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 QIAamp DNA Mini Kit (Qiagen GmbH). BRAF exon 15
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 CDCS 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 mutation.
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


Zhang BH & Guan KL. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. EMBO Journal 2000 19 5429–5439.


References


Acknowledgements

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

<table>
<thead>
<tr>
<th>BRAF mutation</th>
<th>BRAF wild type</th>
<th>P*</th>
</tr>
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<tbody>
<tr>
<td>Median age and range (years)</td>
<td>42.5 (20–70)</td>
<td>42.5 (22–67)</td>
</tr>
<tr>
<td>Female gender</td>
<td>28 (47.5%)</td>
<td>31 (52.5%)</td>
</tr>
<tr>
<td>Familiarity for PTC</td>
<td>6 (54.5%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Tumor size and extension</td>
<td>13 (46.4%)</td>
<td>15 (53.6%)</td>
</tr>
<tr>
<td>T1</td>
<td>6 (60%)</td>
<td>4 (40%)</td>
</tr>
<tr>
<td>T2</td>
<td>19 (48.7%)</td>
<td>20 (51.3%)</td>
</tr>
<tr>
<td>T3</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
</tr>
<tr>
<td>Multicentricity</td>
<td>23 (57.5%)</td>
<td>17 (42.5%)</td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>24 (55.8%)</td>
<td>19 (44.2%)</td>
</tr>
<tr>
<td>Distant metastases</td>
<td>1 (33.3%)</td>
<td>2 (66.7%)</td>
</tr>
<tr>
<td>Stage</td>
<td>28 (50.0%)</td>
<td>28 (50.0%)</td>
</tr>
<tr>
<td>1</td>
<td>5 (41.7%)</td>
<td>7 (58.3%)</td>
</tr>
<tr>
<td>3</td>
<td>7 (46.7%)</td>
<td>8 (53.3%)</td>
</tr>
<tr>
<td>Histological variant</td>
<td>38 (51.4%)</td>
<td>36 (48.6%)</td>
</tr>
<tr>
<td>Classic</td>
<td>1 (33.3%)</td>
<td>2 (66.7%)</td>
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<tr>
<td>Follicular</td>
<td>0</td>
<td>4 (100%)</td>
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<tr>
<td>Oncocytic</td>
<td>1 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse sclerosing variant</td>
<td>0</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Tall cell</td>
<td>0</td>
<td>1 (100%)</td>
</tr>
</tbody>
</table>

*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.