

## High incidence of *BRAF*<sup>V600</sup> mutation in Indian patients with head and neck cancer

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### 1. ABSTRACT

In cancer cells, *BRAF* is frequently mutated at codon 600 (<sup>V600</sup>) leading to the replacement of valine amino acid with other amino acids. The current study was performed to assess the prevalence of *BRAF*<sup>V600</sup> mutation in Indian patients with head and neck squamous cell carcinoma (HNSCC). Among the patients, 27% were homozygous, and 71% were heterozygous for the mutation and only 2% showed a wild genotype. Since identification of *BRAF*<sup>V600</sup> mutation in cancer patients is used for selection of the therapeutic agents, this study shows that all Indian patients with HNSCC must be screened for this mutation.

### 2. INTRODUCTION

Head and Neck Squamous Cell Carcinomas (HNSCC) are the sixth most common cancers worldwide which arise in the oral cavity, oropharynx, larynx or hypopharynx (1). In all cancers, the type of mutations that found in cancer impact disease progression, response to treatment and recurrence of the disease (2). *BRAF*, a major type of oncogene, belongs to the *RAF* family of kinases. The other isoforms of *RAF* includes *ARAF* and *CRAF*. Among all cancers 7% of them have been shown to have mutations in *BRAF*, with *A-RAF* or *C-RAF* mutations occurring rarely (3,4). *RAF* is a serine threonine specific protein kinase that is activated downstream of *RAS*. *RAF* kinases phosphorylates and activates mitogen-activated

protein kinase, which in turn activates extracellular signal-regulated kinase (*ERK1/2*) (5,6).

Many clinical and experimental studies demonstrated that the *ERK* signaling pathway is one of the major pathways associated with tumorigenesis. Among them, mutation in the *BRAF* gene have been identified in a variety of human cancer types (4). Previous studies revealed that *BRAF* mutations were clustered within the P-loop (exon 11) and activation segment (exon 15) of the kinase domain (5). In *BRAF*, the most common mutation across various types of cancers is the classic GTG to GAG substitution at the 1799<sup>th</sup> position of exon 15, which results in the V600E (Valine to Glutamic acid at residue 600) amino acid change that lies within the activation segment of the kinase domain (3,5).

An experimental study has reported that most of the *BRAF* mutations result in an increase in kinase activity (7). The activating *BRAF* mutations, including V600E, can induce cell transformation, promote cell viability, cell proliferation, and tumorigenesis (8). Mutations in *B-RAF* constitutively activate *ERK* signaling through hyper activation of the *RAS-ERK* pathway, resulting in enhanced cell proliferation and survival (6). Additionally, experimental studies using RNA interference demonstrated that *BRAF* suppression inhibits tumor growth and induces apoptosis (9,10). *BRAF* mutations have been identified in a variety of

**Table 1.** The clinicopathological data of all head and neck cancer patients used in this study

Criteria	Patient Cohort	Total (56)
Age	<45 years	26(46%)
	>45 years	30 (54%)
Gender	Male	50 (89%)
	Female	6 (11%)
Diagnosis	Oral cavity	47(84%)
	Oropharynx	2(3.5%)
	Hypopharynx	1 (2%)
	Larynx	2 (3.5%)
	Others	4 (8%)
Stage	Stage I	8 (14%)
	Stage II	0
	Stage III	14(25%)
	Stage IV a	21 (37%)
	Stage IV b	10 (18%)
	Stage IV c	3 (6%)
Histopathology Report	Squamous cell Carcinoma	55 (98%)
	Adeno carcinoma	1 (2%)
	Others	0
Grade	Grade I	8(14%)
	Grade II	46 (82%)
	Grade III	2 (4%)
Tobacco usage	Yes	42 (75%)
	No	14 (25%)
Treatment	Radical Surgery	2 (3.5%)
	Surgery +Post OP RT	52 (93%)
	Radical RT	2 (3.5%)

Abbreviations used: OP: Operation, RT: Radiation Technology

tumors (3,11-13). Therefore, these activating *BRAF* polymorphisms are considered to be oncogenic.

Overall survival (OS) rate of HNSCC is reported to be very low. The five year OS rate is still around 50% (14). Molecular studies are ongoing to determine the fundamental factors that cause cancer and to understand HNSCC carcinogenesis. These findings can shed more light towards the novel targeted therapy modalities (15). Furthermore, former clinical studies have reported that *BRAF* polymorphism frequently occur in melanoma, thyroid cancer, and colorectal cancer (16-18). But only few studies have been reported in Head and neck cancer. It has been reported that *BRAF* mutations are rare in HNSCC (19). *BRAF* mutational status is a strong predictor for overall survival not only in the metastatic setting but also in earlier stage of diagnosis. Thus in current study, we analyzed the mutational status of *BRAF* gene at codon 600 to elucidate a possible role of this single nucleotide polymorphism in Head and Neck cancer.

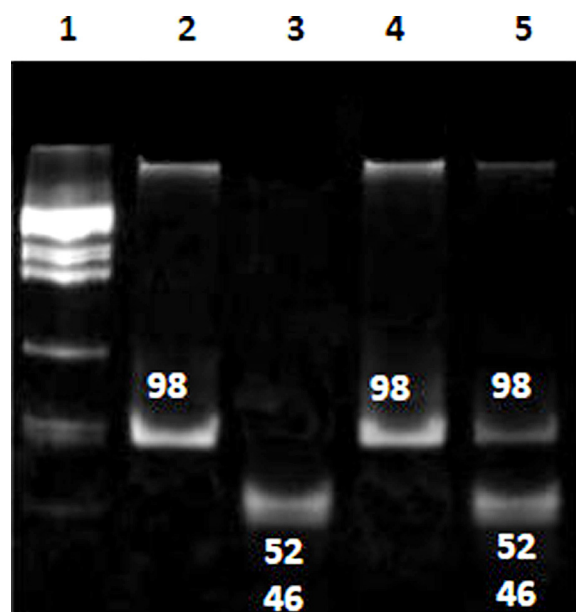
### 3. MATERIAL AND METHODS

#### 3.1. Patients and specimens

Tumor samples used in this study were surgically excised from 56 head and neck cancer patients at Apollo hospital Chennai, India. After resection, the tumors were snap frozen and then transported to VIT University, Vellore. The clinicopathological data of all Head and Neck cancer patients used in this study are summarized in Table 1.

#### 3.2. DNA extraction

DNA extraction from tumor samples were carried out by High Salt Method. The tumor tissue was placed in a microfuge tube containing 1ml of TNEs (10mM Tris, 6M NaCl, 100mM EDTA, 0.6 % SDS) buffer with 60µl of proteinase-k (20 mg/ml) and incubated overnight at 45°C. After incubation time, 277 µl of 6M NaCl was added, mixed and centrifuged. The supernatant was transferred to a fresh microfuge



**Figure 1.** Mutational analysis of *BRAF* codon 600 by PCR-RFLP (digestion with *TspRI* restriction endonuclease). PCR product size: 98 bp, Normal B-raf allele: 52bp and 46 bp, Homozygous Mutant B-raf allele: 98 bp. Heterozygous mutant case: 98bp, 52bp and 46 bp. Lane 1 = molecular weight marker. Lane 2 = Undigested PCR product (98 bp). Lanes 3= Normal sample (52bp and 46bp). Lane 4= homozygous mutant (98bp) and Lane 5 heterozygous mutant (98bp, 52bp and 46bp).

tube, and DNA was precipitated with equal volume of 100% ethanol and centrifuged. After centrifugation, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. The pellet was air dried and suspended in 20-100 µl of sterile distilled water.

### 3.3. PCR-RFLP

PCR-RFLP to screen for V600 mutation was carried out as reported earlier (20). PCR amplification of *B-RAF* gene at codon 600 was performed in 50 µl of reaction mixture containing 5µl of 10x buffer, 10 p mol of each primer, 4µl of dNTP mixture (2.5.mM), 0.4.µl of Taq Polymerase (5units/ml), 50 – 100 ng of template DNA and milliQ water was used to make up the final reaction volume. The profile used in the Eppendorf thermal cycler was: 5min at 95°C, 30 sec at 60°C, 72°C for 30 sec for 40 cycles, 5 min at 72°C. The PCR product was digested with *TspRI* restriction enzyme for one hour at 37°C and the digested products were visualized by ethidium bromide staining under UV light after electrophoresis on a 12% poly acrylamide gel (PAGE). The primers used was (F- 5' TCA TGA AGA CCT CAC AGT AAA AAT 3'; R- 5' TGG ATC CAG ACAACT GTT CAA 3')

## 4. RESULTS AND DISCUSSION

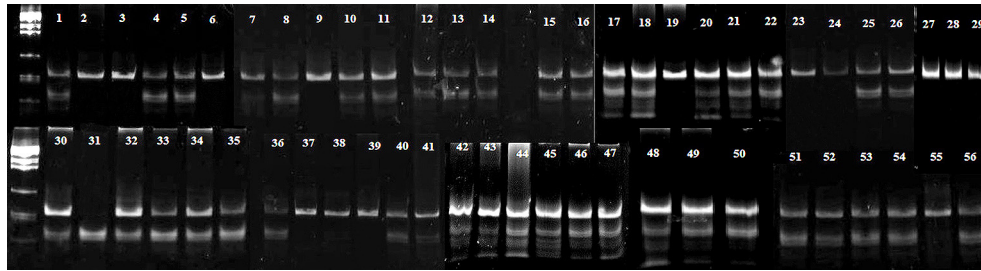
### 4.1. Detection of BRAF V600 mutation by PCR-RFLP

BRAF is a potent activator of ERK signaling pathway leading to activation of necessary

bio-molecules which in turn results in cell division. Uncontrolled and constitutive activation of molecules involved in ERK signaling pathway has been attributed to one of the major reasons for cancer (21). In BRAF, more than 40 different mutations have been reported in which 90% was stated in codon 600 where the valine is substituted with either, glutamic acid (V600E), lysine (V600K), arginine (V600R) or aspartic acid (V600D). Out of these mutations, V600E is the most common one (22). It has been predicted that V600E mutation can activate ERK signaling pathway by more than 500 fold, leading to uncontrolled cell division of cancer cells; which is one of the hall mark of cancer (23). There are no reports available describing about the prevalence of this V600 mutation in head and cancer patients of Indian origin; hence this study was carried out.

Genomic DNA isolated from tumor samples were used as template to amplify 98 base pair (bp) BRAF encoding gene that include the 600<sup>th</sup> codon. The PCR product was digested with restriction enzyme *TspRI* which will digest the fragment only when the wild type codon GTG is present at the 600th position. Thus, upon restriction digestion of the PCR products followed by separation of DNA on PAGE; patient with wild type genome (GTG/GTG) produced two bands of size 52 and 46 bps, patients with homozygous mutation (GTN/GTN) produced a single band of 98 bp and patients with heterozygous mutation (GTG/GTN) produced all the three bands (98, 52 and 46 bps). A representative gel showing the band patterns of PCR product, homozygous wild type, homozygous mutation and heterozygous mutation (Figure 1) and the banding pattern of all the samples analyzed by PCR-RFLP was shown (Figure 2).

PCR-RFLP analysis for mutation in V600 position of BRAF revealed that out of 55 patients, 71.4 % (40/56) were heterozygous, 26.7 % (15/56) were homozygous and 1.7 % (1/56) was wild type. PCR-RFLP using *TspRI* to identify V600 mutation is a widely used technique (24). Though this technique can predict mutation at codon 600 position of BRAF gene, it cannot differentiate between the reported V600 mutations such as V600 E/K/R and D. Further, sequencing the PCR product with Sanger's method is essential to differentiate the reported amino acid substitutions at V600 position. It has been documented that most of the V600 mutations that occur in cancer patients are V600E substitution mutations. Our observation of frequent BRAF V600 mutations in HNSCC patients suggest that *RAS-RAF-MEK-ERK MAP* kinase pathway is involved in HNSCC tumorigenesis. Considering the world wide population of HNSCC cancer patients; only 3% of German, 1.6% of Australian and 2.4% of American carried V600 mutation (25-27). In contrast to this, our studies indicated that a very high incidence (98.21%) of Indian HNSCC population carry either heterozygous or homozygous V600 mutation.



**Figure 2.** Banding pattern of all samples analyzed by PCR-RFLP.

**Table 2.** Correlation between *BRAF* mutations and clinicopathological factors in head and neck cancer

Criteria	Patient Cohort	Total (56)	WT (1)	HTM (40)	HM (15)	p-value
Age	<45 years	26	0	21 (52%)	5 (33%)	0.287
	>45 years	30	1 (100%)	19 (48%)	10 (67%)	
Gender	Male	50	1 (100%)	36 (90%)	13 (87%)	0.883
	Female	6	0	4 (10%)	2 (13%)	
Diagnosis	Oral cavity	47	1 (100%)	35 (87.5%)	11 (73%)	0.853
	Oropharynx	2	0	1 (2.5%)	1 (7%)	
	Hypopharynx	1	0	0	1 (7%)	
	Larynx	2	0	2 (5%)	0	
	Others	4	0	2 (5%)	2 (13%)	
Stage	Stage I	8	0	6 (15%)	2 (13.33%)	0.79
	Stage II	0	0	0	0	
	Stage III	14	1 (100%)	8 (20%)	5 (33.33%)	
	Stage IV a	21	0	18(45%)	3 (20%)	
	Stage IV b	10	0	7 (17.5%)	3 (20%)	
	Stage IV c	3	0	1 (2.5%)	2(13.33)	
Histopathology Report	Squamous cell Carcinoma	55	1 (100%)	39 (97%)	15 (100%)	0.81
	Adenocarcinoma	1	0	1 (3%)	0	
	Others	0	0	0	0	
Grade	Grade I	8	0	3 (7.5%)	5 (33%)	0.937
	Grade II	46	1 (100%)	36 (90%)	9 (60%)	
	Grade III	2	0	1 (2.5%)	1 (7%)	
Tobacco usage	Yes	42	0	31 (77.5%)	11 (73%)	0.206
	No	14	1 (100%)	9 (22.5%)	4 (27%)	
Treatment type	Radical Surgery	2	1 (100%)	1 (5%)	0	0.569
	Surgery +Post OP RT	52	0	39 (95%)	13 (87%)	
	Radical RT	2	0	0	2 (13%)	

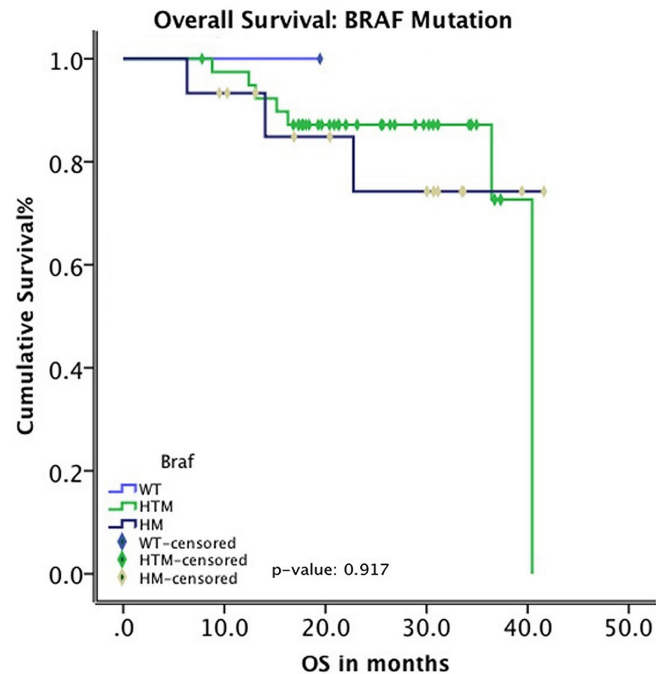
Abbreviations used: WT: Wild type, HTM: Heterozygous mutant, HM: Homozygous mutant, P: Probability, OP: Operation, RT: Radiation Technology. Chi Square analysis using SPSS software was done to evaluate the p-value by comparing the different patient cohort with mutation status.

#### 4.2. Correlations of *BRAF* gene mutations status to clinicopathological features and overall survival factor

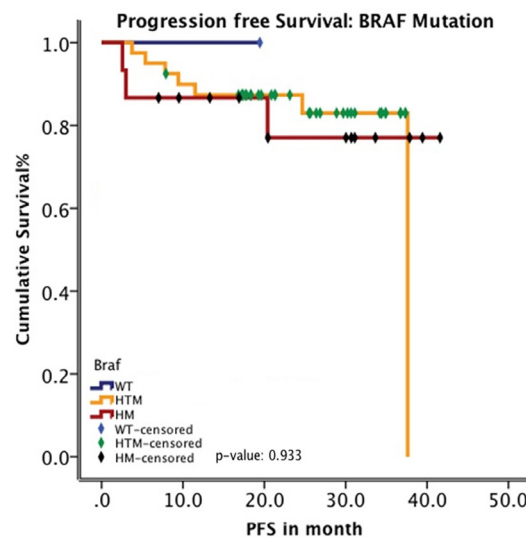
Differences in the categorical variables including age, gender, anatomical location of the tumor, stage, histopathology, grade, tobacco usage and treatment type between patients with and without *BRAF* mutations were evaluated for significance with chi-square tests. None of the variables (Table 2) showed a

significant relationship with *BRAF* gene mutation status due to lack of enough number of patients with wild type genome. For the same reason the overall survival (Figure 3) and progression free survival (Figure 4) could not be correlated with the *BRAF* mutation status.

In conclusion, we describe that generalized treatments such as chemotherapy and radiation therapy to eliminate cancer cells from cancer patients are not the best options to treat them. Molecular



**Figure 3.** Kaplan-Meier overall survival curve in head and neck cancer patients with regard to BRAF gene mutations.



**Figure 4.** Kaplan-Meier progression free survival curve in head and neck cancer patients with regard to BRAF gene mutations.

techniques are slowly proving that each cancer patient is unique and need personalized or specific drugs to treat them depending upon the genetic makeup of the cancer cells. In this regard, this is the first study to demonstrate high prevalence of BRAF gene mutation at codon 600 in HNSCC patients of Indian origin. This observation can be used as a prognostic marker in HNSCC patients and considered for treatment protocols where several inhibitors are already in use for the treatment of cancer patients with V600 mutation, especially the V600E variant.

## 5. ACKNOWLEDGMENT

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**Abbreviations:** HNSCC: Head and Neck Squamous Cell Carcinoma, PCR: Polymerase chain reaction, RFLP: Restriction fragment length polymorphism, WT: Wild type, HTM: Heterozygous mutant, HM: Homozygous mutant, OP: Operation, RT: Radiation Technology

**Key Words:** BRAF, Head, Neck, Cancer, Mutation, PCR-RFLP, V600

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