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Genetic Aberrations in Caspase Family of Genes and Their Possible Association with HNSCC

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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 KN managed the literature searches and performed certain computational analysis. All authors read and approved the final manuscript.

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ABSTRACT

The cell suicide pathway of apoptosis is a necessary event in the life of multicellular organisms. It is involved in many biological processes ranging from development to the immune response. Over expression of interleukin-1β-converting enzyme (later renamed caspase-1) was shown to be sufficient to induce apoptosis in mammalian cells. The present study aims to assess the gene alterations in the Caspase family of cytochromes so as to derive an association with HNSCC. Earlier eleven genes were found in the human genome to encode 11 human caspases, caspase-1 to caspase-10 and caspase-14, which is now populated to 13, whereas 10 genes were found in the mouse genome to encode 10 murine caspases including caspase-1, 2, 3, 6, 7, 8, 9, 11, 12 and 14.

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Caspases share a number of features distinguishable from other proteases. The analysis follows an observational study design, employing several computational tools to identify and predict the possible outcomes of gene alterations identified in HNSCC patients. cBioportal server was used to identify the gene alterations which was further analyzed using tools such as PROVEAN, I-Mutant and gnomAD. Several reported polymorphic variants were also identified. The pathogenicity and protein stability of gene alterations documented in the present study were identified at standard biological conditions. Further experimental studies would provide concrete evidence on the association of the observed genetic abnormalities with HNSCC especially in individuals exposed to habitual carcinogens.

Keywords: Caspase family gene; HNSCC; in silico; mutations; amplification; deletions.

1. INTRODUCTION

Head and neck squamous cell carcinomas (HNSCCs) are an aggressive, genetically complex and difficult to treat group of cancers. It is a heterogeneous group of upper aero digestive tract malignancies which is considered to be the sixth most common type of cancer responsible for over 350,000 deaths every year [1]. Squamous cells are found in the outer layer of skin and in the mucous membranes, which are the moist tissues that line body cavities such as the airways and intestines [2,3]. HNSCC can develop in the mucous membranes of the mouth, nose, and throat. The classification of HNSCC based on its location is as follows: it can occur in the mouth (oral cavity), the middle part of the throat (oropharynx), behind the nose (nasal cavity and paranasal sinuses), near the nasal cavity (nasopharynx), the voice box (larynx), or near the larynx (hypo pharynx). Depending on the location, the cancer can cause abnormal patches or ulcers in the mouth and throat with unusual bleeding or pain in the mouth. It can also lead to sinus congestion that does not clear, sore throat, ear pain and pain while swallowing or difficulty swallowing. Other complications include a hoarse voice, difficulty breathing, or enlarged lymph nodes [4,5].

Apoptosis, or programmed cell death, is an essential physiological process that plays a critical role in development and tissue homeostasis [6]. The process of apoptosis is regulated in an orderly way by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals. Caspases, closely associated with apoptosis, are aspartate-specific cysteine proteases and members of the interleukin-1β-converting enzyme family. The activation and function of caspases, involved in the delicate caspase-cascade system, are regulated by various kinds of molecules, such as the inhibitor of apoptosis protein, Bcl-2 family proteins, calpain, and Ca2+. Caspases, the interleukin-1β-converting enzyme family proteases, are highly homologous to *Caenorhabditis elegans* cell death gene CED-3. Fourteen caspases have been identified so far, all of which share some common properties: they are all aspartate-specific cysteine proteases [7]. Based on their homology in amino acid sequences, caspases are divided into three subfamilies such as apoptosis activator which include caspase2, caspase 8, and caspase 9; apoptosis executioner which includes caspase3, caspase 6, caspase 7: inflammatory mediators which include caspase1, caspase4, caspase5, caspase 11, caspase 12, caspase 13, caspase 14 [8]. Computational approaches have been found to be more appropriate in screening for genetic alterations in a complex phenotype such as cancer. The availability of a huge number of samples and the user-friendly algorithms have made the analysis simple. In line with the above facts, the present study has been designed so as to identify the genetic alterations which would have a putative association with HNSCC.

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 [9,10]. The database is user friendly and hosts genetic, epigenetic and proteomic information of the cases registered. The sample data set includes 528 HNSCC cases (530 samples) of which 504 samples harboured information about copy number variations and sequence data. The demographic details of cases in the head and neck squamous cell carcinoma dataset were recorded.

2.2 Oncoprint Data Analysis

Submission of user defined query of 13 genes belonging to the family of CASPASE (Data not shown) returned a window with OncoPrint data which demonstrated the presence of alterations in genes of the caspase family viz., *CASP1, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8, CASP9, CASP10, CASP12, CASP14, CASP16P*. The somatic mutation frequency and the site of mutation in the candidate genes were documented [10].

2.3 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 [11].

2.4 Protein Stability Analysis

The I-Mutant server was used for prediction of protein stability changes upon single nucleotide mutations leading to change in the amino acid being encoded by the triplet codon. The server uses either protein sequence or structure to predict stabilization and destabilization of protein structure in the majority of cases. The prediction was based on running the query with protein sequence downloaded in the FASTA format from the public domain (https://www.ncbi.nlm.nih.gov/protein/). Upon substitution with the variant amino acid the stability changes were further assessed using the free energy stability change ($\Delta\Delta G$) value. A value less than 0 and greater than 0 implies decrease and increase in protein stability respectively [12].

2.5 Pathogenicity Analysis

PROVEAN (Protein Variation Effect Analyzer) predicts the impact on the biological function of a protein upon substitution with an amino acid. The present analysis employs a user defined query of missense variants entered along with the reference sequence obtained from the NCBI database with a default cut-off value of -2.5. The results returned scores based on amino acid substitutions and classified them as either neutral or deleterious depending on the PROVEAN scores [13,14]. A score less than -2.5 or greater than -2.5 was considered to be deleterious and neutral respectively.

3. RESULTS AND DISCUSSION

The dataset [15] included in the present study had information on 528 HNSCC patients (530 samples). The male: female ratio was found to be 2.7: 1, with age groups ranging from 19 - 90 years. The number of individuals with the history of smoking and alcohol was roughly around 98% and 67%. The dataset had samples from patients of American (85.6%), African (9.1%), Asian (2.1%) and American Indian (0.4%) descent**.** The distribution of patients based on the histologic grade of neoplasm is given in Table 1, of which 59% of patients had grade 2 tumor.

The oncoprint data analysis revealed gene amplification in 13 genes, of which *CASP8* (13%) harboured the highest frequency of gene amplification. The genes *CASP1, CASP2, CASP3, CASP4, CASP5, CASP8, CASP9, CASP10,* and *CASP12* demonstrated deep deletions. Although the site of amplification is not available 8 patients showed amplification in four genes viz., *CASP1, CASP4, CASP5* and *CASP12*. Interestingly, 8 patients had deep deletions recorded in the same set of genes (Fig. 1). *The CASP8* gene harboured the highest number of variations/mutations from among all the genes identified with alterations. Several truncating and mis-sense variants of unknown significance have been documented (Fig. 1, Table 2). A total of 5 previously reported variants were identified using gnomAD analysis viz., *CASP1 (rs2509649), CASP2 (rs898981516), CASP4 (rs776381340), CASP9 (rs201284755), CASP10 (rs151011448)*. The above variants identified in the present study had a minor allele frequency < 0.01, implying the fact that these are rare variants which may be associated with risk of a particular disease.

Stability of the protein largely affects the biological function of the protein. Hence, protein stability was assessed for all the non-synonymous variants identified in the study. Majority of missense variants observed were found to decrease the stability of the protein product, thereby giving away a chance for influencing the catalysis process. Although

presented with decreased stability all the variants were not found to lead to a deleterious phenotype. Interestingly majority of the variants produced neutral effect with exception in a few gene variants exhibiting deleterious outcomes viz., *CASP2, CASP5, CASP6, CASP7, CASP10 AND CASP14* (Fig. 1, Table 3).

Table 1. Demographic details of patients analyzed in the present study (as obtained from the cBioportal site)

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

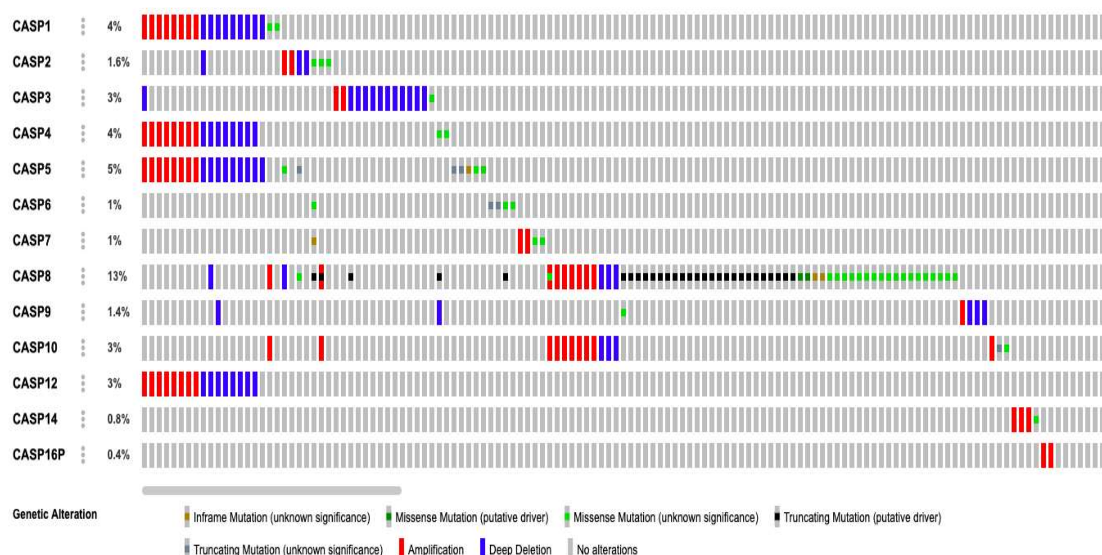

Fig. 1. Oncoprint data depicting alterations in caspase family of genes *viz., CASP1, CASP2, CASP3, CASP4, CASP5, CASP6, CASP7, CASP8, CASP9, CASP10, CASP12, CASP14, CASP16P*

Evasion of apoptosis is considered to be one of the hallmarks of human cancers. This cell death modality is executed by caspases and several upstream regulatory factors, which direct their proteolytic activity, have been defined as either tumor suppressors or oncogenes [16,17]. Apoptosis or programmed cell death process is a potent regulator for controlling death rate in normal cells. Any imbalances in this pathway have been documented to be associated with cancer phenotypes. Based on their implication in apoptotic pathways, caspases have been classified into two types as initiators and executioners. The caspase-2,-8,-9, and -10 belong to the category of initiators and caspase-3,-6, and-7 belong to the category of executioners [18]. Among all the caspase proteins, CASP8 was found to serve as an important component of extrinsic signalling which eventually leads to cell death. Several polymorphic sites were identified in the *CASP8* gene, among which a six-nucleotide insertion/deletion variant was found to have a direct effect on the regulation of CASP8 mRNA. In view of this fact, numerous studies have reported the association of polymorphism with various types of cancer including cancers of neuroblastoma, digestive tract, lung, prostate, breast and bladder [19]. Although this polymorphism produces controversial results in different population groups, it is worthwhile to study the effect of these variants or genetic markers in the South Asian population to derive an association between the same with HNSCC.

A study by Li et al, demonstrated the impact of survivin and caspase 3 on angiogenesis and apoptosis in oral cancer. The expression levels of survivin and caspase-3 was assessed in oral premalignant lesions such as oral leukoplakia with moderate and severe epithelial dysplasia, oral squamous cell carcinoma in comparison to normal oral mucosal tissues employing immunohistochemistry approach. The results gathered from the study revealed that survivin level was increased and that the levels of caspase-3 decreased during malignant transformation [20]. Another study conducted by Liu et al, reported that the protein levels of cleaved CASP3, 4, 8 and 9 were found to be significantly higher when compared to the adjacent normal tissues. Interestingly, the level of expression of CASP8 was high in patients presenting with lymph node metastasis. They concluded that cleaved CASP3 along with CASP3/8/9 can be considered as important prognostic markers for tumorigenesis in patients with oral tongue squamous cell carcinoma [21]. A study directed towards identification of risk factors associated with recurrence in oral squamous cell carcinoma (OSCC) found a significant association with CASP8. This gene was found to be altered in patients with recurrent phenotype of OSCC. In addition, copy number alterations, in cytogenetic location on chromosome 5 viz., 5p15.33 encoding TERT gene coding for telomerase reverse transcriptase was found to show a significant association with recurrent population [22]. Coutinho-Camillo et al, provided evidence on the role of microRNA expression upon target genes involved in apoptosis. Nearly seven different microRNAs were analyzed in OSCC samples in comparison to normal mucosal samples. Computational approaches had identified BCL2, CASP2, CASP7, CASP8 and DIABLO as target genes of the microRNAs observed [23]. Pickering and team identified driver pathways related to mitogenic signalling, Notch, tumor suppressor pathway involving TP53 and cell cycle control elements through comprehensive genome analysis in OSCC cases. Furthermore, two other potential drivers viz., *FAT1* and *CASP8* were also identified. Mutations identified in the CASP8 gene were most often associated with new molecular subtypes in OSCC [24].

Somatic mutations were a common presentation in oral cancer. Arunkumar et al, identified a variant in the *CASP8* gene (*rs1045487*), which was frequent [66.6%] in the oral cancer samples [25]. A study conducted by Chang et al., found that a metabolite in areca nut known as arecoline N-oxide (ANO) was found to induce *CASP8* which was the prime mediator of extrinsic apoptotic pathway. Somatic mutations in CASP8 were identified by whole exome sequencing and the upregulation of CASP8 was demonstrated using immunohistochemical staining. Also co-localization of increased CASP8 and proliferative cell nuclear antigen (PCNA) was observed. The conclusion derived from this experimental approach was that the upregulation of *CASP8* was involved in cell proliferation during the early stages of ANO-mediated tumorigenesis [26]. A very recent study by Singh et al, provided substantial evidence on the association of somatic mutations in *CASP8* gene in case of oral leukoplakia. Allele frequencies of mutated CASP8 were found to be high in case of leukoplakia, which then drives the process of tumor progression [27]. A decade old study carried out by Shinno et al, identified deletion of 1.5 Mbp in the D4S2623, which was identified

Table 2. The genetic loci, protein encoded, frequency of gene alterations in members of caspase gene family

Gene	Protein	Alteration	Loci	% of alteration	Variant allele frequency in tumor sample	gnomAD Frequency data
CASP1	caspase 1	Gene amplification	11q22.3	4		
		Deep deletion				
		G85E			0.02	rs2509649
		R143G			0.40	Novel
CASP2	caspase 2	Gene amplification	7q34	1.6		
		Deep deletion				
		A376T			0.17	rs898981516
		L88F			0.51	Novel
		E253D			0.20	Novel
CASP3	caspase 3	Gene amplification	4q35.1	3		
		Deep deletion				
		R111K			0.31	Novel
CASP4	caspase 4	Gene amplification	11q22.3	4		
		Deep deletion				
		R241H			0.15	rs776381340
		P150L			0.09	Novel
CASP5	caspase 5	Gene amplification	11q22.3	5		
		Deep deletion				
		R10Gfs*21			0.15	Novel
		A263E			0.21	Novel
		A330T			0.31	Novel
		X61_splice			0.13	Novel
		L174del			0.30	Novel
		V39G			0.21	Novel
CASP6	caspase 6	Gene amplification	4q25	1		
		L282V			0.43	Novel
		Q181*			0.22	Novel
		H58Y			0.20	Novel
		G45E			0.20	Novel

Gene	Protein	Alteration	Loci	% of alteration	Variant allele frequency in tumor sample	gnomAD Frequency data
		S280*			0.26	Novel
CASP7	caspase 7	Gene amplification	10q25.3	1		
		G236E			0.24	Novel
		E95K			0.35	Novel
		F182del			0.27	Novel
CASP8	caspase 8	Gene amplification	2q33.1	13		
		Deep deletion				
		Q339*			0.28	Novel
		R68*			0.25	Novel
		S375*			0.18	Novel
		Q97*			0.22	Novel
		R413*			0.37	Novel
		R432*			0.32	Novel
		R435*			0.44	Novel
		L315Efs*19			0.49	Novel
		M1?			0.21	Novel
		X184_splice			0.22	Novel
		L79Pfs*4			0.05	Novel
		Q465*			0.34	Novel
		K461Nfs.10			0.13	Novel
		X138_splice			0.34	Novel
		X137_splice			0.24	Novel
		X184_splice			0.37	Novel
		S115*			0.16	Novel
		Q107*			0.35	Novel
		I98Mfs*14			0.19	Novel
		K51Efs*19			0.25	Novel
		Q462*			0.14	Novel
		N414Tfs*24			0.19	Novel
		X268_splice			0.16	Novel

Gene	Protein	Alteration	Loci	% of alteration	Variant allele frequency in tumor sample	gnomAD Frequency data
		R233W			0.58	Novel
		G11R			0.66	Novel
		L62P			0.21	Novel
		R248W			0.12	Novel
		S99F			0.11	Novel
		R71T			0.33	Novel
		D308G			0.62	Novel
		L7V			0.27	Novel
		G11E			0.23	Novel
		T441I			0.12	Novel
		Y178del			0.19	Novel
		D303V			0.49	Novel
		T272_T273del			0.32	Novel
		I10T			0.41	Novel
		I38M			0.28	Novel
		Q97E			0.19	Novel
		I333M			0.30	Novel
		P394S			0.07	Novel
		Y421H			0.16	Novel
		M146V			0.43	Novel
		L315V			0.12	Novel
		L7R			0.28	Novel
		F24L			0.28	Novel
		L22F			0.11	Novel
		H304D			0.18	Novel
		T469I			0.21	Novel
		C313Y			0.23	Novel
		G268R			0.11	Novel
CASP9	caspase 9	Gene amplification Deep deletion	1p36.21	1.4		

Gene	Protein	Alteration	Loci	% of alteration	Variant allele frequency in tumor sample	gnomAD Frequency data
CASP10	caspase 10	R52W Gene amplification Deep deletion S29* Q511R	2q33.1	3	0.07 0.19 0.07	rs201284755 Novel rs151011448
CASP12	caspase 12 (gene/pseudogene)	Gene amplification Deep deletion	11q22.3	3	-	-
CASP14	caspase 14	Gene amplification R54T	19p13.12	0.8	0.22	Novel
CASP16P	caspase 16, pseudogene	Gene amplification	16p13.3	0.4	-	-

Table 3. Protein stability and pathogenicity analysis of mis-sense mutations identified in the genes of the caspase family

Gene	Protein	Mutation	I-Mutant Score	Protein Stability	PROVEAN Score	Pathogenicity prediction
CASP1	caspase 1	G85E	-0.87	Decrease	1.341	Neutral
		R143G	-0.21	Decrease	-5.005	Deleterious
CASP2	caspase 2	A376T	-1.03	Decrease	-1.460	Neutral
		L88F	0.61	Increase	-2.177	Neutral
		E253D	0.01	Increase	-0.595	Neutral
CASP3	caspase 3	R111K	-2.95	Decrease	-0.842	Neutral
CASP4	caspase 4	R241H	-1.60	Decrease	-3.230	Deleterious
		P150L	-1.08	Decrease	-4.283	Deleterious
CASP5	caspase 5	V263E	-2.10	Decrease	-5.987	Deleterious
		A330T	-0.92	Decrease	-1.475	Neutral
		V39G	-3.43	Decrease	-0.147	Neutral
CASP6	caspase 6	L282V	-0.02	Decrease	-2.777	Deleterious
		H58Y	1.47	Increase	-2.407	Neutral
		G45E	-0.58	Decrease	-7.191	Deleterious

Gene	Protein	Mutation	I-Mutant Score	Protein Stability	PROVEAN Score	Pathogenicity prediction
CASP7	caspase 7	G236E	0.56	Increase	-2.477	Neutral
		E95K	-1.77	Decrease	-0.473	Neutral
CASP8	caspase 8	R233W		Decrease	-6.440	Deleterious
		G11R	-1.14	Decrease	-3.029	Deleterious
		L62P	-1.59	Decrease	-5.948	Deleterious
		R248W	-0.57	Decrease	-3.487	Deleterious
		S99F	-0.54	Increase	-4.398	Deleterious
		R71T	1.02	Decrease	-3.899	Deleterious
		D308G	-0.45	Decrease	-6.007	Deleterious
		L7V	-0.52	Decrease	-2.395	Neutral
		G11E	-1.18	Decrease	-1.917	Neutral
		T441I	-1.34	Decrease	-3.728	Deleterious
		D303V	-0.98	Decrease	-6.641	Deleterious
		I10T	-0.93	Decrease	-4.108	Deleterious
		I38M	-4.16	Decrease	-1.617	Neutral
		Q97E	-1.13	Increase	-1.283	Neutral
		I333M	0.01	Decrease	-2.760	Deleterious
		P394S	-1.20	Decrease	-7.053	Deleterious
		Y421H	-1.69	Decrease	-4.465	Deleterious
		M146V	-0.51	Decrease	-2.793	Deleterious
		L315V	-0.168	Decrease	-2.860	Deleterious
		L7R	-1.49	Decrease	-4.982	Deleterious
		F24L	-1.19	Decrease	-5.290	Deleterious
		L22F	-2.57	Decrease	-3.460	Deleterious
		H304D	-0.33	Decrease	-7.962	Deleterious
		T469I	-0.74	Decrease	-5.204	Deleterious
		C313Y	-0.00	Decrease	-10.131	Deleterious
		G268R	-0.48	Decrease	-0.059	Neutral
			-1.65			
CASP9	caspase 9	R52W	0.25	Increase	-4.098	Deleterious
CASP10	caspase 10	Q511R	-2.02	Decrease	0.809	Neutral
CASP14	caspase 14	R54T	-0.46	Decrease	-0.650	Neutral

using fine mapping technique. The deleted region was found to harbor caspase 6 which was considered to be the tumor suppressor gene [28]. Although the present study has not reported any deletions in *CASP6* gene, missense and truncating mutations of unknown significance were identified. Computational analysis has aided in identifying potential genetic alterations in crucial genes [29]. Dysregulation of the caspase pathway will have a direct role to play in case of cancer and other inflammatory disorders [30].

The limitations of the study were (a) the dataset selected had individuals from different ethnic groups or populations. More specific analysis on a single group or population is required to establish the actual effect of gene alterations with HNSCC, as differences in ethnic groups may contribute to variations in allele frequencies associated with the variants identified. Each of the individuals might have been exposed to different group of chemical carcinogens or pollutants based on the geographical locations hence a precise association could not be derived in such situations, (b) since both genetic and epigenetic factors act simultaneously to exhibit a disease phenotype, more information on these factors are also necessary to relate the regulation of altered genes with the disease phenotype.

4. CONCLUSION

Advancements in computational approaches in biology have led to the development of methods which can be used for identification of genetic abnormalities within a short span of time in a cost effective manner. The present study is one such attempt to accumulate information related to the genetic abnormalities in the Capsase family and HNSCC. Probing into the genetic alterations related to HNSCC will open new avenues towards identification of potential targets which can be used to develop diagnostic as well as therapeutic leads in relation to HNSCC.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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