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SCREENING OF BRAF c.1799 T > A MUTATION IN PAPILLARY THYROID CARCINOMA

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ABSTRACT

Thyroid cancer is a highly prevalent endocrine malignancy with steadily increasing incidence in most parts of the world, possibly due to over diagnosis. The most common type of thyroid malignancy is the papillary thyroid carcinoma (PTC), which accounts for 85-90% of all thyroid cancers. Genetic alterations associated with PTC include those in the mitogen activated protein kinase (MAPK) pathway. Proteins such as BRAF, involved in this pathway, have an important role in cell proliferation, cell differentiation, and programmed cell death. Somatic point mutations in the BRAF gene are the most common events that are specific to PTC. Here, we have focused on thymine-to-adenine transversion mutations that occur in exon 15. Its prevalence has been found to be variable in different studies, from 29-83%. In this study, we analyzed the BRAF V600E mutation in central Kerala population using mutant allele-specific PCR amplification (MASA). We screened 14 Formalin Fixed Paraffin embedded (FFPE) thyroid tissue samples for BRAF gene mutations at codon 600. This mutation was detected in 4 out of 14 samples (28.6%) from patients with PTC. This method proved to be a specific, sensitive, and reliable method for early diagnosis and accurate detection of thyroid cancer.

KEYWORDS: Thyroid Carcinoma, Papillary Thyroid Carcinoma, FFPE, MASA-PCR, BRAF V660E

The tyrosine kinase receptor RAS-RAF-MEK-ERK (MAPK pathway) cascade is a key regulator of cell growth, survival, proliferation, and differentiation in all eukaryotic cells (Sapio et al. 2006). The MAPK pathway serves as a link between extracellular signals and nuclear protein activation. Among the components of this pathway, RAF kinase is considered the strongest activator of downstream MAPK signaling. *BRAF* (v-raf murine sarcoma viral homolog B1) is a proto-oncogene and a member of the rapidly accelerated fibrosarcoma (RAF) kinase family of proteins (Matallanas 2011). There are three types of RAF kinase proteins: A-RAF, B-RAF, and C-RAF (RAF-1). Among these, BRAF, located on chromosome 7q34 (Huang et al. 2013), is the most effective activator of the MAPK pathway (Rahman et al. 2014). The most common *BRAF* mutation is the BRAF V600E, which accounts for over 90% of all *BRAF* mutations (Sapio et al. 2006; Chakraborthy et al. 2012; Yarchoan et al. 2015; Rowe et al. 2007). The BRAF V600E mutation causes uncontrolled and inappropriate cell growth, division, and proliferation, driving tumorigenesis in the thyroid gland. This mutation is found almost exclusively in papillary thyroid carcinomas (PTC), and is absent in normal thyroid tissues, benign thyroid tumors, and follicular and medullary carcinomas (Navarro-Locsin et al. 2016). In PTC, the c1799 T>A mutation has been reported to be associated with aggressive clinico-pathological features such as extra thyroidal extension, lymph node metastasis, advanced tumor stages, disease recurrence, and patient mortality. It has also been shown to cause dedifferentiation, resulting in the loss of expression of thyroid genes. The mutation also upregulates several classic angiogenic and tumor-promoting factors and is associated with hyper methylation, resulting in the inactivation of tumor-suppressor genes (Navarro-Locsin 2016).

Thyroid was the primary site in one out of every ten cancers diagnosed in Thiruvananthapuram, the capital city of Kerala, a south Indian state; large number of cases was under 40 years of age (Mathew & Mathew, 2017). There are only a few studies reported on BRAF V600E mutation in Kerala population.. Hence, in the present study, we assessed the BRAF V600E point mutation of PTC in the local population using the mutant allele-specific PCR amplification method (MASA).

MATERIALS AND METHODS

A total of 14 formalin-fixed paraffin-embedded (FFPE) PTC tissues were obtained from the Department of Pathology, Jubilee Mission Medical College & Research Institute (Thrissur, India). Pathological features of the patients with confirmed PTC were retrieved from their medical records.

Isolation of DNA: Eight sections of 5-µm thick FFPE tissue samples (n = 14) were cut using a microtome. Genomic DNA was isolated using the QIAamp DNA FFPE Tissue kit (Qiagen; Hilden, Germany), according to the manufacturer's instructions. The isolated DNA was separated on a 0.7% agarose gel, and the bands were

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observed. DNA quantification was performed using the Qubit 2.0 Fluorometer (Invitrogen; Carlsbad, CA).

Detection of BRAF V600E mutation using MASA: Two sets of primers were used to amplify a 125-bp fragment of exon 15 of *BRAF*, which contains the site of BRAF 1799 T >A mutation. Two forward primers with a difference in a single base at the 3′ end were used. The reverse primers used were also similar in this respect.

Wild type forward primer: 5′-GTGATTTTGGTCTAGCTACAGT-3′;

Mutant forward primer: 5′-GTGATTTTGGTCTAGCTACAGA-3′;

Reverse Primer5′-GGCCAAAAATTTAATCAGTGGA-3′

These primers were used to amplify the wild type allele and the mutant allele with the *BRAF* transversion mutation, respectively. The PCR was performed in a 100 Peltier Thermal Cycler with the following PCR conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 47 °C for 30 s, and extension at 68 °C for 15 s. The PCR products were electrophoresed in 1.5% agarose gel. The fluorescent bands were observed . All samples were screened for BRAF V600E twice.

Pathological features of the patients with confirmed PTC were retrieved from their medical records.

RESULTS

DNA isolated from the samples was separated on a 0.7% agarose gel. Sheared fluorescent bands were observed, as shown in Fig. 1. Electrophoresis of the PCR product obtained through MASA-PCR (Fig. 2) revealed fluorescent bands in both wild type (W) and mutant (M) wells, indicating the presence of the mutant allele (heterozygous). This was considered a positive result.

Fluorescent bands observed in the W, but not in the M well indicated the absence of mutated DNA, which was considered a negative result. Table 1, which lists the features and histology of the 14 samples used, shows that *BRAF* V600E is present in both classic and follicular variants of PTC (FvPTC). Out of four samples which showed the presence of mutation, three had FvPTC morphology and one showed classic PTC morphology.

Table 1: Histology, BRAF mutation status and pathological features of the samples

Sample	Gender	Age (years)	Histology	BRAF	Tumor size (cm)
T1	Female	23	NA	Negative	5.0
T2	Female	47	Other	Negative	2.5
T3	Male	18	Classic	Negative	4.2/3.2
T4	Female	56	FvPTC	Positive	1.3
T5	Female	33	FvPTC	Negative	1.2
T6	Male	48	Classic	Negative	3.2/2.6/2.5
T7	Female	67	Micro	Negative	0.5
T8	Male	34	FvPTC	Positive	6.0/4.7/4.0
T9	Female	66	FvPTC	Negative	5.5
T10	Female	38	Classic	Negative	2.1
T11	Female	60	NA	Negative	1.5/1.0
T12	Female	60	FvPTC	Negative	10
T13	Female	27	FvPTC	Positive	1.4/0.7/0.4
T14	Female	30	Classic	Positive	1.6/1.5/1.0

NA: Data not available; Other: Warthin-like variant; FvPTC: Follicular variant of PTC; Micro: Microcarcinoma

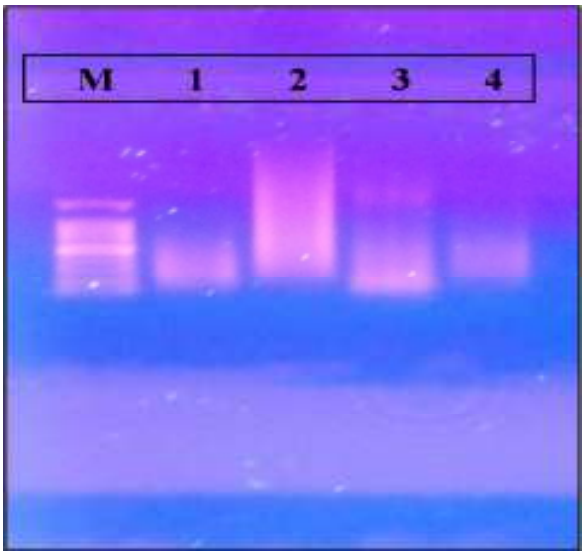

Figure 1: Isolated DNA from formalin-fixed, paraffin-embedded tissues, separated on agarose gel electrophoresis (M- 100 bp ladder; 1-4: DNA from 4 different patients)

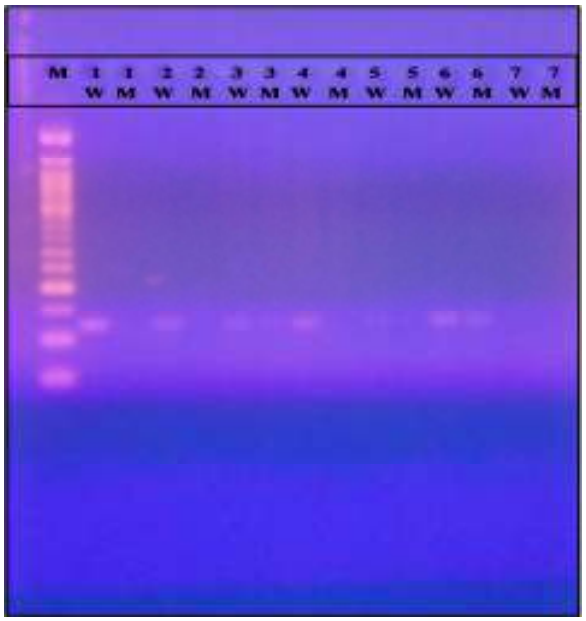

Figure 2: Representative picture of DNA from patient samples amplified using MASA-PCR method and separated on agarose gel electrophoresis (M- 50 bp ladder. 1-6 are wild (W) and mutant (M) PCR products of 6 different PTC patients. 7 is the control)

DISCUSSION

The human genome project and advances in molecular biology have improved our understanding of the genetic changes underlying carcinogenesis and provided opportunities for better diagnosis. MASA is a valuable tool in the field of genetics. It is a well-established diagnostic procedure used for early evaluation of indeterminate thyroid nodules. Previous studies have shown that patients with BRAF mutations are more likely to have clinicopathological characteristics of aggressive disease, extra thyroidal invasion, lymph node metastasis, and advanced tumor stage at initial surgery.

A previous study (Sapio et al. 2006) on the detection of BRAF mutations in PTC using the MASA method revealed that the assay was more specific, sensitive, and reliable than single-strand conformation polymorphism (SSCP) and direct DNA sequencing of PCR products. In their study, the DNA was extracted from different sources, including cytologic samples, either fresh or archival glass slides. Mutations in *BRAF* was detected in 19/43 (44.2%) of PTC samples.

The sheared bands obtained after DNA isolation could be attributed to the fragmentation of DNA during formalin fixation. The quantity of DNA obtained was also small. In our study, 28.6% (4/14) of the PTC samples were found to be *BRAF*-positive.

A previous study on a population in Kerala (Nair et al.2017) revealed that 51% of the population was *BRAF*-positive. Their study population comprised of patients showing classic and FvPTC morphology. They had used Sanger sequencing for their study on DNA, isolated from FFPE samples.

A large study with data from 2099 patients from 16 medical centers across 8 countries suggested that the BRAF V600E mutation has prognostic value for recurrence of PTC (Xing et al. 2014). The authors showed a significant association between the presence of the BRAF V600E mutation and an increased risk of PTC recurrence. Within the subtypes of classic PTC and FvPTC, recurrence rates were statistically higher in BRAF mutation-positive PTC than BRAF mutation-negative PTC.

In our study, only 1 of the 14 samples showed lymph node involvement, and four had classic PTC, while six showed FvPTC morphology.

To conclude, MASA-PCR is a specific, sensitive, and reliable method for detecting BRAF transversion mutation in small quantities of DNA from FFPE tissues. We propose this method as a promising tool for early detection and improved accuracy of thyroid cancer diagnosis.

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