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The Impact of Specific High-Pathogenic Tp53 Mutations on Survival in Head and Neck Cancers

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ABSTRACT

Head and neck cancers (HNC) constitute about 30-40 % of all cancers in India. tp53 tumor suppressor gene is the most common gene mutated in HNC but has not been demonstrated to have any diagnostic or prognostic significance. therefore, we hypothesize that the survival rate and prognosis of head and neck cancer patients are linked with certain specific high-pathogenic tp53 hot spot mutations.

To evaluate the hypothesis, we conducted a retrospective study on a cohort of 100 HNC patients between April 2013 and April 2018. targeted deep sequencing was performed to identify specific somatic mutations. Kaplan–Meier analysis and log-rank tests were performed. Tp53 mutations were identified in 47 of the 100 cases, with dual mutations in 4 patients.

Functional characteristics displayed loss of function in 54.9% and gain of function in 27.5 % of cases. the majority of cases displayed missense aberration (74.5%), followed by nonsense mutations (11.76%). Of these pathogenic mutations, 33.3% fell into 6 hot spot residues while mini hot spot residues constituted 20.5% of the mutations.

There was no statistically significant difference in the median survival rate between patients with and without the out tp53 mutation. however, the median survival rate was linked with poor prognosis in patients with specific pathogenic hot spot mutations in 247, 248, 245, and 175 residues (p value= 0.028).

The majority of hot spot mutations (66%) showed a gain of function which may have been contributory to the pathogenicity of the disease. Results provide evidence that the existence of high-pathogenicity hot spot residues appears to be the only mutation that warrants further research to identify the therapeutic and prognostic significance of the tp53 gene in the treatment of HNC. further systemic studies are recommended to validate this hypothesis.

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Introduction

Head and neck cancers (HNC) constitute about 30–40% of all cancers in India and represent a major health problem with a mortality rate of around 50% [1,2]. The comprehensive genomic analysis by the cancer genome atlas (TCGA) has confirmed that mutations in gene tp53 are among the most common somatic mutations in HNC [3,4].

Despite this it has not been considered important for prognostication and treatment of the disease. The p53 gene consisting of 11 exons, 393 amino acids, and 25,772 bases forms several domains with different functions. More than 90% of pathogenic mutations in tp53 have been found in its DNA binding domain. Of these more than 30% occur at the six mutational hot spots which impair the transcriptional activity of p53 [5].

These mutations lead to the loss of function (LOF) of tumor suppression activity and result in a dominant-negative (DN) effect; conversely, the gain of function (GOF) also leads to an increase in oncogenic potential [6]. The p53 database of the international agency for research on cancer (IARC) has classified these mutations as missense, non-sense, silent, splice variants, frameshift, and others [7,8]. Although the association of tp53 mutations with carcinogens in tobacco has been well documented, these mutations have been reported independent of exposure to tobacco in literature [9].

Understanding the specific disruptions within the tp53 genes and correlating that with clinical findings may help in discovering potential avenues for future research.

The Hypothesis

The study hypothesizes that the survival rate and prognosis of head and neck cancer patients are linked with certain specific high-pathogenic tp53 hot spot mutations. To evaluate the hypothesis, a

retrospective study was conductedon a cohort of 100 HNC patients between April 2013 and April 2018. Targeted deep sequencing was performed to identify specific somatic mutations. We aimed to characterize tp53 mutation in our cohort of HNC based on types of mutations, their hot spot residues, its functional characteristics (GOF/LOF), location of codon on DNA binding domains, its association with habits, and the impact on overall survival for HNC in India. We also evaluated the median survival rate of patients with and without tp53 mutations and also compared different mutations.

Evaluation of Hypothesis

Method

To test our hypothesis, we conducted a retrospective study on a cohort of 100 HNC patients aged 26 to 75 years at a tertiary cancer hospital in south India between April 2013 and April 2018. The study was approved by the institutional ethics committee. A cohort of 100 patients with HNC who were subjected to mutational analysis was selected. Samples that displayed tp53 mutations (n=47) were further analyzed for understanding the type of substitutional change, change in function of mutation, functional analysis, and the location of codons on the specific exons. Out of the 100 patients included in the study, complete clinical data was retrieved for only 46 patients and these patients are subjected to further analysis including the clinical data on an anatomical subsite, stage at presentation, habit history, treatment protocol. The patient's survival status was calculated based on the follow-up update at the time of data collection.

Histopathology Reporting

The histopathology reporting for tumor type and tissue staging was done after preparing formalin-fixed paraffin-embedded (FFPE) blocks by following proper protocols of tissue preparation in the department of pathology. The percentage of viable tumor nuclei was also scored based on areas of neoplastic cells with respect to the total area on the slide. Estimated tumor nuclei≥20 percent was considered for the study.

DNA Extraction Protocol

Genomic DNA was isolated from 10-µm sections of the selected FFPE blocks in accordance with the manufacturer's instruction using a DNA extraction kit (QIAAMP ®DNA mini kits, QIAGENTM), quantified using qubit, and qualified using the Illumina Infinium assay kit (Illumina, San Diego, ca, USA).

Library Preparation, Sequencing, and Analysis

Amplicon sequencing was performed using TRUSEQ® amplicon-cancer panel (Illumina, San Diego, ca, USA), a highly multiplexed targeted resequencing assay for detecting somatic mutations across mutational hotspots. The PCR products were purified using agencourt ampure XP beads (Beckman coulter, Brea, ca, USA). The quality of the DNA libraries was assessed with an Agilent 2100 bioanalyzer (Agilent technologies, Santa Clara, ca, USA).

The normalized libraries were pooled and sequenced using the Illumina MISEQ system in 151-base-pair (bp) paired-end reads. paired-end NGS reads obtained from the Illumina MISEQ sequencer were aligned against the hg19 reference genome using the MISEQ reporter software from Illumina and the aligned files were imported into AVADIS NGS 1.5 for qc and downstream analysis.

Tp53 Mutation Subgrouping

The tp53 mutations were grouped according to the location of mutation or the functional effect of the mutation into:

- Specific exon in which the p53 mutation is located
- Mutations in the DNA binding domain
- Mutation type - missense/nonsense/deletion/frameshift
- Mutation function: gain or loss of function
- Mutations within hot spot residues: r175, r213, r245, r248, r273, and r282.

Statistical Analysis

The Kaplan–Meier technique was used to estimate survival, and log-rank tests were used to compare survival across groups. cox proportional hazard regression models were used to evaluate the simultaneous impact of several variables on survival. All stated hazard ratios (HRs) are based on these estimated models. all endpoints except disease were treated as censored observations in disease-free survival (DFS). Statistical significance was defined as a p-value <0.05.

Results

Descriptive Analysis and Mutational Landscape

Tp53 gene was mutated in a total of 47 primary tumors of 100 HNC patients. functional characteristics and types of mutations are analyzed in these 47 patients. (Table 1) the detailed mutational spectrum of tp53 genes (n=47/100) is provided in Table 1. the analysis revealed a preponderance of loss of function (56.5%) rather than the gain of function (28.2%) and some unknown mutations (19.5%). missense mutations (73.9%) were found to be most common while nonsense (13.0%), unknown (17.4%), deletion (2.1%), and frameshift (2.1%) mutations were also noted. the greatest number of mutations 44 (95.6%) of the total 47 were noticed between the exons 5-8, which is the DNA binding domain region of the p53 gene. 4 patients displayed mutations on two different (multiple) codons. One mutation was found on exon 4, 14 on exon 5, 6 on exon 6, 10 on exon 7, 13 on exon 8, and 2 on exon 10. There were 15.2% mutations at 273 codon, 8.6% at 248 codon, 6.5% at 245 codon, 6.5% at 196 codon, and 4.3% at 175, 179, 173, and 339 codons.

Table 1: Mutational Spectrum in Head and Neck Cancers

No	Site	C. DNA Change	Amino acid Change	Function	Type of mutation	DNA Binding Site	Exon
1	Tongue	c.536A>T	his179leu	GOF	Missense	YES	5
2	Tongue	c.517G>A	val173met	LOF	Missense	YES	5
3	Oral cavity	c.818G>A	arg273his	GOF	Missense	YES	8
4	Oral cavity	c.8189G>C	arg273pro	LOF	Missense	YES	8
5	Tongue	c.1015G>T	gly339ter	LOF	Nonsense	NO	10
6	Tongue	c.586C>T	arg196ter	LOF	Nonsense	YES	5
7	Tongue	c.592C>T	arg198ter	LOF	Nonsense	YES	6
8	Oral cavity	c.586C>T	arg196ter	LOF	Nonsense	YES	5
9	Oral cavity	c.844C>T	arg282trp	GOF	Missense	YES	8
10	Oral cavity	c.733G>A	gly245ser	LOF	Missense	YES	7
11	Oral cavity	c.646G>A	val216met	LOF	Missense	YES	7
12	Oral cavity	c.1024C>T	arg342ter	LOF	Missense	YES	10
13	Tongue	c.328C>T c.750T>A	arg110cys cys135ser	UK LOF	Missense Missense	NO YES	4 5
14	Oral cavity	c.722C>T	arg273his	GOF	Missense	YES	8
15	Tongue	c.722C>T	ser241phe	LOF	Missense	YES	7
20	Oral cavity	UK	pro301glnf-ster44	LOF	Frameshift	YES	8
17	Oral cavity	c.772C>T	ser241phe	LOF	Missense	YES	7
18	Oral cavity	c.404G>T	cys135phe	LOF	Missense	YES	5
19	Oral cavity	c.527G>T	cys176phe	LOF	Missense	YES	5
20	Oral cavity	c.818G>A c.742C>T	arg273his arg248trp	GOF GOF	Missense Missense	YES YES	8 7
21	Oral cavity	c.916C>T	arg306ter	LOF	Missense	YES	8
22	Oral cavity	c.848G>C	arg283pro	LOF	Missense	YES	8
23	Oral cavity	c.524G>A	arg175his	GOF	Missense	YES	5
24	Oral cavity	c.437G>A	trp146ter	UK	UK	YES	5
25	Oral cavity	UK	672_672+5de-linscctc	UK	Del	UK	UK
26	Oral cavity	c.664G>A	gly245 asp	LOF	Missense	YES	7
27	Tongue	c.742C>T c.472C>T	arg248trp arg158cys	GOF LOF	Missense Missense	YES YES	7 5
28	Oral cavity	c.743G>A	arg248gln	GOF	Missense	YES	7
29	Oral cavity	c.830G>T	cys277phe	LOF	Missense	YES	8
30	Tongue	c.818G>A	arg273his	GOF	Missense	YES	6
31	Tongue	c.586C>T	arg196ter	LOF	Nonsense	YES	6
32	Oral cavity	c.503A>C	his168pro	UK	UK	UK	5
33	Oral cavity	c.541C>T	arg181cys	LOF	Missense	YES	5
34	Oral cavity	c.733G>A	gly245ser	LOF	Missense	YES	7
35	Oral cavity	c.838C>T	arg280ter	UK	UK	UK	8
36	Oral cavity	c.817C>T	arg273cys	LOF	Missense	YES	6
37	Oral cavity	UK c.578A>T	thr211ile his193leu	UK LOF	UK Missense	YES YES	6 6
38	Oral cavity	c.542G>A	arg175his	LOF	Missense	YES	5
39	Oral cavity	c.818G>A	arg273his	GOF	Missense	YES	8
40	Oral cavity	c.743G>A	arg248gln	GOF	Missense	YES	8
41	Oral cavity	c.733C>T	arg282trp	GOF	Nonsense	YES	8
42	Oral cavity	c.517G>T	val173leu	LOF	Missense	YES	5
43	Oral cavity	c.535C>T	his179tyr	LOF	Missense	YES	5

44	Oral cavity	UK	ala84profster39	UK	Frameshift	UK	UK
46	Oral cavity	c.818G>A	arg273his	GOF	Missense	YES	8
47	Oral cavity	c.743G>A	arg248gln	GOF	Missense	YES	7 UK: Unknown

Out of the 100 patients included in the study, complete clinical data was obtained for only 46 patients and these patients are subjected to further analysis. Of these 46 patients included in the further analysis, 21 (45.46%) patients displayed mutations in the tp53 gene (study group), and 25 patients (54.4%) showed no mutations (control group). The association between tp53 gene mutation and various clinicopathological features of head and neck cancer are compared in these 46 patients and is provided in Table 2.

Table 2: Descriptive statistics of clinicopathological Correlation

	Total number of patients (n)	Tp53 Mutation N/N	Percentage
All patients	46	21/46	45.65%
Sex			
Male	38	18/38	47.37%
Female	8	3/8	37.50%
Age			
<= 39	11	4/11	36.36%
40-65	27	14/27	51.85%
>=66	8	3/8	37.50%
Anatomical Diagnosis			
Oral Cavity	31	13/31	41.94%
Tongue	10	7/10	70.00%
Pharynx	5	1/5	20.00%
TNM classification survival statistics (46)			
T I-II	19	08	38.09%
T III-IV	27	13	61.90%

TMajority of the cases in our cohort were located in the oral cavity (61.9%) in the tp53 group and 65% in the control group., the maximum frequency of pathogenic mutations was noted in the tongue (70%) therefore these were classified separately. 61.90 % of patients in the tp53 group and 55 % of patients in the control group presented in the late stages. most of the patients were treated using multiple modalities including surgery, radiation, and chemotherapy. More patients with tp53 mutations had a history of tobacco and alcohol use with a hazard ratio of 1.885 (0.7445-4.766). (Table 3)

Table 3: Hazard Ratio of Tp53 Mutation with Habits

	Tp53 mutation	No Mutation	Hazard ratio (ci)
Habit	13 (56.50%)	10 (43.5%)	1.885 (0.7445-4.766)
No Habit	8 (34.8%)	15 (65.2%)	reference category

The study found median overall survival of our cohort is 1.667 years (p-value = 0.6459) with a hazard ratio of 1.615 in the p53 group over the no mutation group. (Table 4) There was no statistically significant difference in the overall median survival (OS) between the two groups.

The median disease-free survival (DFS) of our cohort was 1.916 years with no statistical difference between the two groups. (Table 5)

Table 4: Survival Analysis of Tp53 Mutations

	Alive	Dead	Hazard Ratio (Ci)
Tp53 mutation	13 (61.9%)	8 (38.1%)	1.615 (0.333-3.105)
No Mutation	18 (72%)	7 (28%)	reff. category

Table 5: Overall Mean and Median Survival Time of Tp53 Mutations

	Mean			Median		
	Survival Time	95% Confidence Interval		Survival Time	95% Confidence Interval	
		Lower bound	Upper Bound		Lower bound	Upper Bound
No Mutation	3.102	1.488	4.716	2	0	4.086
Tp53 Mutation	1.736	1.388	2.083	1.334	0.913	1.755
Overall	2.332	1.561	3.104	1.667	1.163	2.171

Of the 21 patients with tp53 mutations, 11 (52.38%) patients had hot spot mutations. (Table 6) the analysis of this group revealed a statistically significant difference in the OS and DFS between patients with hotspot mutations and no hotspot mutations (p<=0.028). (Table 7).

Table 6: Hot spot Tp53 Mutation Hazard Subgroup

	Alive	Death	Total
Hotspot	4 (36.40%)	7 (63.60%)	11
No hotspot	9 (90%)	1 (10%)	10

Table 7: Hot Spot Mutation Tp53 Survival Analysis

Mutation	Meana				Median			
	Survival time (yrs)	Std. Error	95% confidence Interval		Survival time (yrs)	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower bound	Upper bound
No hot spot	2.528	0.443	1.661	3.396	3.411	0.12	0.598	1.402
Hot Spot Mutation	1.676	0.485	0.726	2.627	1	0.205	0.548	1.402
Overall	2.179	0.362	1.468	2.889	1.5	0.69	0.148	2.852

Discussion

The tp53 gene located on chromosome 17p13.1, also known as "guardian of the genome," encodes a transcription factor for the regulation of cell cycle arrest, cell differentiation, and DNA repair which ensures genomic stability [10]. It is formed by three subunits: n-terminal, core domain, and c-terminal. The n-terminal is composed of a transactivation Al domain (amino acids 1–44) and a proline-rich domain (58–101) which mediates p53 response to DNA damage through apoptosis [4]. The central core contains the DNA-binding domain (102–292) and is the target of 90% of p53 mutations found in human cancers. The c-terminal domain is characterized by a tetramerization domain (325–356) which mediates the dimerization of two p53 dimers to form a tetramer [12,13].

Tp53 is the most frequent genetic abnormality in all HNCS [4,14]. Somatic tp53 mutations are more frequent, occurring in more than 50% of all cancers while germline tp53 mutations are linked to cancer predisposition syndromes such as known as li-Fraumeni syndrome [6]. Our study, which investigated the somatic mutations in 100 HNC patients showed an incidence of 46.0% of tp53 mutations. Majority of the these were found in the oral cavity, followed by the tongue (which included the oral and base of the tongue). This is in concordance with previous studies that reported the incidence of tp53 mutations ranging between 34-80% of all cases of HNC [15,16].

The association of tp53 mutations with tobacco and transversions of g:c-t: a is a well-established mechanism of mutation [2,17]. in our study, 48.7 % of the study population had a positive habit history. of these, 56% belonged to the tp53 group and 43.5% belong to the control group with a hazard ratio of 1.885. This was statistically insignificant, therefore could not corroborate the association of tp53 mutations with the use of tobacco in our cohort. other studies have corroborated these findings [11,18,19].

Most patients in our study presented in late stages, as is common in the Indian subcontinent. however, the advanced disease was not found to be linked with the presence of tp53 mutations. Majority of our patients received multimodality treatment in concordance with standard international guidelines in both groups. Hence, the effect of tp53 mutations on response to treatment was elicited using parameters related to disease-free survival and overall survival.

The tp53 Mutational Landscape

Mutations in the tp53gene have been categorized into the following types: missense, non-sense, silent, splice variants, and frameshift according to the IARC data base [21]. The present study showed that the majority of tp53 mutations are missense (74.5%) followed by nonsense mutations (11.76%), unknown (7.8%), frameshift (3.9%), and deletions (1.9%) (Table 2). studies reported that missense being the most frequent mutation in their study on HNC, followed by frameshift, stop mutation and splice-site variants [5].

Although tp53 mutations can occur spontaneously in all coding exons of the gene, the majority have been reported in exons 5 to 8 between codons 102 to 293 in its DNA binding domain [12]. Mutations between exons 2-4 and 9-11 in hncs are uncommon [22-25]. Missense mutations disrupt protein binding to DNA and may not significantly alter the protein's function outside this region [26]. Conversely, nonsense mutations, deletions, frameshift mutations, and unknown changes are common in peripheral coding regions and these may have a more dramatic impact on the overall protein structure. This difference in the site-specific distribution of mutations can be attributed to functional or conformational characteristics of the coding regions of the tp53 gene in our cohort,

86.27% of mutations were seen in the DNA binding domain between the exons 5-8, and the majority of these mutations were found to be missense (Table 2)

The mutant tp53 protein not only shows loss of function (LOF) of tumor suppressor function but also increases oncogenic potential by a gain of function mutations (GOF), independent of wild-type tp53 [22].in a heterozygous situation, the mutant allele can antagonize the function of wild type (WT) in a dominant-negative (DN) manner. It is common to see the loss of heterozygosity (LOH) following tp53 mutation, leading to the deletion of wild type allele [23]. the present study showed a majority of LOF mutations (54.9%) and only 27.45% with GOF, while 13.7% were unknown. (Table 2)

Interestingly, GOF in tp53 plays a significant role in the positive selection of missense mutations during tumorigenesis. Recent studies have also shown that GOF of tp53has a major role in tumorigenesis by exerting its effect on cell proliferation, metastasis, genomic instability, differentiation, metabolic reprogramming, tumor microenvironment, immune response, and cancer therapy resistance [7,13,24].

The commonly occurring mutations at specific codons also known as hot spot residues comprise 30% of all mutations reported in the literature (175, 213, 245, 248, 273, and 282) [27]. In the present study, 33.33% of cases showed hotspot mutations mostly in codon 273 (n=7), codon 248 (n=4), in codon 245 (n=3), in codon 175 (n=2). other important mini hot spot codon mutations noted were in codon 196 (n=3), in codon 179 (2), in codon 173 (n=2) and 339 (n=2). few unknown variants in head neck cancers were also found. their significance for HNC has to be further elucidated following multiple sequencing studies.

Influence of mutational landscape on survival and overall prognosis

in our study, the patients without hot spot mutations had a median overall survival of 3.4 years when compared to patients with hot spot mutations with a survival of 1 year with a significant p-value of 0.028. gain of function was noted in 61.90% of hot spot mutations with a hazard ratio of 63.60%. (Table 6)

Mutations Associated with Poor Prognosis

Four patients in our cohort displayed more than 1 mutation in the tp53 gene and three of four died of the disease. a total of seven patients with a hot spot mutation at codon 273, all showed recurrence of the disease. Of these only one patient survived after salvage surgery. hence mutation in 273 codon is found to be highly pathogenic in our cohort. This was similarly reported in other studies [28]. Mutation in codon 245, codon 173, and codon 339 showed poor survival of fewer than 2 years and was treatment refractory. Patients with mutation in codon 196 conferred a good prognosis and patients are alive with no reoccurrence of the disease. The patients were lost for follow-up with a mutation in codon 248.

Tp53 mutation occurs in various stages of malignant transformation leading to additional tumor growth and invasion [20,28]. It appears that the timing of the mutations during the pathway of initiation and progression is extremely variable from one cancer to another. the present cohort showed the maximum mutations in stages III & IV of HNC. the pathogenic codons 273, 245, 173, 339, and 179 were seen in the late stage while codon 196 were seen in stage I & II. Tp53 mutations have been found in the late stage of tumorigenesis in other cancer types like breast, colorectal, hepatocellular carcinoma, and bladder cancer [29-32], while astrocytoma showed an early loss or mutation in tp53 [33].

Although the presence or absence of tp53 mutations did not significantly affect the overall survival of patients in our study. however, it was noted that those patients who succumbed to the disease had hot spot mutations specifically in the DNA binding codon of the tp53 gene (exon 5-9, mostly missense). This could provide avenues for future research for prognostication of tp53 mutated cancers based on specific codons in certain cancers which might also may also help in developing a highly specific primed dendritic cell vaccine designed against HNCs.

Conclusion

Traditionally, p53 mutations are known to be associated with poor prognosis and have been associated with resistance to radiotherapy and chemotherapy at other anatomical sites. A noteworthy finding obtained by this study is that, there exists a statistically significant diagnostic and predictive value only among hot spot residues at specific codons among tp53 gene mutations. the gain of function in these hot spot residues contributes to the pathogenicity and tumor progression. therefore, the results are consistent with the idea that that the existence of the highly pathogenic hot spot residues seems to be the only mutations that warrant further studies to identify the potential for therapy/ prognostication of head and neck cancers. It is recommended that this hypothesis be validated by further studies in large populations and prospective studies.

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