathway in oral squamous cell carcinoma patients

Gene	Type of mutation	Frequency	Amino acid change	gnomAD analysis	Mutant protein stability	MutPred analysis
NOTCH1	Truncated mutation (PD)	2.5%	Q290* (Stop)	Novel	*	*
SPEN	Missense (US)	5%	Y626C	Novel	Decrease	0.449
			S725R	Novel	Increase	0.102
FBXW7	Missense (US)	2.5%	R505G	Novel	Decrease	0.849**
EP300	Missense (US)	2.5%	C1385Y	Novel	Decrease	0.838**
NUMB	Missense (US)	2.5%	R630H	Novel	Decrease	0.202
RBPJL	Missense (PD)	2.5%	A253= †		†	†

US – Unknown Significance, PD – Putative driver.
** - Stop codon – truncated protein, † – Splice site mutation*
*** - Highly pathogenic*

3. RESULTS AND DISCUSSION

The OSCC dataset obtained from the cBioPortal site included 40 completely sequenced samples from patients with a diagnosis age between 26-85 years. All the demographic details were made available for the users in the cBioPortal database. Number of male participants (70%) was more in the study group when compared to females (30%). Among the 40 individuals, 72.5% were smokers, 27.5% were non-smokers and 22.5% were alcoholic. The HPV (human papillomavirus) statuses of the participants were recorded for 12, out of which one was positive and the others tested negative. The query submitted in the cBioPortal pipeline produced results, revealing mutations in the *SPEN, FBXW7, Notch1, EP300, NUMB,* and *RBPJL* genes. The gene alterations were of missense, nonsense and splice site mutations. Mutation frequency was observed to be highest in the *SPEN* gene (5%), whilst the other genes showed the same frequency of mutation (2.5%). Mutation in *Notch1* and RBPJL genes produced truncated or nonfunctional proteins due to nonsense and splice site mutations respectively (Figs. 1 and 2).

The protein interaction network reveals the major interactions of *SPEN* with genes such as *RBPJ, HEY1, Notch1, MAML2, MAML1, EP300, NCOR2, RBBP8, KDM1A, CTBP1* which are crucial regulators of signal transduction pathways (Fig. 3). The *SPEN* gene encodes a transcriptional regulator which negatively regulates Notch1. EP300 gene encodes E1A binding protein p300 which functions as histone acetyltransferase and regulates transcription via chromatin remodeling, Notch1 encodes receptor which binds to membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination, HEY1 is a downstream regulator of Notch signaling pathway. MAML1 and MAML2 act as a transcriptional coactivator of the Notch pathway. RBPJ is a transcriptional activator of Notch target genes. RBBP8 is a retinoblastoma binding protein 8, which plays an important role as a cell cycle arrest during DNA damage. KDM1A is a lysine specific demethylase, which is indirectly involved in the repair of double stranded breaks via homologous recombination and CTBP1 possesses dehydrogenase activity and is involved in cell cycle regulation. Protein stability analysis performed using I-Mutant software revealed that the substitution of amino acid decreases the stability of the protein in all the mutation encoded proteins except S725R mutation of *SPEN* gene. Additionally, the MutPred score identified several mutations to be highly pathogenic (score >0.50) (Table 2).

Notch signalling is an evolutionarily conserved process which operates in different developmental stages of the cell. Dysregulation in these signalling molecules are often associated with cellular transformation which leads to cancer [18]. The pathway holds a number of receptors (Notch 1-4) and ligands such as Jagged1, Jagged2, DLL1, DLL3 and DLL4) in mammals. Activation of Notch receptors is ligand mediated, which releases Notch intracellular domain (NICD) into the nucleus.

In the nucleus, NICD binds to RBPJ (DNA binding protein) and co-activator MAM (Mastermind transcriptional co-activator) thereby stimulating transcription of target genes [19]. However, the role of Notch signalling in cancer is

Fig. 1. Oncoprint data demonstrating alterations in the genes involved in Notch signaling pathway in OSCC cases

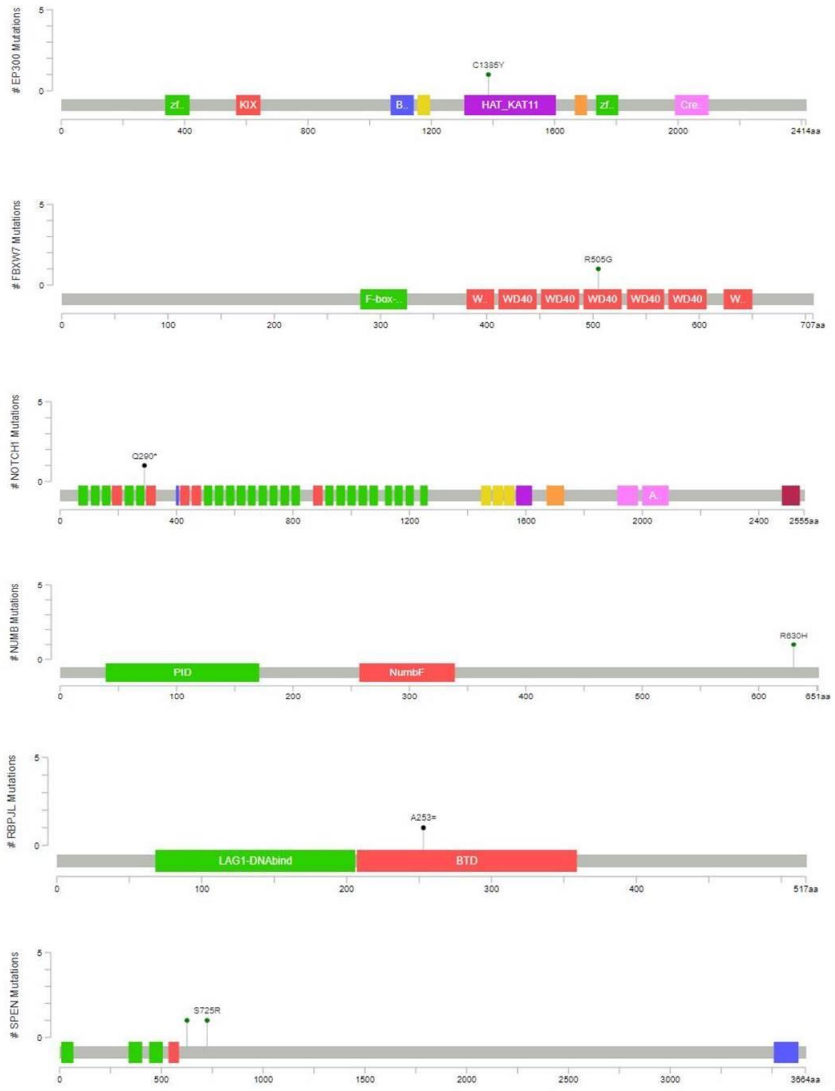

Fig. 2. Mutations located in the genes in the Notch signaling pathway with highest frequency

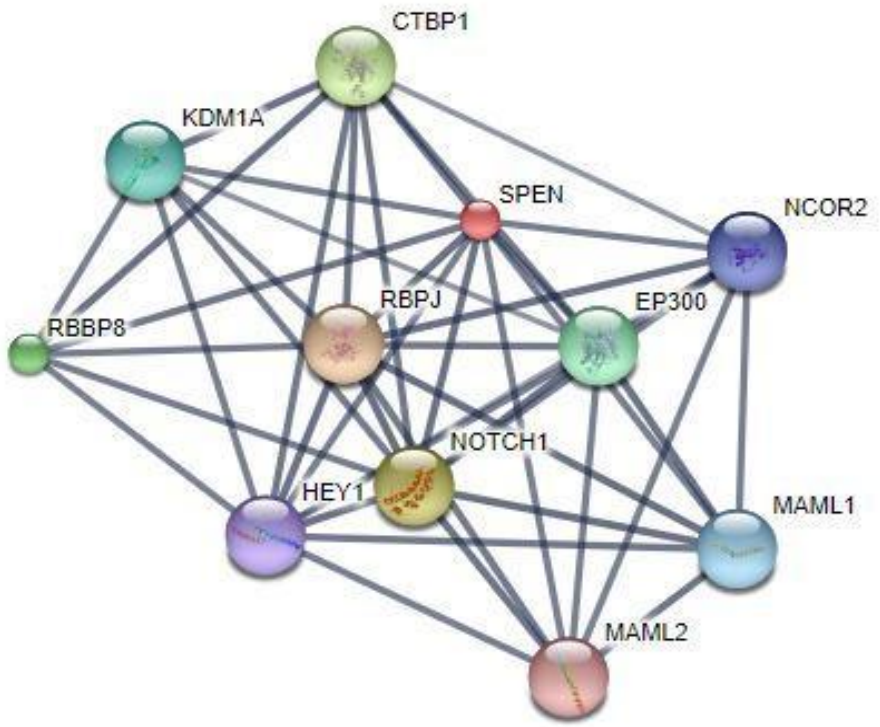

Fig. 3. The protein network interactions of SPEN gene in the Notch1 signaling pathway

cell dependent. Notch*1* is considered to be a tumor suppressor in some types of cancers such as hepatocellular carcinoma, small-cell lung cancer, prostate cancer and cervical cancers [20,21]. It activates p53 leading to cell cycle arrest and apoptosis [22]. In contrast, overexpression of NOTCH1 was demonstrated in cutaneous squamous cell carcinomas [23]. Also, inactivating mutations are detected in 10-15% of HNSCC cases [24].

Although numerous studies have substantiated the role of Notch signalling genes in association with OSCC, there remain several discrepancies related to the opposing roles of Notch. There is evidence which elucidates the oncogenic potential and tumor suppressor effect of Notch [25]. A cDNA microarray study conducted by Leethanakul et al., 2000, reported overexpression of Notch1, Notch2, Jag1 etc., in microdissected tumour cells of HNSCC cases [26]. This finding was justified by other researchers who found the expression of Jagged1 to be increased in dysplastic tissues in comparison with normal epithelial tissues [27]. Furthermore, stronger associations were identified when increased protein levels of *Notch1*, Hes1 and Jag1 was reported in oral dysplasia compared to normal mucosa [28,29].

The next generation and exome sequencing had slowly aided in unraveling the tumour suppressive effect of Notch signalling genes. A study reported that the overexpression of NICD1 suppressed cell growth in tongue carcinoma cell line which was mediated by G0-G1 cell cycle arrest followed by apoptosis [30]. Another group of researchers found that Notch1 was the second most frequently mutated gene accounting for approximately 15% of all mutations in HNSCC. Our results report a frequency of 2.5% in Notch1 and other related genes, with the highest frequency being attributed to SPEN gene. SPEN gene mutation has been reported in a rare case of salivary adenoid cystic carcinoma [31]. A large proportion of mutations were predicted to produce truncated proteins. Additionally, mutations were identified in regions of FBXW7, which is a negative regulator of Notch1 [32]. The present study also reports a novel missense mutation R505G in *FBXW7* gene, which was found to decrease the stability of the protein and scored high (0.859) among all mutations observed. We identified a novel nonsense mutation Q290* in Notch1 and a splice site mutation in the *RBJPL* gene which were designated as putative driv

Several studies have reported about the involvement of Notch signalling pathway in inducing epithelial-mesenchymal transition (EMT). Expression of Notch1 remains to be crucial for the dysplastic changes happening in the oral squamous epithelium. Significant downregulation was observed in the case of oral neoplasia.[10] A recent study by Zhang et al., 2018, demonstrated that Notch signalling not only promotes EMT but also aids in the metastasis of OSCC cells [33]. Blockage of Notch pathway with g-secretase inhibitor resulted in the downregulation of EMT marker, Snail and vimentin and upregulation of E-cadherin. The present study throws light on one of the vital pathways associated with OSCC. Identification of mutations in the OSCC population and comparison of the same with the reference genome database has clearly identified most of the mutations as novel and pathogenic [34]. Experimental studies should also be designed so as to provide concrete evidence on the association of Notch1 mutations with the disease phenotype.

4. CONCLUSION

The mutations identified using computational tools serves as a primary resource of information to further probe into the disease condition. Notch1 signalling is a less understood pathway and its association with OSCC has not been dealt with in an extensive manner. The present study has some limitations such as, (a) the population studied does not include a larger proportion of Asians, (b) the mutations identified may not always precipitate the disease phenotype, but can indirectly influence the pathways associated with the disease phenotype. Hence, the missense mutations identified in the present study has to be replicated in south Asian population so as to arrive at a conclusion about the role of the pathway in establishing the disease phenotype.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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