RESEARCH ARTICLE

BRAF Mutations in Iranian Patients with Papillary Thyroid Carcinoma

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Abstract

Background: Papillary thyroid cancer or papillary thyroid carcinoma (PTC) is the most common thyroid cancer. The fact that it occasionally occurs in women aged 30-40 years old suggests that genetic alterations are involved its genesis. Recently, activator mutations in *BRAF* gene have been relatively frequently discovered. Materials and Methods: In this study, we tested 63 DNA samples from PTC patients to identify the V600E mutation frequency in the Ahvaz population. DNA was isolated from formalin fixed paraffin-embedded (FFPE) PTC tumor tissues. Genotyping was performed by PCR-RFLP and confirmed by direct DNA sequencing of a subset of PCR products. PCR-RFLP data were reported as genotype frequencies and percentages. Results: Forty nine out of 63 patients (77.8%) had a mutated heterozygote form while 14 (22.2%) showed normal genotype but none demonstrated a mutant homozygote genotype. The frequency of V600E mutation was significantly high in PTC patients. Conclusions: These findings support involvement of V600E mutations in PTC occurrence in Iran. Assessment of correlations between *BRAF* V600E mutations and papillary thyroid cancer progression needs to be performed.

Keywords: Papillary thyroid carcinoma - *BRAF* proto-oncogene - V600E mutation - PCR-RFLP method

Asian Pacific J Cancer Prev, **14 (4)**, 2521-2523

Introduction

Papillary thyroid cancer or papillary thyroid carcinoma (PTC) is the most common thyroid cancer that is account for 80-90% of all thyroid cancer cases (Kimura et al., 2003; Xing et al., 2005; Yoon et al., 2013). This thyroid cancer is occasionally occurs in women aged 30-40 years old and it is also prevalent in children and patients whose exposed to radiation in head and neck area. These types of tumors are frequently un-encapsulated and show more propensities to metastasis into lymph nodes that may lead to production of cystic structures near thyroid which are diagnosed sorely. Genetic alterations are involved in thyroid carcinogenesis. Recently, occurrence of activation mutations with high frequencies is discovered in *BRAF* gene (Kimura et al., 2003; Soares et al., 2003; Xu et al., 2003; Jeong et al., 2012; Li, 2012). About 43 somatic mutations in *BRAF* gene were discovered which are associated with human cancers and located in exon 11 and 15 (Davies et al., 2002; Pakneshan et al., 2013).

Serine/Threonine- protein kinase B-RAF or proto-oncogene B-RAF or in brief B-RAF is a human protein which is coded by *BRAF* gene (Sithanandam et al., 1990; 1992). Raf kinase is a member of Ras-Raf-MEK-ERK-MAP pathway which plays role in cellular proliferation, differentiation and programmed cell death (Peyssonnaux and Eychène, 2001). Three types of Raf genes are present in mammalian, among them *BRAF* is the strongest one in respect to activation of signal pathway which is located on chromosome 7 (Sithanandam et al., 1992; Avruch et al., 2001; Mercer and Pritchard, 2003). Some inherited mutations in *BRAF* may cause birth defect; alternatively other acquired mutations may lead to cancers such as: non-hodgkin lymphoma, colorectal cancer, malignant melanoma, papillary thyroid cancer, non-small cell lung carcinoma and adenocarcinoma of lung (Davies et al., 2002). *BRAF* mutations probably are involved in tumor initiation. Most of the PTCs are removed by surgery in early stages regardless to genotype. Many studies indicate that PTC carrying *BRAF* mutations are predominant among cases representing during advanced stage. Two mutation hot spots were discovered in *BRAF* gene exon 11 and 15, the most common point mutation is T1796A transversion which occurs in exon 15 while lead to glutamine for valine substitution (V600E). V600E somatic mutation codes constitutively active B-RAF kinase. Among differentiated neoplasm, this mutation is most discovered in PTC and approximately involved in 44% of all cases (Xing et al., 2005). Consequently, we examine *BRAF* V600E mutation frequencies in PTC patients to determine presence of relationship between occurrences of above mutation and PTC.

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Materials and Methods

Subjects

Totally, 63 formalin fixed paraffin embedded (FFPE) Papillary Thyroid Carcinoma tumor tissues filed at Imam Khomeini hospital of Ahvaz, southwest Iran, were collected. These FFPE samples were obtained from PTC patients whom were diagnosed between 2000-2010 during biopsy or surgery. Thirty six out of 63 patients were female while other 44 patients were male. Most cases regardless their sexes were in 30-40 age range. Some of these patients whose were diagnosed in Ahvaz city were not living in Ahvaz but they came from other neighbor cities. This study was approved by the Review Board of the School of Medicine, Ahvaz Jundishapur University of Medical Sciences.

Genotyping

FFPE blocks were sectioned to 10 μm thickness by microtome and collected in 1.5 ml tubes. These micro-sectioned paraffin embedded tissues were treated by xylene to remove the paraffin. Genomic DNA was extracted by QIAamp DNA FFPE Tissue kit (Cat No: 56404).

PCR-RFLP

Isolated DNA was amplified by PCR. The PCR was carried out using a specific pair of primers for exon 15 of the *BRAF* gene: 5'TCA TGA AGA CCT CAC AGT AAA AAT3' (Forward) and 5'TGG ATC CAG ACA ACT GT T CAA3' (Reverse) as designed previously by Takahashi et al.(2007) using the following PCR thermal program: initial denaturation at 95˚c for 5 minutes, followed by 40 cycles for denaturation at 95˚c for 30 seconds, annealing at 60˚c for 30 seconds and elongation at 72˚c for 30 seconds. After the last cycle, a final extension at 72˚c for 5 minutes was performed. PCR products were digested by TspRI (New England Biolabs, Ipswich, MA) restriction enzyme at 60˚c for 16 hours. TspRI is a restriction enzyme which recognizes and cuts the ACAGTGAAA restriction site in the wild type genotype. However, T1799A point mutation replaces T by A so enzyme cannot distinguish the new restriction site and intact 98 bp PCR products will remain. While in the presence of the normal genotype two distinct fragments with 46 and 52 bp lengths are produced. RFLP products were visualized on 8% poly acrylamide gel electrophoresis (PAGE) after ethidium bromide staining. DNA bands were distinguished by use of 100bp ladder (Gene on GmbH, Germany).

DNA Sequencing

DNA sequencing of the PCR-amplified product was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) (Bioneer, South Korea) using the same primers. The results were using Chromas lite version 2.0 software.

Statistical analysis

The results were represented as the frequency and percentage of each genotype. Results were shown as a genotype frequencies and percentages in Table 1. We also considered relationships between genotypes and age as well as sex. Data were analyzed using SPSS 16.0 computing program and relationships tasted by χ^2 test (Table 2). According to the tests results no significant relationships observed between sex or age and *BRAF* genotypes.

Results

We tested 63 isolated DNA from paraffin-embedded papillary thyroid cancers patient tissues to identify V600E mutation frequency in Ahvaz PTC patients. Using the PCR-RFLP and sequencing of PCR products, mutation were found in 49/63 (77.78%) of PTC cases (Table 1). All mutations were in heterozygote form while all of them contain T→A transversion at nucleotide 1796 during sequencing. Other samples showed normal homozygote genotype that was accounted for 14 out of 63 cases (22.22%) cases and we didn't observed any samples with mutant homozygote genotype as mentioned in following table. According to RFLP results heterozygote samples showed 3 bands: 46, 52 and 98bp when normal homozygote produced 2 fragments only include: 46 and 52 bp (Figure 1). Although there was a 1.5 fold increase in the risk of PTC related to the sex (OR: 1.507, 95%CI: 0.455-4.994, P=0.708), but the impact was not statistically

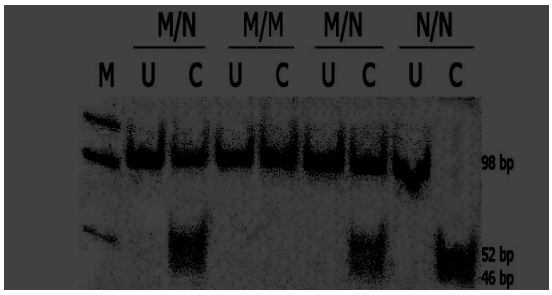

Figure 1. Position of RFLP Bands after Cutting by TspRI Restriction Enzyme to Identify V600E *BRAF* Mutation, from left F side 2nd, 6th and 8th Samples are Heterozygous while 4th Sample Shows Homozygous Genotype. M: marker, U: uncut, C: cut, M: mutant, N: normal, bp: base pair

Table 1. Genotypes Frequencies and Percentages in 63 PTC Patients

Variables		Number (%)
Age	20-29	8 (12.70%)
	30-39	26 (41.27%)
	40-49	16 (25.40%)
	>50	13 (20.63%)
Sex	Male	32 (50.79%)
	Female	31 (49.21%)
Genotype	Mutant (A/T)	49 (77.78%)
	Normal (A/A)	14 (22.22%)

Table 2. Relationships between Age or Sex and *BRAF* Genotypes

Variables	OR (95%CI)	df	χ^2	P value
Sex and genotype	1.507 (0.455-4.994)	1	0.14	0.708
Age and genotype	0.919 (0.192-2.092)	1	0.02	0.655

significant (Table 2). These findings suggest that the *BRAF* V600E mutation is involved in the carcinogenesis in most PTC patients especially whose diagnosed in Iran so it can be used as a selective marker for prediction of patient's prognosis and their response to chemotherapy.

Discussion

The *BRAF* mutations were reported in most of carcinomas while it's ranged 36-69% of papillary thyroid cancer (Kim et al., 2004). We have calculated the frequency of V600E mutation in Ahvaz PTC population during 2000-2010, which was equal to 77.78%. These findings confirmed results of many studies which had proved association between *BRAF* V600E mutation and PTC occurrence. Wide range for V600E frequency might be due to ethnical differences, environmental factor, genetic disposition of specific population, varies in disease stage, different mutation determination method and differences in samples size. However, estimation of *BRAF* mutation frequency in thyroid cancer patients may influence on thyroid cancer diagnosis and prognosis as a molecular biomarker. In many studies *BRAF* mutations which effect on response to chemotherapy were considered. These finding were revealed that presence of *BRAF* mutation was associated with absence of Cetuximab and Panitumab therapy. Cetuximab (Erbitux) and Panitumumab (Vectibix) are two monoclonal antibodies that inhibit EGFR which are used to treat CRC and PTC patients with chemotherapy (Meyerhardt and Mayer, 2005; Xing et al., 2005). PTC harbor *BRAF* mutations have a high recurrence rate and metastatic recurrence may defect radio-iodine avidity (Xing et al., 2005; Durante et al., 2007). Other studies also represented same results and mentioned that *BRAF* mutation-positive patients required more aggressive surgical and external radiation treatments for their recurrent disease (Dralle and Machens, 2008; Lalami and Awada, 2011). In conclusion, these results indicated that presence of *BRAF* mutation plays role in PTC mortality and morbidity, while it can effect on its treatment.

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