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Research Article

BRAF V600E VARIANT IDENTIFIED AS A TARGETABLE SOMATIC VARIANT PICKED UP BY NEXT GENERATION SEQUENCING IN THYROID TISSUE SAMPLES FROM SOUTH INDIA

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ABSTRACT

Molecular targeted therapy is custom tailored treatment aiming at the molecular variations that drive the cancer growth. BRAF (B-homologue of the Rapidly Accelerated Fibrosarcoma) V600E variant is one such variant seen in several cancers that serves as a promising target for therapy. The aim of the present study was to evaluate the percentage of Thyroid Cancer (TC) cases harboring BRAF V600E mutation, from patients of two tertiary care hospitals of south India to plan specific molecular therapy irrespective of the tumor pathology. Samples were recruited into the study after the histopathological confirmation as, Papillary TC (PTC), Follicular TC (FTC), Medullary TC (MTC) and Anaplastic TC (ATC) cases based on the morphology. In the study, 106 different FFPE thyroid cancer samples were recruited of which 13 samples were subjected to Next Generation Sequencing using Comprehensive Onco CEPT panel. BRAF V600E variation was analyzed using Polymerase Chain Reaction followed by Restriction Fragment Length Polymorphism. 2 samples out of 13 sequenced harbored BRAF V600E variation, which was tested on the rest of the samples. In total 38% of the TC samples were found to harbor BRAF V600E variation located in the exon 15 implying that these could be benefitted from the targeted therapy. It is the most common variation seen in TC and also in several other cancers. It serves as a promising target for therapy and a better prognosis for radio-iodine refractory thyroid carcinomas. Tyrosine Kinase inhibitors like Dabrafenib and Trametinib can be given to these patients.

Keywords: Next Generation Sequencing, BRAF, Targeted therapy

1. INTRODUCTION

Thyroid cancer (TC) is the most common malignant disease of the endocrine system. Its incidence has almost tripled worldwide in the last three decades [1]. The global trend in its increasing incidence indicates that TC would become the fourth common cancer replacing Colorectal cancer in the near future [2]. The incidence of TC is three to four times more in females than in males. On the basis of morphology and histopathology it is categorized as Papillary TC (PTC), Follicular TC (FTC), Medullary TC (MTC) and Anaplastic TC (ATC) accounting for 80%, 15%, 3% and 2% of the TC cases, respectively [3, 4]. PTC and FTC are together known as differentiated TCs while the others are undifferentiated, hence more aggressive. Biologically and clinically TC is heterogeneous, ranging from being very indolent in nature in well differentiated TCs, to very aggressive in poorly differentiated types [5]. Most of the Thyroid tumors are found in the reproductive age group starting from the second decade of life [6].

Thyroid nodules both palpable and intangible are very common. About 6% of women and 2% of men have palpable nodules predominantly in elderly and iodine deficient areas and majority of them are asymptomatic [7]. They are usually detected on ultrasound, CT or MRI and their evaluation is necessary as 5% of thyroid nodules and 5-13% of Thyroid incidentalomas may be malignant [8, 9]. Genetic and Molecular variations are a hallmark of cancer and are considered to be the triggering agents for tumor initiation, differentiation and progression. The molecular variants that add advantage to the cell in its proliferation are known as "Driver mutations". These drivers help in the tumor molecular profiling and in predicting its clinical outcome [10]. Tumor sequencing known as somatic testing is used as a diagnostic tool in recent years to

target the sequence variant that causes tumor initiation or progression [11]. Surgical removal (hemi/total thyroidectomy) followed by Radioactive Iodine (RAI) therapy comprise the first line of treatment, however, some tumors does not respond to RAI limiting this treatment modality. Hence, molecular assessment of the tumor provides us information for targeted therapy with mutation specific drugs.

The aim of the present study was to first evaluate a set of TC samples with somatic Next Generation Sequencing (NGS) panel and subsequently assess relevant targetable sequence variants in the available TC cases, to identify those who could benefit from established targeted therapies.

2. MATERIAL AND METHODS

This was a retrospective study on available paraffin tissue blocks. Histopathological reports were reviewed and confirmed cases of Thyroid cancer only were included in the present study. From the available data base of 215 thyroid tissues from two hospitals, 106 cases had a confirmed histopathological diagnosis of thyroid malignancy. Ethics committee approval was obtained from the Institutional Ethics Committees of Kamineni Hospitals (Registration Number: ECR/58/Inst/AP/2013) and MNJ Institute of Oncology and Regional Cancer Centre (Registration Number: ECR/227/Inst/AP/2013/RR-16) Hyderabad, India before collecting samples.

2.1. Next Generation Sequencing through Onco CEPT panel

Thirteen Thyroid tissue samples with different histopathological diagnosis (PTC n= 06, MTC n=04, ATC n=01, 01 Nodular Hyperplasia with Hashimotos Thyroiditis and 01 Multinodular Goitre) were selected and subjected to somatic comprehensive panel testing with Onco CEPT panel (Comprehensive Evaluation for Personalized Treatment) at a commercial diagnostics lab, which identifies the molecular variations that can be targeted for treatment (Neuberg Supratech micropath laboratory and research institute private limited, Ahmedabad, India). The panel consists of 161 genes which analyses hotspot regions of 86 genes, complete sequence of 48 genes and fusion driver mutations from 51 genes.

The variants reported by the NGS testing were assessed with OncoKB (oncokb.org), a precision knowledge base that gives information about the effects and treatment implications of specific cancer gene alterations. OncoKB provides the information about the individual somatic alterations in the tumors tested to support optimal treatment decisions [12]. The BRAF V600E variant identified by somatic testing was further assessed in other TC samples.

2.2. Genomic DNA isolation

Tissue sections of 5-10 micron thickness were microdissected from the remaining 93 FFPE blocks for DNA isolation by the method published from our group [13]. Briefly tissue sections were treated with Xylene at room temperature for wax removal. Genomic DNA isolation was done employing standard salting out method after 2mg/ml Proteinase K digestion. The quality of the isolated DNA was checked and quantified using NanoDrop™ 2000/2000cc (ThermoScientific, MA, USA model number: ND-2000) and the DNA was stored at -80 ºC Ultra freezer until further use.

2.3. Designing of Primers for PCR

DNA primers for the BRAF V600E (rs 113488022) sequence variant of exon 15 were designed using NCBI (National Centre for Biotechnology Information) database and primer 3 plus. These primers were synthesized at BioArtis Lifesciences Pvt Ltd, Hyderabad, India.

Details of the primer are mentioned below:

Forward Primer: 5' TCATAATGCTTGCTCTGATAGGA 3' and

Reverse Primer: 5' GCCTCAATTCTTACCATCCACA 3'

Melting temperature (Tm) of primers and estimates of annealing temperatures were calculated using Tm calculator application (Thermo Fisher Scientific™).

2.4. PCR and RFLP for BRAF V600E evaluation

Emerald AMP GT PCR Master mix (DSS Takara Bio India Pvt Ltd) was used for amplifying the region required using thermal cycler (PCR Genemate series, model number:960). Restriction enzyme TSPRI (New England Biolabs, Catalogue Number:101229-314) was used for restriction digestion.

A three step PCR was performed for 40 cycles with an initial denaturation at 95 °C for 5 min followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 45 seconds. Amplified PCR products were checked for the expected band on 2% Agarose gel electrophoresis and the subsequent restriction digested (RD) products after digestion with TSPRI enzyme at 65 °C for 1 hour were analyzed on 10% PolyAcrylamide Gel Electrophoresis

to visualize the BRAF variant. All the samples had amplifiable DNA and after PCR gave a 196 bp band, while TSPRI restriction enzyme recognized and cleaved the amplified PCR product into 121 bp and 75 bp bands if it has GTG nucleotide- as seen in wild type and fails to cleave if it has GAG the mutant type.

3. RESULTS

This study included a total of 106 TC cases, of which 70% were females and 30% were males. The mean age of the TC patients at diagnosis was 41.12±16.21 years, whereas mean age of females at diagnosis was 42±16.45 years and that of males was 37±15.45 years. Individuals below the age of 45 years were 55%. Of the total TC cases, 88 were of PTC and the rest were of other types including 9 MTC, 4 FTC, 2 ATC and 03 were of poorly differentiated TC.

Onco CEPT testing revealed variations in BRAF, HRAS, NRAS, KRAS TP53, KIT, IDH and KDR genes. Of the 6 PTCs one sample showed BRAF V600E alone, other PTC sample showed BRAF V600E along with TP53, other 2 PTC samples showed TP53 and KRAS each, while 2 PTCs showed no variations. Among the 3 MTC samples one MTC showed both KIT and TP53, one showed both KDR and IDH mutations. Two PTC samples, one MTC sample, one ATC sample and the multi nodular goiter tissue sample revealed no variations. Amongst the above mentioned molecular alterations, activating mutations of the BRAF gene were considered to be the most common molecular defect currently known in thyroid tumors and are considered as driver mutations for this tumor type.

Since BRAF V600E is an actionable variant, it was selected for evaluation in rest of the tumor samples to evaluate what percentage of individuals from this cohort would benefit from the therapy that targets this variation.

Of the samples tested for BRAF 1799 T>A (V600E) mutation, 38% of the cases had the V600E variant and 62% were negative for it (details in Table 1). In this cohort interestingly the disease was predominantly seen in younger patients (60%, < 45 years) compared to older patients (40%, >45 years).

Of the 40 cases that had the BRAF V600E variant 62.5% were females and 37.5% were males. The mean age of cases that were positive for the BRAF V600E variant was 37± 14.7 years. Females positive for BRAF V600E variant had a mean age of 37.72±16.64 years and males had a mean age of 36.06±11.18 years.

Of the 32 male subjects included in the study 15 (47%) were positive and among the 74 female subjects 25 (34%) were positive for the BRAF V600E variation. Of the different types of TC included in this study, BRAF variant is predominantly seen in PTC 82.5% (33/40 Mutants) and in TCs other than PTC only 17.5% (7/40 Mutants) were positive for it.

Table 1: TC types with (mutant) and without (non mutant) BRAF 1799 T>A mutation

TC type	Non mutant	Mutant
PTC (n=88)	55 (62.5%)	33 (37.5%)
MTC (n=9)	4 (44%)	5 (56%)
FTC (n=4)	3 (75%)	1 (25%)
ATC (n=2)	1 (50%)	1 (50%)
POORLY DIFFERENTIATED CANCER (n=3)	3 (100%)	0 (0%)
TOTAL	**66 (62%)**	**40 (38%)**

4. DISCUSSION

Mutations occur in somatic cells throughout life, of which few mutations exhibit a cell proliferation advantage and these are considered important "driver mutations" for developing tumors or cancer [14]. Studies have shown that such driver mutations are potential targets for drug therapy to treat cancer. Surgical management and Radioactive Iodine therapy remained as a mainstay in treating Thyroid cancers for a long time [15]. Differentiated TC is usually indolent in nature and can effectively be treated with surgery followed by radioiodine therapy. However, C-cell derived Medullary TC and the tumors that have lost the ability to differentiate fail to trap radioiodine and do not respond to this therapy, and are considered to have poor prognosis.

Advanced scientific approaches have illustrated the molecular pathways that result in Thyroid cancers. MAPK pathway is one of the extensively studied pathways in many cancers [16]. Mutations in the BRAF gene have been reported in about 7%-15% of all human cancers, with highest incidence of about 40%-70% in melanoma [17]. According to Davies et al [18] BRAF gene alterations were observed in colorectal cancer (5-22%), serous ovarian cancer (<30%) and thyroid cancer (40-45%) apart from malignant melanoma (27-70%).

BRAF V600E mutation was reported in TC initially by Kimura et al [19] and since then, many studies evaluated BRAF sequence variants [20, 21]. More than 40 different variants have been identified in BRAF,

however T1799A is the most common one accounting for above 90% of all the BRAF variants [22].

BRAF V600E variation was reported with different frequencies where the percentage ranged from 29%-83% [23]. It was reported to be 90% from a Korean study by Kyung Hee [24], 69% and 35.8% from two independent studies from US by Cohen et al [25] and Kimura et al [19] respectively, 49% from a multicentre study by Xing et al [23], as 38% from a Philippine study by Xu et al [26] and 25% from an Indian study by Khan et al [27]. 38% of TC cases were found to be positive for BRAF V600E in the present study, which indicates a slightly higher percentage compared to the other Indian study.

Several studies have reported that BRAF mutations were only restricted to papillary type of TC and other types of TC does not harbor them. A study by Goutas etal [3] reported that 68.2% of the MTC samples included in their study have showed BRAF V600E variation. Our study also has showed that 55.5% of MTC samples were positive for BRAF V600E, although the numbers of MTC samples in this group were less.

Tumors with a BRAF V600E mutation can be targeted with Tyrosine kinase inhibitors [16]. Based on our results, 38% (irrespective of histopathology classification) of the TC cases may benefit from the targeted molecular therapy using FDA approved drugs like Dabrafenib, Trametinib, Vemurafenib. Identifying a BRAF V600E variant in TC tissue by molecular testing can offer promising therapy for 38% of TC from this part of the globe.

5. CONCLUSION

Our study has identified that 38% of TC individuals in our cohort harbored the BRAF V600E variant and would benefit from FDA approved targeted therapy. We propose that a simple molecular test on tissue sample will be useful for treatment stratification to help in clinical management of TC appropriately.

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Conflict of interest

The authors declare no competing interests.

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