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CASE REPORT

Characterization of a novel complex *BRAF* mutation in a follicular variant papillary thyroid carcinoma

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Abstract

Introduction: Activating mutations of the *BRAF* oncogene are frequently detected in papillary thyroid carcinoma (PTC) and have been associated with a worse prognosis. The amino acid substitution V600E accounts for 90% of all oncogenic *BRAF* mutations and is typically detected in classic PTCs, whereas other less frequent *BRAF* mutations seem to be associated with other PTC histotypes.
Case: Screening for activating *BRAF* mutations in a series of 83 PTCs identified the most common V600E mutation in 39 cases (histologically, 38 classic PTCs and 1 sclerosing variant PTC) and a complex in-frame mutation involving amino acids V600–S605 in a stage III multicentric follicular variant PTC, occurring in a 50-year-old female patient, who was affected by hypothyroidism in autoimmune thyroiditis and had a family history of PTC and autoimmune thyroiditis. Since the identified *BRAF* mutation was novel in the literature, bioinformatic modeling was performed to predict its impact on BRAF activity. Although the mutation resulted in loss of a phosphorylation site in the activation loop of BRAF, it was predicted to increase BRAF kinase activity by mimicking an activating phosphorylation.
Conclusions: This study, which reports a new *BRAF* mutation, highlights the usefulness of bioinformatic modeling in the prediction of functional effects of new mutations and indicates that mutation-specific screening tests might miss some rare *BRAF* mutations. These facts should be taken into consideration in the molecular diagnosis of thyroid cancer and in the design of therapeutic protocols based on inhibitors of the BRAF pathway.

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Introduction

The *BRAF* oncogene plays a crucial role in the development of numerous cancer types, such as melanoma, thyroid, colon, ovarian, and stomach cancer (1). It encodes a downstream effector of the RAS G-protein that is part of the ERK/MAPK signal transduction pathway, a cascade involved in the regulation of cell proliferation, differentiation, and apoptosis. *BRAF* mutations are the most common alterations found in thyroid cancer, with a prevalence that varies from 23 to 62% (2), and, together with *RET/PTC* rearrangements (20–40%), they account for almost the totality of genetic alterations at the basis of papillary thyroid carcinoma (PTC).

There is virtually a unique type of *BRAF* mutation, the amino acid substitution V600E, which accounts for about 90% of all oncogenic *BRAF* mutations (1). This mutation causes a 500-fold increase in BRAF kinase activity and it is thought to facilitate the acquisition of secondary genetic events in cancer progression (3). Other less frequent *BRAF* mutations have been described, such as K601E (4), K601del (4, 5), and V599ins (6), but all of them involve amino acids close to V600, suggesting that this is a crucial site to maintain the inactive conformation of the protein. The V600E mutation has been typically detected in classic PTC and tall cell PTC variant, whereas follicular variant PTC has been characterized by the presence of mutations different from V600E, such as K601E (4, 7) or the complex mutation reported by Lupi *et al.* (8), thus suggesting a genotype–phenotype correlation might exist.

We here report a case of follicular variant PTC carrying a novel complex *BRAF* mutation. Bioinformatic modeling was used to predict the effect of the mutation on BRAF activity.

Methods

Detection of BRAF exon 15 mutations

Genomic DNA was isolated from frozen tissues by using QIAmp DNA Mini Kit (Qiagen GmbH). *BRAF* exon 15

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was amplified by PCR using oligonucleotide primer sequences reported by Davies *et al.* (1). Bidirectional sequencing of PCR products was performed by using an ABI PRISM BigDye terminators v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA), and sequences were run on an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) and compared with the reference sequence CCDS 5863.1.

In order to better define the complex heterozygous mutation identified, the PCR amplicon carrying the mutation was subcloned into a pGEM-T Easy vector (Promega), transformed in competent DH5α cells and plated onto LB agar with ampicillin and X-gal selection. Then, 15 distinct blank colonies were picked, plasmidic DNA was extracted and submitted to amplification and sequencing of *BRAF* exon 15 as described above, to separate the two alleles.

Bioinformatic analysis

Mutant and wild-type activation loops (A-loops) of BRAF protein were modeled on the template X-ray structure of BRAF retrieved from pdb databank (pdb code: 1UWH). The A-loop region of the wild-type BRAF encompassing 12 residues from 600 to 611, with the amino acid composition KSRWSGSHQFEQ, and the A-loop of the mutated protein encompassing nine residues from 599 to 607, with the amino acid composition DVGSHQFEQ, were modeled using RAPPER (9). One thousand different loop models were built for each protein and the best energy minimized ones are reported in Fig. 1 and superimposed in Pymol (http://pymol.sourceforge.net/).

Clinical case

Out of 83 consecutive patients (Table 1) who underwent thyroidectomy at our department in the period from January 2005 to July 2007 and had a pathological diagnosis of PTC, 39 carried the most common V600E somatic mutation, due to T1799A transversion, whereas one PTC showed a novel complex mutation, which has not previously been reported in the literature.

The patient carrying the novel *BRAF* mutation was a 50-year-old female who underwent total thyroidectomy and node dissection of the central compartment. Preoperative cytologic analysis of fine needle aspiration biopsy (FNAB) indicated the presence of a follicular variant PTC, with positive galectin-3 immunoreactivity, which was confirmed at histological examination (10). The tumor was classified as stage III, according to the sixth TNM classification, since it was multicentric (consisting of a 2.1 cm tumor mass in the isthmus and a 0.6 cm nodule in the left lobe), infiltrated the perithyroid tissues, invaded the vasculature, but did not have nodal or distant metastases. The patient had a family history of PTC, since her father was operated on for PTC. Moreover, the patient was affected by hypothyroidism in autoimmune thyroiditis, diagnosed about 8 years before, and had two sisters with autoimmune thyroiditis.

The *BRAF* mutation detected in this case of follicular variant PTC consists of the in-frame deletion of 16 nucleotides and the simultaneous insertion of 4 nucleotides (Fig. 1A), thus resulting in the replacement of amino acids from V600 to S605 (VKSRWS) by an aspartate (D) and a valine (V). As a consequence, S602, which is a phosphorylation site in the A-loop, is lost and the interaction between F468 and V600 seems to be disrupted by the V600D substitution (Fig. 1B). Since the loss of a phosphorylation site could lead to decreased BRAF kinase activity instead of activation, we performed bioinformatic modeling to predict the effects of this mutation on BRAF kinase activity, and, more in particular, on the conformation of the A-loop of BRAF and its interaction with the P-loop. In the model, as compared with wild-type BRAF, the deletion of four amino acids shortens the A-loop and hinders it from covering efficiently the enzymatic cleft of the protein (Fig. 1C). This leads to a destabilization of the inactive conformation of the BRAF kinase domain, promoting the active state of BRAF. Altogether, the anomalies of this complex mutation are predicted to increase BRAF kinase activity.

Discussion

This study represents an example of the diagnostic usefulness of bioinformatic modeling in the prediction of the effects of a new mutation on the encoded protein, such as in the case of mutations of the *BRAF* oncogene.

While performing genetic analysis of *BRAF* mutations in our series of PTCs, we detected, in agreement with the literature (2), the most common V600E mutation in 48.2% of cases, mostly classic PTCs, and also a new in-frame deletion/insertion mutation in follicular variant PTC. This type of *BRAF* mutation is really very unusual, since a similar, but not identical, in-frame insertion/deletion has been reported only in a cutaneous melanoma (11). It involved the activation loop (A-loop region) of the C-lobe of the BRAF protein. This region plays an important role in regulating the kinase activity of BRAF as it forms a loop that covers the catalytic cleft of the enzyme stabilizing its inactive state (3). Upon substrate recognition and the subsequent phosphorylation of Thr599 and Ser602 (12), the A-loop disrupts its hydrophobic interaction with the ATP-binding site (P-loop region) exposing the active site (Fig. 1B and C). Over 80% of the mutations are V600E (1) and are likely to result in increased BRAF activity because the charged glutamate residue mimics the structure of a phosphorylated A-loop (13). It is conceivable that the replacement of V600 by aspartate (V600D) in the context of our extended mutation brings about the same effect of the simple V600D missense

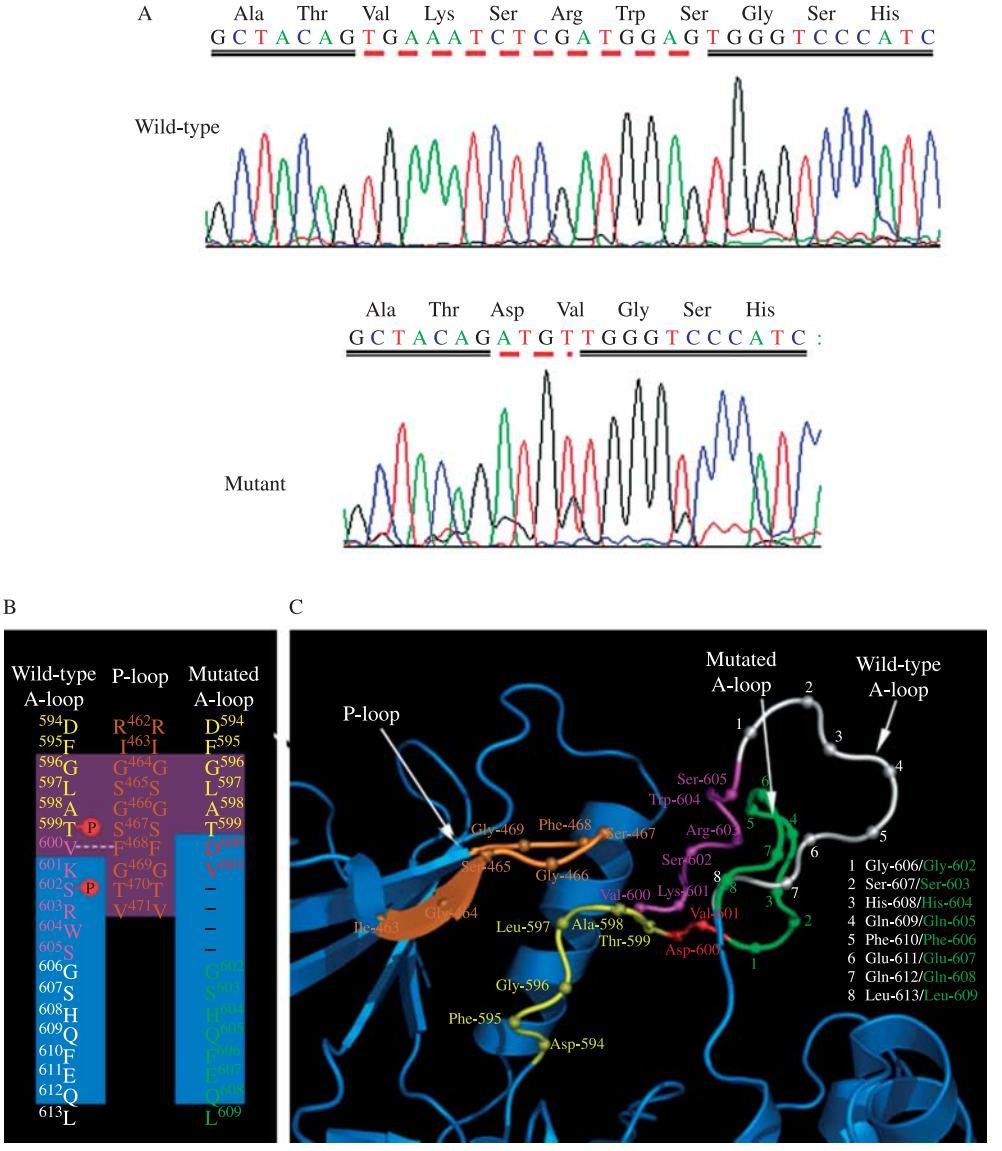

Figure 1 (A) Electropherograms of the wild-type allele and the allele carrying the mutation. Sequences underlined in black are conserved in both wild-type and mutated alleles. Nucleotides deleted and substituted by mutant sequence are underlined in red. (B and C) Comparison of the modeled A-loops from the wild-type and mutated BRAF protein. (B) P- and A-loops interact via a hydrophobic interface (shaded violet) and the V600D mutation disrupts an important contact between F468 and V600 weakening the interaction. Phosphorylation of T599 and S602 is reported. The modeled residues are shaded in blue and superimposed in (C) showing that both the steric hindrance and the length of the A-loop is reduced due to the deletion of four amino acids in the mutated form.

mutation, which has been functionally characterized and demonstrated to increase by 700-fold the kinase activity of BRAF and to enhance ERK activation by about 4-fold (3). The effect of the deletion involving residues from position 601 to 605, which are replaced by the hydrophobic valine, is less clear. In this case, the missing phosphorylation site of Ser602 might decrease BRAF activity. However, Ser602 is considered a minor phosphorylation site with respect to Thr599, which is the major activation segment phosphorylation site (12). A biochemical study would have allowed us to determine the effect of the mutation on BRAF kinase activity, but we decided to bypass time-consuming experiments and to perform *in silico* analysis to get a quick answer. Bioinformatic modeling showed that the deletion of four amino acid residues drastically shortens the A-loop and hinders it from covering efficiently the enzymatic cleft of the protein. Probably, the combination of V600D with the deletion in the A-loop, which further contributes to the destabilization of the inactive conformation of the BRAF kinase domain, strongly increases BRAF activity.

In conclusion, our case, which reports a new activating *BRAF* mutation, underlines the potential usefulness of bioinformatic analysis of new mutations to

Table 1 Clinical and pathological features in 83 patients with papillary thyroid carcinoma (PTC) and their relationship with the presence of *BRAF* mutations.

	***BRAF* mutation** ($n=40$)	***BRAF* wild type** ($n=43$)	***P****
Median age and range (years)	42.5 (20–70)	42.5 (22–67)	0.58
Female gender	28 (47.5%)	31 (52.5%)	0.83
Familiarity for PTC	6 (54.5%)	5 (45.5%)	0.65
Tumor size and extension			
T1	13 (46.4%)	15 (53.6%)	0.93[a]
T2	6 (60%)	4 (40%)	
T3	19 (48.7%)	20 (51.3%)	
T4	2 (33.3%)	4 (66.7%)	
Multicentricity	23 (57.5%)	17 (42.5%)	0.24
Lymph node metastases	24 (55.8%)	19 (44.2%)	0.21
Distant metastases	1 (33.3%)	2 (66.7%)	0.97
Stage			
I	28 (50.0%)	28 (50.0%)	0.86
II	5 (41.7%)	7 (58.3%)	
III	7 (46.7%)	8 (53.3%)	
Histological variant			
Classic	38 (51.4%)	36 (48.6%)	0.19[b]
Follicular	1 (33.3%)	2 (66.7%)	
Oncocytic	0	4 (100%)	
Diffuse sclerosing variant	1 (100%)	0	
Tall cell	0	1 (100%)	

*Mann–Whitney *U* test and χ^2 test were used to calculate *P* values.
[a]Comparison of T1–T2 versus T3–T4.
[b]Comparison of classic PTC versus other PTC histological variants.

quickly predict their effect at protein level. The definition of the oncogenic potential of a mutation, such as in the case of *BRAF* mutations, has important diagnostic, prognostic and therapeutic implications, with the availability of new kinase inhibitors targeting the BRAF pathway. This study also supports the hypothesis that some *BRAF* mutations might be associated with PTC histological variants, since the *BRAF* V600E mutation is generally detected in classic PTCs, whereas other types of mutations, such as the complex insertion/deletion mutation here described, are found in follicular variant PTCs. Finally, we would like to point out that this *BRAF* mutation would have been missed if mutation-specific screening tests were used.

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