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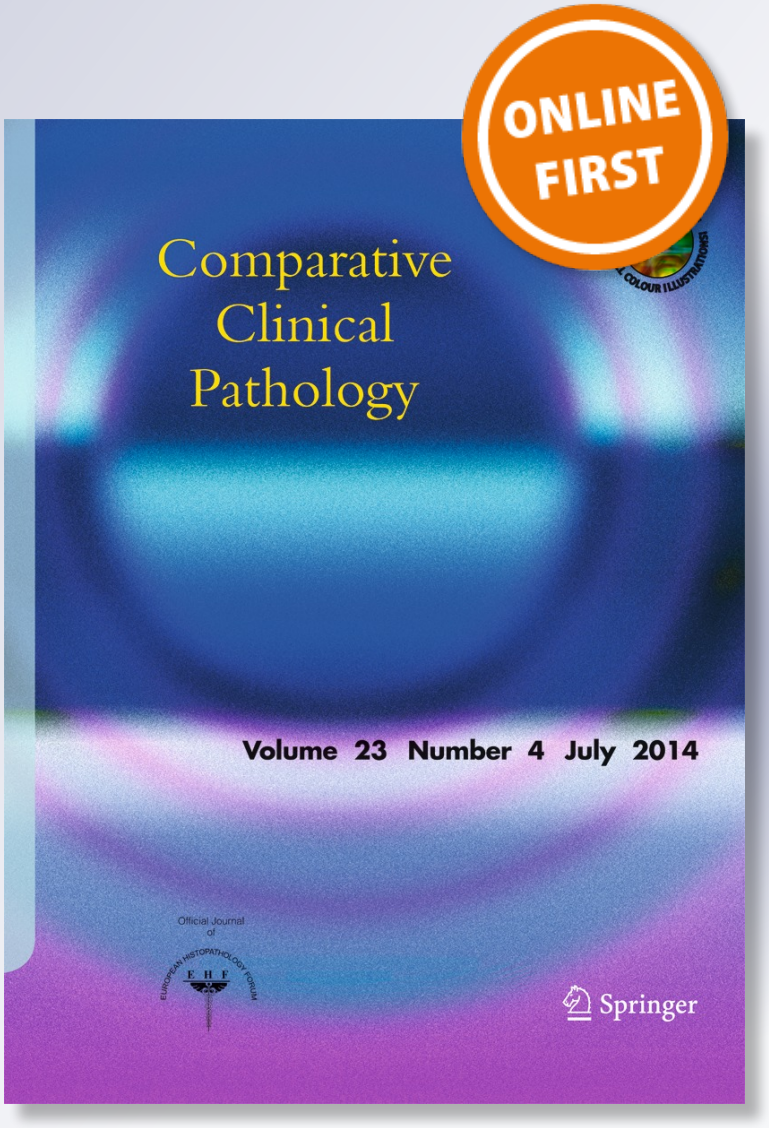

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ORIGINAL ARTICLE

The status of BRAFV600E mutation among Egyptian patients with papillary thyroid carcinoma

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Abstract The T1799A activating point mutation in v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) is considered to be the most common genetic change in papillary thyroid cancer. It causes a change in the amino acid valine at position 600 to glutamic acid in the BRAF protein kinase, causing abnormal activation of the signaling pathway mitogen-activated protein kinase. Being associated with aggressive clinicopathological outcomes, it is now considered as a strong prognostic molecular marker for poorer prognosis of papillary thyroid carcinoma. The aim of the study was to investigate the frequency of the BRAFV600E mutation among the Egyptian patients with papillary thyroid carcinoma and to correlate with the clinicopathological status of the patients. The study included 50 formalin-fixed paraffin-embedded thyroid tumor tissue samples collected from the Department of Surgical Pathology, Faculty of Medicine, Cairo University, and the Egyptian National Cancer Institute. Patients' data archived from records included age, sex, multifocality, lymph node metastasis, vascular invasion, and distant metastasis. The V600E mutation was tested by polymerase chain reaction-restriction fragment length polymorphism and direct sequencing. One (2 %) out of the 50 tumor tissue samples was found to be positive for the BRAFV600E mutation. Large-scale studies and inclusion of multiple molecular markers are recommended to elucidate the molecular basis of papillary thyroid carcinoma among Egyptian patients.

Keywords Papillary thyroid carcinoma · Mutation · BRAF · Sequencing

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Introduction

Thyroid cancer is considered to be the most common endocrine malignancy (Xing 2005), with steadily increasing incidence in USA and several countries (Hodgson et al. 2004; Davies and Welch 2006; Albores-Saavedra et al. 2007). In Egypt, the recorded numbers for incidence and prevalence of thyroid carcinoma are very similar to those of the Western records (Salim et al. 2009), as it represents 1.2 % of total cancers (Sherman et al. 2004). Also, similar to other Arabian and Western countries, thyroid cancer is more common in females (Hussain et al. 2013) with female to male ratio of ≈2:1 (Salim et al. 2009).

Papillary thyroid cancer (PTC) is the most common type of thyroid cancer according to the report of the National Cancer Institute, Cairo University (Mokhtar et al. 2007). Also, PTC is more frequent than the other types, accounting for 85–90 % of all cancers (Dal Maso et al. 2009; Xing et al. 2013).

Being involved in the pathogenesis of thyroid cancer, many molecular biomarkers are well studied, being potential therapeutic targets and their potential diagnostic and prognostic values (Melck et al. 2010). These include the rearrangement of the receptor tyrosine kinase (RET) gene or neurotrophic tyrosine receptor kinase (NTRK) (Xing 2005), point mutations of the rat sarcoma (RAS) proteins accounting, and BRAF genes (Ricarte-Filho et al. 2009). These genes are

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involved in the activation of the mitogen-activated protein kinase (MAPK) signaling pathway (Nikiforov 2011). This intracellular pathway regulates cell growth, differentiation, and apoptosis through to the interaction of the different growth factors, hormones, and cytokines with the cell surface receptor tyrosine kinases (Mercer and Pritchard 2003). Mutations of any single effector of this pathway is sufficient for the activation of MAPK pathway (Kimura et al. 2003), leading to tumorigenesis through mitogen-independent proliferation, insensitivity to inhibitory signals, angiogenesis, evasion of apoptosis, and even resistance to therapy (Shelis 2005).

The v-Raf murine sarcoma viral oncogene homolog B1 (BRAF) gene, located on the long arm of chromosome 7 (Garnett and Marais 2004), is an important MAPK pathway activator (Feng et al. 2011). BRAF mutations have been reported in numerous types of human cancer with its highest frequency in thyroid cancer (Cantwell-Dorris et al. 2011).

More than 45 BRAF mutations had been reported in thyroid cancers; however, the exon 15 T1799A mutation is considered to be the most common BRAF mutation in PTC (Xing 2005). It causes the change of valine (V) to glutamine (G) (V600E) at amino acid residue 600 resulting in the activation of the MAPK pathway in PTC (Xing 2005). The V600E mutation is also needed for the maintenance and progression to more aggressive forms thus considered to be a potential prognostic factor for PTC (Xing 2007). Many studies have shown an association of the V600E mutation with aggressive clinicopathological characteristics of PTC such as extrathyroidal invasion and lymph node metastasis (Xing et al. 2005, 2013).

Concerning its potential diagnostic, the BRAFV600E mutational analysis increased the sensitivity of cytology for PTC from 77 to 87 % (Xing 2005; Kim et al. 2005), supporting its use as a complementary adjunct to routine cytological analysis (Jin et al. 2006). Determination of the V600E mutation status allows for better risk stratification, proper preoperative planning and postoperative decisions (Mazzaferri and Kloos 2001).

The BRAFV600E frequency among Egyptian patients with PTC had not been previously investigated. This study aims to investigate the frequency of the BRAFV600E mutation among a cohort of Egyptian patients with PTC and to correlate with the clinicopathological status of the patients.

Subjects and methods

This study included 50 archived formalin-fixed paraffin-embedded (FFPE) thyroid tumor tissue samples belonging to patients diagnosed with PTC, who underwent total and subtotal thyroidectomy. FFPE tissue blocks were obtained from the Department of Surgical Pathology of Faculty of Medicine, Cairo University, and the Egyptian National Cancer Institute. These samples included 35 females and 15 males, with age ranging from 18 to 80 years.

Methods

Data collection

The clinicopathological data of the patients were collected from their archived records. Histopathological review was done by two experienced pathologists. The diagnoses were reassessed according to the WHO classification of thyroid cancer (DeLellis and Williams 2004). Hematoxylin and eosin-stained sections were reexamined, taking into consideration the known risk factors for thyroid cancer, such as tumor size, multifocality, extrathyroidal tumor extension, lymph node metastasis, and distant metastasis. Tumors were staged according to the tumor, node, metastases (TNM) classification staging system, recommended by the American Joint Commission on Cancer (AJCC) and the Union for International Cancer Control (UICC) (DeLellis and Williams 2004; Lang et al. 2007).

Molecular genetic studies

DNA extraction from FFPE tumor tissue blocks Serial 5-μm sections were dissected from paraffin blocks from the primary tumor. DNA extraction was performed using the QIAamp Mini Kit (Qiagen, Inc., CA, USA). The quality and quantity of the extracted DNA were determined by agarose gel electrophoresis and spectrophotometry using NanoDrop 3.0 (NanoDrop, Inc., Wilmington, DE, USA), respectively.

Determination of BRAFV600E mutation using polymerase chain reaction-restriction fragment length polymorphism analysis DNA template was amplified by polymerase chain reaction (PCR) using forward and reverse primers particular for the region flanking the BRAF T1799A mutation according to Mohammadi-Asl et al. (2009). The primers' sequence was as follows: 5′TCA TGA AGA CCT CAC AGT AAA AAT 3′ (forward) and 5′TGG ATC CAG ACA ACT GTT CAA 3′ (reverse). The PCR amplification was carried out under the following conditions: initial denaturation at 95 °C for 2 min, 40 cycles of amplification, denaturation at 94 °C for 30 s, annealing at 54 °C for 60 s, and extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The PCR amplified products were digested using TspRI restriction enzyme under the conditions provided by the supplier (Fermentas, USA). The TspRI enzyme cuts the wild allele (ACA GTG AAA), but not the BRAF T1799A mutant sequence (ACA GAG AAA). The wild-type BRAF fragment is cut into two bands, 47 and 52 bp, while the mutant BRAF fragment is not digested and produces

a 98-bp band. The digested products were checked by 12 % polyacrylamide gel electrophoresis using the Bio-Rad Mini-Protean Tetra gel system (Bio-Rad, Hercules, CA, USA) at 120 V for 30 min and were then stained with 0.5 mg/ml ethidium bromide. A 50-bp DNA ladder (MBI Fermentas) was used as a size marker. The gel was visualized using UV transilluminator (Bio-Rad, Hercules, CA, USA).

Sequencing analysis Direct sequencing was done to confirm the presence of the V600E mutation. The template of BRAF exon 15 encompassing the nutation was PCR amplified using forward and reverse primers as described previously. PCR products were purified using MinElute Gel Extraction Kit (Qiagen, Inc., CA, USA). The purified PCR products were directly sequenced on both strands using the 310 Capillary Array Sequencer and Big Dye Terminators Chemistry (Applied Biosystems, Foster City, CA, USA). BRAF sequence analyses were performed using the BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA, USA) and comparing the results to reference sequence (GenBank access no. NM_004333).

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) (Advanced Statistics version 20.0) (SPSS Inc., Chicago, IL, USA). Numerical data were expressed as mean and standard deviation or median and range as appropriate. Qualitative data were expressed as frequency and percentage. Chi-square test was used to examine the relation between qualitative variables. For quantitative data, comparison between the two groups was done using independent sample *t* test. A P value <0.05 was considered significant.

Results

The current study included 50 archived FFPE thyroid tumor tissue blocks. The samples belonged to 35 females (70 %) and 15 males (30 %) with their age ranged from 18 to 80 years with a mean of 45±16.1. Total thyroidectomy was performed in 44 (88 %) patients of the studied group, and subtotal thyroidectomy was performed in 6 (12 %) patients.

Pathological examination of the samples revealed 32 (64 %) classical variants of PTC, 10 (20 %) follicular variants, 7 (14 %) tall cell variants, and 1 (2 %) sclerosing variant. Concerning the tumor size, 45 (90 %) patients had macrocarcinoma with a mean diameter of 3.8 cm, whereas 5 (10 %) patients had a microcarcinoma with mean diameter of 0.58 cm. On the basis of surgical and histopathological examination of the studied group, multifocality was seen in 22/50 (44 %), capsular invasion in 15/50 (30 %), vascular invasion in 27/50 (54 %), lymph node metastasis in 27/50 (54 %), and distant metastasis in 10/50 (20 %) patients. The demographic data and histopathologic features of the studied group are summarized in Table 1.

Screening for the BRAFV600E mutation in PTC patients revealed only one case (2 %) with heterozygote BRAFV600E mutation (Figs. 1 and 2). The BRAFV600E-positive case belonged to a 53-year-old female patient, and her pathological report revealed a multifocal macrocarcinoma for which she underwent total thyroidectomy. Histopathological examination of the BRAFV600E-positive sample showed multifocality, vascular invasion, capsular invasion, and lymph node metastasis, but with no distant metastasis to surrounding tissues.

Discussion

Thyroid cancer accounts for more than 90 % of all endocrine cancers (Sherman et al. 2004). PTC is the most prevalent histological type with an excellent overall survival; however,

Table 1 The demographic and histopathological data of the studied group (N=50)

Variable	N (%), N=50	P value
Age (years)		
>45	24 (48)	0.09
≤45	26 (52)	
Sex		
Females	35 (70)	0.001
Males	15 (30)	
Histological variants		
Classical	32 (64)	0.01
Follicular	10 (20)	
Tall	7 (14)	
Sclerosing	1 (2)	
Size of the tumor		
Macro	45 (90)	0.001
Micro	5 (10)	
Multifocality		
Yes	22 (44)	0.1
No	28 (56)	
Vascular invasion		
Yes	27 (54)	0.02
No	23 (46)	
Capsular invasion		
Yes	15 (30)	0.001
No	35 (70)	
Lymph node metastasis		
Yes	27 (54)	0.02
No	23 (46)	
Distant metastasis		
Yes	10 (20)	0.001
No	40 (80)	

Data are presented as number (N) and percentage (%)

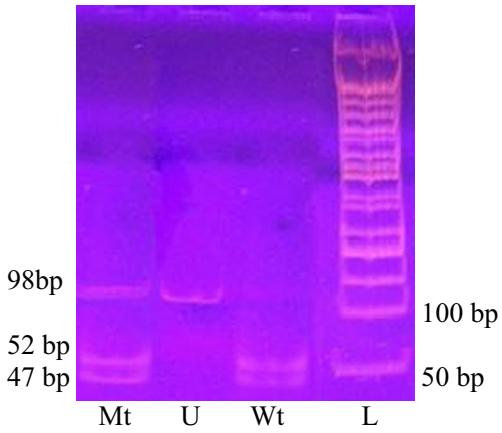

Fig. 1 RFLP analysis showing heterozygote BRAFV600E-positive mutation. *Lane 1* 50-base pair (*bp*) ladder, *lane 2* wild-type (*Wt*), *lane 3* uncut PCR product, *lane 4* mutant type (*Mt*)

up to 10 % of patients eventually die as a result of the disease (Zheng et al. 2012).

A single hot-spot mutation (T>A) at nucleotide 1799 of the BRAF gene had been identified as the most common genetic event in PTC, with a prevalence of 29–83 % (Cantwell-Dorris et al. 2011). Preoperative diagnosis of PTCs using a specific molecular marker as the BRAFV600E would be helpful for the better management and follow-up of patients (Xing et al. 2013).

This study included 50 archived FFPE of PTC tissue samples that were investigated for the BRAFV600E point mutation. Only one case (2 %) had heterozygote BRAFV600E mutation indicating a low prevalence of this mutation among Egyptian patients with PTC. This result was in contrast to studies from different ethnic populations such as Iran, Korea, and Japan (Mohammadi-Asl et al. 2009; Kim et al. 2010; Fukushima et al. 2003) who found high positive rate of this mutation in PTC samples, 71.4 and 52.6 % respectively. The low positive rate encountered in the present study was much lower than the rates observed in other Mediterranean countries such as Italy (55.3 %) (Zatelli et al. 2009) and Greece (36.2 %) (Mitsiades et al. 2007). To the best of our knowledge, this is

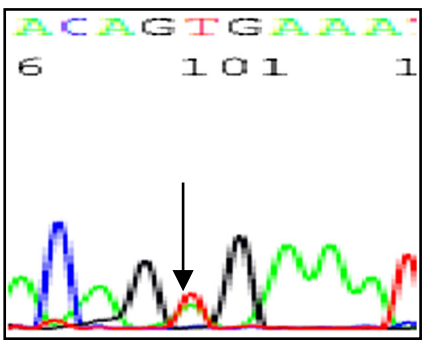

Fig. 2 Heterozygote BRAFV600E mutation confirmed by direct sequencing (*arrow*)

the first study reporting the status of BRAFV600E among Egyptian PTC patients. Also, there are no other similar studies in the Middle East and Arabian countries.

Several factors may contribute to the difference in the prevalence of the BRAFV600E mutation in PTC, as geographic area, ethnicity, and iodine consumption were proven in a Korean study that mentioned the prevalence of BRAFV600E mutation to be 80 % in PTC patients with high iodine consumption (Kim et al. 2013).

Also, the variation of detection methods may be another reason to explain the great variation in the prevalence of this mutation that has been reported in different studies on PTC (Kim et al. 2009).

An important explanation of the molecular results of the present study is that other genetic mutations were suggested to contribute in the tumorigenesis of PTC. Goutas et al. (2008) studied the prevalence of RAS and BRAF mutations among a group of 99 thyroid tumor samples, 55 of which were PTC. K-RAS was positive in 30/55 (54.5 %), while BRAF mutation was positive in 15/55 (27.3 %) of the PTC samples.

The BRAFV600E-positive sample belonged to a 53-year-old female. Histopathological examination of the sample showed that it was a classical PTC variant. This was in agreement with Trovisco et al. (2005); Zatelli et al. (2009), and Kim et al. (2013) who pointed that the V600E is highly associated with the classical variant of PTC in comparison to all the other variants. This could be explained by the different oncogenic mechanisms that might be involved in each PTC variant, the different diagnostic criteria used by different pathologists (Lloyd et al. 2011).

As for the age and gender, the present study showed that the V600E-positive case belonged to a 53-year-old female. Similarly, a study of Mohammadi-Asl et al. (2009) included 28 PTC samples. The V600E mutation was positive in 16/22 (72.2 %) females in comparison to 4/6 (66.7 %) males and in 11/15 (73.3 %) of patients with ages >45 years in comparison to 9/13 (69.2 %) in patients <45 years. On the contrary, Lee et al. (2007) found no correlation between the V600E mutation and patient age.

The V600E-positive case in the present study was found to have poor clinicopathological characteristics as large tumor size (3.5 cm), multifocal lesions, lymph node metastasis (3 nodes out of 10 involved), and capsular and vascular invasion, but with no distant metastasis. These poor clinicopathological features were found to be commonly associated with V600E mutation in various studies (Ito et al. 2009; Kim et al. 2011, 2013; Basolo et al. 2010; Lee et al. 2013). On the contrary, few studies reported no significant correlation between V600E mutation and any of the common high-risk pathological characteristics (Fugazzola et al. 2006; Durante et al. 2007; Ito et al. 2009; Kim et al. 2010).

On the contrary to the present study results, Lloyd et al. (2011) showed a high prevalence of V600E mutation in highly

aggressive microcarcinomas with a possibility of rapid progression to a large sized tumor.

The conflicting results of these studies might be due to variations in the study populations in terms of the genetic factors, histological variants, environmental factors, molecular methods, and clinicopathological criteria used in V600E analysis (Xing et al. 2013).

Limitation of the present study is the small number of the studied patients. Large-scale studies and a study of other molecular markers are highly recommended for better elucidation of the genetic basis of PTC among Egyptian patients.

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Conflict of interest Authors disclose no conflict of interest.

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