BRAF V600E mutation in differentiated thyroid carcinoma

Mutation BRAF V600E dans le cancer différencié de la thyroïde

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ABSTRACT

Background: The BRAFV600E mutation is a common genetic alteration in papillary thyroid carcinoma (PTC). This mutation has been widely reported, more recentely, in north African population.

Our objectives were to determine the prevalence of BRAF V600E in a Tunisian population with differentiated thyroid carcinoma and to investigate its association with aggressive clinicopathologic characteristics.

Methods: DNA was extracted from paraffin-embedded thyroid tumor specimens taken from 13 patients with PTC and 7 patients with follicular carcinoma. BRAF V600E mutation was determined using multiplex real-time polymerase chain reaction (PCR) assay based on the TaqMan MGB probe system. The fluorescence absorbance of probes was calculated separately.

Results: The BRAF mutation was present in 7 of 13 PTC (53,8%) but was not detected in follicular carcinoma. The age distribution, tumor size, multifocality, lymph node metastasis and staging did not differ significantly between patients with and without the BRAF(V600E) mutation.

Conclusion: The BRAF V600E mutation was detected in more than half Tunisian patients with PTC which in accordance with the literature. However, its presence was not significantly associated with poor prognostic factors. Our findings need confirmation by large Tunisian and African series.

Keywords: Tunisia, prognosis, genetic markers, DNA mutational analysis, carcinoma, papillary.

RÉSUMÉ

But: La mutation BRAF V600E est une altération génétique fréquente dans le cancer papillaire de la thyroïde (CPT). Cette mutation a été identifiée dans différentes populations et récemment quelques études africaines.

Notre objectif est de déterminer la prévalence de la mutation BRAF dans une population tunisienne porteuse d'un cancer différencié de la tyroïde et d'évaluer son association avec des critères d'agressivité clinico-pathologiques.

Matériel et Méthodes:

De l'ADN a été extrait à partir de tissu thyroïdien tumoral paraffiné prélevé chez des 13 patients porteur d'un CPT et 7 patients ayant un carcinome folliculaire. La mutation BRAF V600E a été détectée en utilisant la technique de réaction de polymérisation en chaine en temps réel basé sur le système de sondes TaqMan MGB. L'absorption de la fluorescence des sondes a été calculée séparément.

Résultats: La mutation BRAF V600E a été identifiée dans 7 cas de CPT (53,8%) mais n'a pas été retrouvée dans les cancers folliculaires. Il n'existait pas de différence statistiquement significative entre les patients porteurs de la mutation BRAF V600E et les autres patients concernant l'âge, la taille tumorale, la multifocalité, les métastases ganglionnaires et le stade tumoral.

Conclusion: La mutation BRAF V600E a été retrouvée dans plus de la moitié de la population Tunisienne ayant un CPT, ce qui rejoint les données de la littérature. Cependant, sa présence n'était pas significativement associée à des facteurs de mauvais pronostic. Mais nos données nécessitent une validation par un échantillon plus large.

Mots clés: Tunisie; pronostic; marqueurs génétiques; analyse de mutations d'ADN; carcinome papillaire.

INTRODUCTION

The term differentiated thyroid carcinoma (DTC) defines papillary (PTC) and follicular (FTC) thyroid carcinomas and accounts for about 90% of all thyroid carcinomas [1]. DTC has usually a favorable course, with survival rates of approximately 85%-90% after five years [2]. However, more than 10% of patients eventually die of DTC and an even greater proportion faces the morbidity of recurrences [2]. The recognition and evaluation of prognostic factors combined with data from molecular factors that evaluate

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the risk of recurrence and may stratify DTC patients, have been used for the selection of specific treatment modalities [3].

BRAF V600E mutation (hereafter referred to as "BRAF mutation"), the most potent activator of the mitogen-activated protein kinase pathway, plays a central role in the regulation of cell growth, division, and proliferation [4,5]. It accounts for more than 90% of all mutations found in the BRAF gene [6].

The reported prevalence of the BRAF mutation in PTC ranges from 27,3 to 90,2% [7,8]. The average prevalence rate was 56,3% [8]. In Middle Eastern population, only one study analyzed the prevalence of BRAF mutation [9]. The mutation was identified in 51,7% of studied PTC, and the authors found a significant association between BRAF mutation and either metastasis and disease-free survival [9].

Many studies including meta-analyses have shown a correlation between BRAF mutation and at least one poor prognostic factor [8, 10]. Conversely, other investigations were not able to confirm these correlations, even by analyzing a large number of patients [11]. Therefore, the clinical significance of BRAF V600E mutation in PTC is still controversial.

The aim of the present study was to evaluate for the first time in Tunisia, the prevalence of the BRAF mutation in Tunisian patients diagnosed with DTC and to analyze the association between high-risk clinicopathologic characteristics and this mutation.

METHODS:

Patients

Under an institutional review board-approved protocol, a retrospective study was conducted of a cohort of patients with DTC, treated and followed up using the same protocol at our hospital in 2010. The diagnosis of DTC and histopathological classification of the tumours were carried out according to the standards of the World Health Organization [12]. The hospital records were retrospectively reviewed in relation to various prognostic factors, and the information was entered into the database. The following data were collected for each patient:

-Demographic characteristics: age at diagnosis, gender, family history of thyroid disease and/or DTC and personal history of external head and neck radiotherapy.

-Tumor characteristics at diagnosis: main histological type and histological subtype (if any) according to the WHO classification; size, focality, number of tumors, the presence of thyroiditis in the surgical specimen, the existence of nodal metastases and distant metastases.

-Characteristics related to the treatment used: initial surgery (total thyroidectomy, total thyroidectomy in two stages), lymphadenectomy and its extent, the number of doses of 131I ablation therapy.

-All patients were staged based on the above data using the staging system of the AJCC (American Joint Committee on Cancer) based on the TNM classification system (7th edition) and age [13].

-Date of the final follow-up and date and cause of death.

BRAF mutation analysis

The BRAF mutation analysis was performed in the Laboratory of Histology in Medicine school of Sfax in southern Tunisia.

Somatic DNA was extracted from two 15-mm thick paraffin-embedded sections obtained from each sample, using the QIAamp DNA Mini Kit (Qiagen; ref 51306, CA, USA) according to the manufacturer's instructions. Areas of tumor were identified on hematoxylin and eosin stained slides and marked by pathologists to determinate the percentage of tumoral cells. It was between 10 % and 100 %.

Real-time PCR, endpoint genotyping, was performed using the LightCycler 480 (Roche Diagnostics, Indianapolis, IN, USA) in 384 template to detect the BRAFV600E (1799T>A) somatic mutation of the BRAF oncogene in a background of wild-type genomic DNA. It was determinate using a multiplex real-time PCR assay based on the TaqMan MGB probe system.

The sequences of primers and probes are listed in (Table I). The PCR conditions were as follows: denaturation at 95°C for 10 minutes, followed by 38 cycles of amplification at 95°C for 15 seconds, 60°C for 1 minute 30 seconds, and a cooling step at 40°C for 1 minute.

Table I: Primers and probes sequences

	Forward primer	Reverse primer	Reporter 1 VIC	Reporter 2 FAM
Bra fV600E	CTACTGTTTTCCTTAC TTACTACACCTCAGA	ATCCAGACAACT GTTCAAACTGATG	CTAGCTACA GTGAAATC	TAGCTACA GAGAAATC

In a final volume of 5 μl, the PCR mixture was composed of 1 μl of extracted DNA at 10 ng/μL, 0.125μl of each of mutation and control assay mix 40× designed by Applied (USA) and 2.5μl of Taqman Genotyping Master mix 2× from Applied (USA).

All samples were studied in duplicate. The BRAF mutation assay was labeled with 6-carboxyfluorescein (FAM), and contained a specific probe for the discrimination of the V600E mutated allele. The internal control assay, labeled with VIC (define acronym), was used for the wild type one. The fluorescence absorbance of probes was calculated separately. The result was displayed in the form of a scatter plot as ordinate the fluorescence absorbance of mutant assay FAM and as abscissa the fluorescence absorbance of control assay VIC. Samples situated on median were considered positive for the V600E BRAF mutation as it is heterozygosis.

Statistical analysis:

All statistical analyses were performed using PASW Statistics ver. 18.0 (SPSS Inc., Chicago, IL, USA). The chi-square test and Fisher exact test were used for analysis of the relationship between the BRAF mutation and clinicopathologic factors for univariate analysis. A $p<.05$ was considered statistically significant.

RESULTS:

Thirty patients with primary DTC were treated in our department during 2010. Seven were excluded due

to incomplete data. In three cases, the DNA quality of samples was deficient to analyze BRAF V600E mutation. These patients were excluded from the subsequent analysis resulting in a final study cohort of 20 patients.

BRAF V600E mutation was found in 7/20 (35%) DTC (figure 1). Our cohort included 13 PTC and 7 FTC. As expected, no BRAF mutation was observed in FTC samples. Seven PTC samples contained the mutated type allele (53,8 %) whereas 6 PTC samples contained the wild type allele. Patients and tumor characteristics of the final cohort are summarized in (Table II).

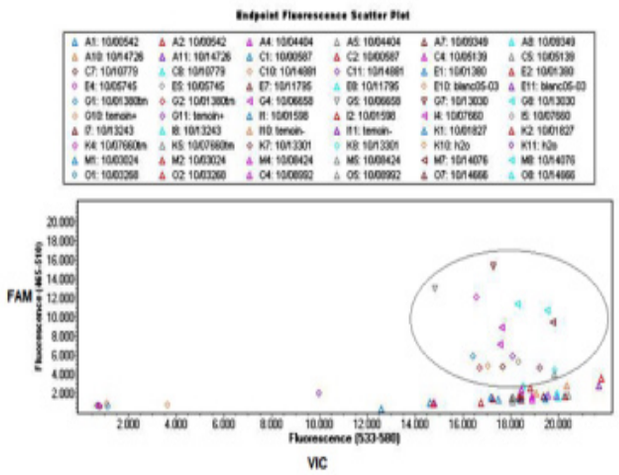

Figure 1: Endpoint fluorescence scatter plot: Encircled samples are positive for BRAF V600E mutation.

Table II: Clinical and pathological characteristics of patients

Characteristics	No. (%)
Age (yr) <45 ≥45	 7 (53,8) 6 (46,2)
Sex Male Femal	 3 (23) 10 (77)
Thyroidectomy Total or near total Completion	 7 (53,8) 6 (46,2)
LN dissection Central compartment dissection Central + lateral neck dissection	 12 (92,3) 3 (23)
LN metastasis	6 (50)
Thyroiditis None Lymphocytic thyroiditis Hashimoto's thyroiditis	 11 (84,6) 2 (15,4) 0
Focality Unifocal Multifocal	 6 (46,2) 7 (53,8)
BRAF mutation Negative Positive	 6 (46,2) 7 (53,8)
T category pT1 pT2 pT3	 8 (61,6) 2 (15,4) 3 (23)
TNM stage I II III	 6 (66,7) 2 (22,2) 1 (11,1)

The mean age was 41 ±18 years old. Female predominance was noted (F/M: 3/1). One female patient had personal history of external head and neck radiotherapy and familial history of thyroid cancer. No suspected cervical lymph node metastasis or distant metastasis at the time of initial presentation was noted in our patients.

All of patients underwent surgery consisting of total thyroidectomy. Papillary carcinoma was present in 13 cases. Patients had either pure (7 cases (53,8%)) or follicular variant of papillary carcinoma (4 cases (30,7%)) or microcarcinoma (2 cases (15,3%)).

For the 17 patients who underwent cervical lymphadenectomy, we could analyze the BRAF mutation status in both primary tumor and lymph node metastasis in only two cases. The BRAF mutation was present in the two primary tumors. However, BRAF mutation was detected in lymph node metastasis in only one case.

After surgery, 131I ablation therapy was administered in nine cases (69,2%). The mean dose was 210 mCI. For patients who did not have 131I therapy, one presented a microcarcinoma without high risk clinicopathological factors. For the remaining cases, the patients were lost during the follow up after the surgery. Distant metastases were found during the first dose of 131 I ablation therapy in one patient. The metastases occurred in the lung. Mean follow-up time was 47 months.

The univariate analysis showed no significant association between BRAF mutation status and high risk clinicopathological factors (including age of patients, tumor size, multifocality, lymph node metastasis and TNM stage) (table 3).

Table III: BRAF mutation and clinicopathologic factors: Univariate analysis

Variable	BRAF (+)	BRAF (-)	p-value
Mean Age (Yr)	43	38.8	0,53
MeanTumor size (cm)	2,45	2,3	0,44
Multifocality Yes No	 5 2	 2 4	0,28
T1 (low risk group) T2 + T3 (high risk group)	4 3	4 2	1
N0 N1	1 5	5 1	0,08
TNM stage 1+2 3+4	 4 1	 2 2	0,52

DISCUSSION

The thyroid cancer is a relatively uncommon disease, afflicting only 4 per 100 000 persons around the world. However, this rate varies over the decades and among different countries, with a higher incidence in Europe and North America [14]. In North Africa, published studies from Algeria showed an incidence of thyroid

cancer of 1,4 per 100 000 for men and 6,2 per 100 000 for women between 2006 and 2010 [15]. In Morocco, the incidence of thyroid cancer for men was 1,4 per 100 000 and 6,7 per 100 000 for women between 2005-2007 [16]. In Libya, the uptaded report from the Benghazi cancer registry (Eastern Libya) showed a world age-standardized incidence rate of 0,8 per 100 000 for men and 3,8 per 100 000 for women [17]. In Tunisia, there were 3 registers for cancer. One register accounts for northern population in Tunisia, a second register for the center (Sousse) and the last register for the south (Sfax). For North Tunisia, the incidence was 1 for the men and 3,26 for the women per 100 000 habitants between 2004-2006 [18]. In Sousse province, the incidence for the female was 2,8 per 100 000 between 1993 and 2006 [19]. For the south of Tunisia and particularly in Sfax province, the incidence was 0,8 for the men and 2,96 for female, per 100 000 habitants between 2000-2002 (20).

In our Tunisian cohort, BRAF mutation was seen in more than half of PTC samples studied (53,8%). This is in accordance with previous reports [7] including the only Arabic population study [9]. However, unlike Abubaker et al. results study [9], we failed to demonstrate a significant correlation between BRAF mutation and PTC prognostic factors.

Actually, little information is known in PTC about which molecular targets of the BRAF mutation and its consequent downstream phosphor-MEK 1/2 and phospho-ERK 1/2 kinases deregulation leads to this worse prognosis. Until now, it is not clearly understood how BRAF mutation leads to more aggressive PTCs. Nucera et al. after analyzing 539 gene sets, identified 17 upregulated and one downregulated gene sets exclusively linked to the genomic signature of BRAF V600E positive PTCs [21]. These new genes are involved in cellular signaling cross-talks and in the regulation of the extracellular matrix remodeling. The authors concluded that their findings may help to explain how this single gene mutation triggers thyroid tumor cell migration and invasion thus causing a worse clinical picture for patients harboring the mutation [36].

Another argument, supporting the clinical aggressiveness of this mutation, is that the expression of BRAF in thyroid cells in culture demonstrated a strong oncogenic potential and increased matrix cell invasion with respect to the proto oncogene RET (rearranged during transfection)/PTC, the other most frequent genetic alteration in thyroid cancer [22,23].

Experiments in animal models demonstrated the pathogenetic role of BRAF mutations and its important role in the aggressive behavior of PTCs [24]. Nucera et al. showed that an orthotopic mouse model of anaplastic thyroid carcinoma cells harboring BRAF mutation displays tumor aggressiveness and lymph node as well as lung metastases that recapitulate an advanced human thyroid cancer [25].

Although the studies in favor of a correlation between the BRAF mutation and more aggressive PTC prevail, the results of many other studies [11,26-32], including our study, did not confirm the association between the BRAF mutation and high-risk clinicopathological factors. Differences in the diagnostic criteria, clinical data collection, methods to detect BRAF mutation, genetic, geographical and environmental factors had been suggested to explain these apparently conflicting results [33]. However, there is no definitive explanation for these inconsistent results.

Guerra et al. [34] were the first to use the pyrosequencing analysis to quantify the occurrence of the BRAF mutation in the cancer cells. They demonstrated that the correlation with the disease outcome is not a feature of any PTC harboring the BRAF mutation but that this correlation is instead restricted to PTC with a high percentage of BRAFV600E alleles [35]. They also proved that the direct sequencing of BRAF using the BigDye Terminator method was less sensitive than pyrosequencing, indicating that samples with a lower percentage of mutant alleles (less than 20%) were considered wild type by direct sequencing. Guerra et al. concluded that their results might explain why even some large studies failed to find a correlation between BRAF V600E and clinicopathological features [35]. These results were confirmed by Kim et al. on the Korean patients [36]. However, Gandolfi et al. using the pyrosequencing analysis to investigate the impact of the V600E mutation on the metastasis tendency of primary PTCs, found no correlation between the occurrence or the percentage of the BRAF V600E mutated alleles at the primary site with the development of either distant or lymph node metastases (LNM) [32].

Concerning the correlation between the BRAF mutation and LNM [32,37,38], literature results are still conflicting. Lu et al. [38] found a high concordance in BRAF V600E mutational status in primary and matched metastatic lesions. However, in 3 cases the mutation was positive in primary lesions while their matched LNM were wild type. They assumed that whether it's BRAF mutant or not, certain cells were preferentially selected by factors other than BRAF V600E mutation during microevolution and invaded regional lymph nodes, grew faster or became multifocal [38,39]. The authors concluded that BRAF V600E mutation was not involved in the process of LNM and that BRAF V600E functions solely as an initiator in tumorgenesis of PTC [38]. In the current study, we analyzed the mutation status of both primary tumor and the lymph node metastasis in only 2 patients. We found that BRAF mutation was present in the two primary tumors. However, the mutation was lost in one case and present in lymph node metastasis in the other sample. Others previously published works reported this phenomenon, who accounts between 6 and 20% of the described cases [30,32,40].

CONCLUSION:

This is the first African study that investigates the BRAF mutation status in DTC. The prevalence of this mutation was 53,8% of Tunisian patients with PTC. No

correlation was found between BRAF mutation and poor prognostic factors. However, this study had limitations including the small size of our cohort. We couldn't also perform the analysis between the association of the presence and percentage of BRAFV600E alleles with recurrence and survival data due to lack of resources. But despite these limitations, we think that our results offer some valuable data about this part of the world. Another well-designed study with long-term follow up would be required to clarify the associations of the BRAF mutation with the aggressiveness of PTC in the Tunisian population.

Compliance with ethical standards

Conflict of interest: The authors stated that there is no conflict of interest.

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