Med Oncol (2013) 30:336
DOI 10.1007/s12032-012-0336-3

ORIGINAL PAPER

Expression analysis of B-Raf oncogene in V600E-negative benign and malignant tumors of the thyroid gland: correlation with late disease onset

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Received: 28 August 2012 / Accepted: 26 September 2012

Abstract B-Raf, a member of the Raf serine/threonine kinase family, is an intermediate molecule in the mitogen-activated protein kinase pathway, which relays extracellular signals from the cell membrane to the nucleus via a cascade of phosphorylation events, ultimately promoting cancer development. This pathway is usually activated in human neoplasias. The purpose of this study was to investigate the role of B-Raf in thyroid pathology. We scanned for the presence of mutations at codon 600 (V → E) of the B-Raf gene, using a PCR–RFLP assay. In tumors with no mutation (32 benign and malignant thyroid tumors) and in their adjacent normal tissue, we measured the expression levels of B-Raf gene, using a quantitative real-time PCR (qPCR) assay. B-Raf expression in V600E-negative tumors deviated from the normal pattern, since it was overexpressed in 42 % of benign samples and downregulated in 54 % of malignant specimens. Hashimoto's thyroiditis also seemed to play an important role, since benign specimens with Hashimoto's thyroiditis had a 2.2-fold higher B-Raf expression than samples without thyroiditis (1.71 ± 0.63 vs. 0.78 ± 0.13). Statistical analysis revealed that B-Raf deregulation postponed disease onset by more than 10 years in both benign and malignant thyroid (benign: 55.6 ± 3.9 vs. 45.3 ± 3.3, $p = 0.049$; malignant: 52.2 ± 3.5 vs. 33.0 ± 7.9, $p = 0.020$). From the above results, we deduce that in the absence of mutation activation, B-Raf overexpression or downregulation is a protective event, since it delays the development of both malignant and benign thyroid tumors.

Keywords Mutation · mRNA · Real-time PCR · Hashimoto's thyroiditis · Disease onset

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Introduction

Although thyroid cancer is the most common endocrine malignancy, it is a relatively rare disease, accounting for ~1 % of new cancer cases each year, with a male:female ratio 1:3. The most prominent histological type is papillary thyroid carcinoma (PTC), with 80 % frequency among malignant thyroid tumors [1]. The medical treatment for PTC is thyroidectomy, followed by radioiodine ablation. 10-year survival rates are ~89 % [2].

The Raf serine/threonine kinase family has three members, A-Raf, B-Raf, and C-Raf, which share three conserved regions, CR1, CR2, and CR3. Raf is activated when phosphorylated Ras binds to the Ras-binding domain (RBD) and cysteine-rich domain (CRD) of CR1 [3, 4]. After its activation, which can also occur by Ras-independent elements, Raf relays the upstream signals, ultimately mediating cell proliferation and migration and thus playing a significant role in angiogenesis, tumorigenesis, and metastasis [5].

Somatic B-Raf mutations are observed in 60–70 % of melanomas, colorectal carcinomas, and ovarian malignancies [6, 7], and at lower rates in gliomas, sarcomas, lymphomas, leukemias, and neoplasms of the breast and lung [8, 9]. The most common mutation, accounting for 80 % of all B-Raf mutations, is a single-base substitution which changes the amino acid Valine (V) with Glutamic acid

Published online: 22 December 2012

(E) at codon 600 of the B-Raf coding region (V600E) at exon 15 and leads to the activation of B-Raf, independently of the upstream signal from Ras [6].

B-Raf mutations have been also detected in PTCs [10]. The V600E mutation in particular, with a frequency between 35 and 55 % [11–13], has been linked to disease aggressiveness and progression, frequent recurrences and poor patient survival [14, 15].

Since no data exist regarding B-Raf expression, especially in tumors with no mutations, we aimed to investigate the role of the B-Raf oncogene, by evaluating its mRNA expression in benign and malignant thyroid tumors that have not been activated by the V600E mutation, and to correlate the results with patients' clinical and pathological characteristics.

Methods

Study subjects

Benign or malignant and adjacent normal tissue samples were collected from patients that underwent surgery for thyroid tumors at the Department of Surgery, 251 Hellenic Air Force Hospital, Athens, Greece. Specimens were immediately frozen after surgical removal and stored at −80 °C until used. All tumors were located only on one thyroid lobe, and normal tissues were obtained from the other, unaffected lobe. Histopathological analysis revealed that all malignant tumors were papillary thyroid carcinomas (PTCs). The clinical and histopathological characteristics of the patients used, after excluding those with V600E mutations, are listed in Table 1. The Ethics Committees of the University of Crete and the 251 Hellenic Air Force Hospital approved this study, and written informed consent was obtained from all participants.

Table 1 Clinicopathological characteristics of the study subjects

	Benign ($n = 19$)	Malignant ($n = 13$)
Age, years (mean ± SD)	49.6 ± 11.9	47.8 ± 13.9
Gender		
Male	4 (21.1 %)	2 (15.4 %)
Female	15 (78.9 %)	11 (84.6 %)
Hashimoto thyroiditis		
Yes	8 (42.1 %)	–
No	11 (57.9 %)	–
TNM		
T1–T2	–	9 (69.2 %)
T3–T4	–	4 (30.8 %)
Cancer stage		
I	–	8 (61.5 %)
II–IV	–	5 (38.5 %)

DNA extraction

DNA extraction was performed using the Genomic DNA Purification Kit (Genomed, Löhne, Germany) according to the manufacturer's instructions. DNA was stored at −20 °C until use.

PCR–RFLP analysis

The primers used for the identification of the V600E mutation are listed in Table 2. PCR was carried out at an annealing temperature of 58 °C, using *Pfu* polymerase for higher accuracy (Fermentas, Vilnius, Lithuania).

PCR products (10 μl) were incubated at 37 °C with 20 units of XbaI endonuclease (Fermentas) for 16 h (Table 2). Restriction fragments were resolved on 2 % (w/v) agarose gels and stained with ethidium bromide. Results were confirmed in representative samples by direct sequencing.

RNA extraction and cDNA synthesis

RNA was isolated from the 32 B-Raf V600E-negative tumors using the PARIS™ Protein and RNA Isolation System (Ambion, Austin, TX) according to the manufacturer's protocol. RNA concentration and purity was determined by measuring its absorption rate at 260/280 nm with a ND1000D spectrophotometer (NanoDrop, Wilmington, DE).

cDNA was synthesized by reverse transcription (RT) with the RETROscript® Kit (Ambion) according to the manufacturer's manual. cDNA was stored at −20 °C until used.

Quantitative real-time PCR

B-Raf mRNA expression was measured using a real-time qPCR assay with SYBR® Green I dye, with β-actin as internal control (Table 2). cDNA (1 μl) was amplified in a PCR (final volume 20 μl) containing 2× Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) and 300 nM of each primer. A representative pool of all samples was diluted in a series of six 2× dilutions and was run in the same plate, in order to construct a standard curve for the quantification process. After initial denaturation at 95 °C for 10 min, samples were subjected to 45 cycles of amplification, comprised of denaturation at 95 °C for 20 s, annealing at 60 °C for 30 s and elongation at 72 °C for 20 s, followed by a melt curve analysis, in which the temperature was increased from 60 to 95 °C at a linear rate of 0.2 °C/s. Data were collected during annealing (two measurements) and at all times during melt curve analysis. Experiments were conducted on Mx3000P real-time PCR thermal cycler using the software version 4.1 (Stratagene,

Table 2 Primer sequences used for PCR–RFLP and quantitative real-time PCR

Gene	Primer pair sequence (5′–3′)	Tm (°C)	PCR product (bp)	$V^{600}E$ XbaI RFLP
B-Raf (DNA)	TCATAATGCTTGCTCTGATAGGA	58	224	V:124 + 87 + 13
	GGCCAAAAATTTAATCAGTGGA			E:211 + 13
B-Raf (RNA)	GGGGCAGTCGCGCCTGTGAA	60	101	–
	CCGGCGCCCACCACCACCAG			
β-actin (RNA)	CGGCATCGTCACCAACTG	60	70	–
	GGCACACGCAGCTCATTG			

La Jolla, CA). To normalize the mRNA expression of B-Raf, its value was divided by the same sample's β-actin mRNA value. The normalized values of benign and malignant thyroid tumor samples were divided by the normalized values of their adjacent normal samples. Twofold increased (a value ≥ 2) or decreased (a value ≤ 0.5) expression was considered biologically significant (overexpression or downregulation, respectively).

Statistical analysis

Pearson's correlation or the nonparametric Spearman rank correlation was used to examine the association of B-Raf mRNA levels with continuous variables (age). Additionally, their association with categorical data (gender, cancer Stage and TNM, Hashimoto thyroiditis) was examined using Student's *t* test or its nonparametric equivalents Mann–Whitney *U* and Kruskal–Wallis *H* tests. Moreover, the chi-square (χ^2) test, using Fisher's exact test when indicated by the analysis, was used to examine B-Raf expression status with the various clinicopathological parameters after stratification. Finally, one minus survival plots were created with Kaplan–Meier, using the Breslow test to compare the equality of survival distributions in each one. All statistical analyses were two-sided and performed with SPSS 11.5 (SPSS, Chicago, IL). Statistical significance was set at the 95 % level (p value <0.05).

Results

In this study, we measured the mRNA expression levels of B-Raf gene in 32 benign and malignant thyroid tumors, matched with normal adjacent tissue, in which no mutations at codon 600 (V → E) were detected.

B-Raf was overexpressed in 8/19 (42.1 %) benign samples, downregulated in 6/19 (31.6 %), while in 5/19 samples (26.3 %), its expression was normal. On the contrary, B-Raf was overexpressed in 3/13 (23.1 %) malignant samples, downregulated in 7/13 (53.8 %), while in 3/13 (23.1 %), its expression was normal (Table 3). Interestingly, the mean average expression of B-Raf in malignant samples was only ~15 % of its expression in benign samples (0.21 ± 0.06 vs. 1.36 ± 0.33, $p = 0.042$; two-tailed Mann–Whitney *U* test) (Fig. 1). Further analysis revealed an expression difference even within benign tumors: in specimens with Hashimoto's thyroiditis, B-Raf expression was 2.2-fold higher than in samples without thyroiditis (1.71 ± 0.63 vs. 0.78 ± 0.13) (Fig. 2).

Statistical analysis showed that B-Raf expression correlated with age in benign samples, since patients who

Table 3 mRNA expression status of B-Raf in benign and malignant thyroid tumors

	Overexpression	Normal expression	Downregulation
Benign ($n = 19$)	8 (42.1 %)	5 (26.3 %)	6 (31.6 %)
Malignant ($n = 13$)	3 (23.1 %)	3 (23.1 %)	7 (53.8 %)
Total ($n = 32$)	11 (34.4 %)	8 (25.0 %)	13 (40.6 %)

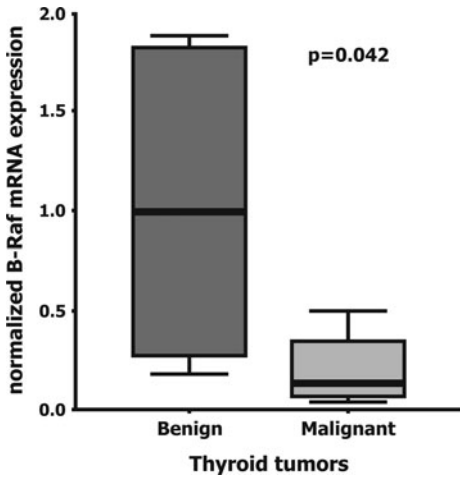

Fig. 1 *Box* and *whisker plots* depicting the mRNA expression of B-Raf gene in V600E-negative benign and malignant thyroid samples. Expression in benign samples was 6.5-fold higher than in malignant specimens (1.36 ± 0.51 vs. 0.21 ± 0.10, $p = 0.042$). Statistical analysis was conducted with two-tailed Mann–Whitney *U* test

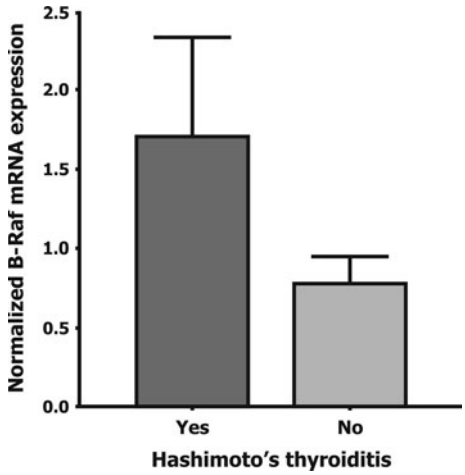

Fig. 2 B-Raf expression in benign thyroid tumors with or without Hashimoto's thyroiditis. Samples with Hashimoto's thyroiditis have a 2.2-fold higher expression than samples without thyroiditis (1.71 ± 0.63 vs. 0.78 ± 0.17)

overexpressed B-Raf were on average 10 years older than those with normal or reduced B-Raf expression (55.6 ± 3.9 vs. 45.3 ± 3.3, $p = 0.049$; Kaplan–Meier Breslow test) (Fig. 3a). A similar phenomenon was observed in malignant samples, since patients who overexpressed or downregulated B-Raf were on average 19 years older than patients with normal B-Raf mRNA expression (52.2 ± 3.5 vs. 33.0 ± 7.9, $p = 0.020$; Kaplan–Meier Breslow test) (Fig. 3b). No other statistically significant findings were observed in benign or malignant samples in comparison with the other clinicopathological characteristics (gender, TNM, cancer stage).

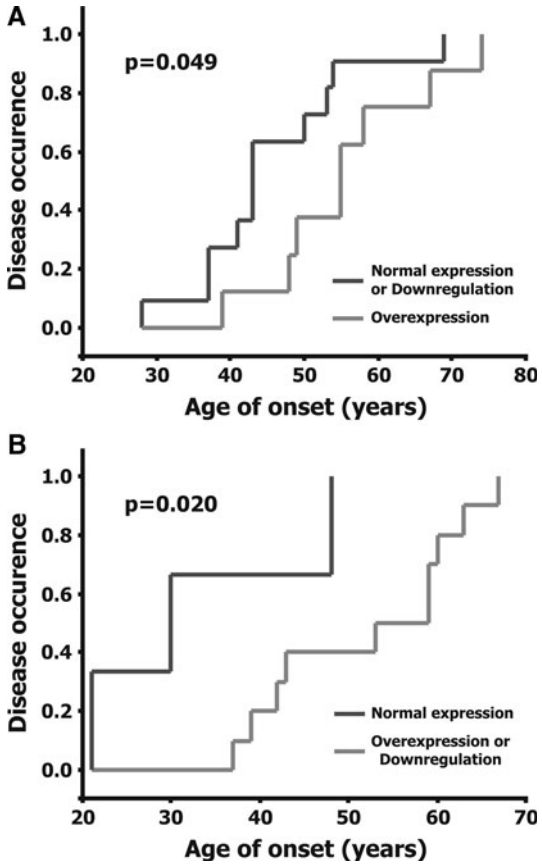

Fig. 3 Kaplan–Meier one minus survival plots of B-Raf expression and age of disease onset in **a** benign and **b** malignant thyroid samples. **a** B-Raf overexpression delays benign disease onset by ~10 years, when compared to samples that have normal or reduced B-Raf expression (55.6 ± 3.9 vs. 45.3 ± 3.3, $p = 0.049$). **b** B-Raf expression deregulation (overexpression or downregulation) delays malignant disease onset by ~19 years, when compared to samples that have normal B-Raf expression (52.2 ± 3.5 vs. 33.0 ± 7.9, $p = 0.020$). Statistical analysis was conducted with the Breslow test

Discussion

In this study, we demonstrated that in V600E-negative tumors, B-Raf was predominately overexpressed in benign specimens (especially those with Hashimoto's thyroiditis) and downregulated in malignant samples and that this deviation from the normal expression pattern in the absence of mutations delays tumor development and onset.

Since B-Raf is a downstream mediator of Ras signaling, and since mutant Ras proteins are present in ~33 % of all human neoplasias [16], the detection of mutant B-Raf alleles supports the involvement of the Ras/Raf/MEK/ERK signaling pathway in tumorigenesis. However, the role of B-Raf V600E mutation in thyroid cancer progression remains unclear. While numerous reports suggest that B-Raf mutations are associated with thyroidal invasion, lymph node metastases, advanced tumor stage, and tumor recurrence [14, 17], several studies have failed to detect such an association [18, 19]. Since 40–60 % of thyroid tumors do not have B-Raf mutations, other genetic events, such as RET/PTC rearrangements [20], contribute to the development and progression of these tumors. Additionally, although oncogenic alterations, such as Ras mutations, have been observed in benign thyroid adenomas, their role is unclear, as the probability of malignant transformation of these benign tumors is low [21].

Our study is the first to examine B-Raf expression in benign and malignant thyroid tumors not activated by the V600E mutation and to find that in both diseases, its transcription levels deviate from the normal pattern. The fact that the average B-Raf expression in malignant samples was only 1/6th of its expression in benign samples is also noteworthy. The literature lacks data regarding mRNA or protein expression of the Ras/Raf/MEK/ERK pathway genes. Only the expression of the upstream Ras genes has been examined thus far in laryngeal tumors. Expression levels were elevated [22].

Interestingly, in our study, the average B-Raf expression of benign tumors with Hashimoto's thyroiditis (HT) was 2.2-fold higher than in samples without thyroiditis. Hashimoto thyroiditis is the most common form of thyroiditis, with a female:male ratio 20:1. The majority of patients is hypothyroid and with elevated thyrotropin serum levels. Some patients with HT develop nodules that share morphological features and immunohistochemical patterns with PTC [23]. Although our results could enhance the link between Hashimoto's thyroiditis and the neoplastic transformation of these lesions, their significance remains unclear, since only a fraction of HT cases evolve into clinically significant papillary carcinomas [24].

We also observed that, in the absence of V600E-activating mutations, B-Raf expression deregulation is associated with delayed benign and malignant thyroid tumor onset by at least 10 years. Although genetic events associated with both early and late disease onset have been observed in several malignancies, such as colon [25], prostate [26], and breast [27], this is the first report for thyroid tumors. The only molecule of the Ras/RAf/MEK/ERK pathway that has been associated with early disease is K-Ras, whose mutations have been linked to early lung cancer onset in mice [28]. Therefore, this is the first study to associate B-Raf with any disease onset. Large cohort studies are needed to verify our preliminary results.

In conclusion, we deduce that B-Raf expression plays an important role in both benign and malignant thyroid disease, by delaying their development and progression in the absence of activating mutations. Nevertheless, the precise molecular pathways that are involved as well as B-Raf's potential role as a therapeutic target or as a biomarker for early disease detection still need to be investigated.

Conflict of interest The authors declare that they have no conflict of interest.

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