Peptide nucleic acid clamp polymerase chain reaction reveals a deletion mutation of the BRAF gene in papillary thyroid carcinoma: A case report

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Abstract. The BRAF point mutation is the most common genetic event in papillary thyroid carcinoma (PTC), occurring in 29-69% of such tumors. The V600E mutation accounts for up to 95% of all BRAF mutations. Therefore, the majority of diagnostic assays have been developed to detect only the V600E mutation of the BRAF gene. A peptide nucleic-acid (PNA)-clamp quantitative polymerase chain reaction (qPCR) was developed to detect the V600E mutation and other mutations in the BRAF gene. In this study, a 3-bp deletion mutation (c.1799_1801delTGA) was detected in a subject with a PTC by PNA clamp qPCR, in contrast with the results of allele-specific (AS)-PCR. The mutant allele was not detected by AS-PCR, but was detected using PNA-clamp PCR. The atypical 3-bp deletion mutation (c.1799_1801delTGA) was identified by confirmatory PCR combined with sequencing. The conversion of codons 600 (GTG) and 601 (AAA) into a single codon (GAA) resulted in the insertion of a glutamic acid residue into the activation segment of the B-raf protein (p. V600_K601delinsE). In cases where PTC is highly suspected but no mutation is detected by AS-PCR specific for V600E, PNA clamp qPCR, which is complementary to other sequencing methods, should be performed in order to detect other mutations in the BRAF gene.

Introduction

Various oncogenes and tumor suppressor genes regulate the development and progression of papillary thyroid carcinoma (PTC). However, few of these have been identified to date (I). Numerous tumors have a thymine (T) to adenine (A) transversion at nucleotide position 1799 (T1799A) of the BRAF gene, resulting in a valine (V) to glutamate (E) substitution at residue 600 (V600E) (2,3). This BRAF point mutation is the most common genetic event in PTC, occurring in 29-69% of such tumors (4). The incidence rate of BRAF V600E in Korean patients with PTC is relatively high compared with that in other countries (5-7). Few other BRAF mutations have been documented. Therefore, the majority of diagnostic assays, including allele-specific (AS) amplification and quantitative polymerase chain reaction (qPCR) assays, have been developed to detect only the V600E mutation of the BRAF gene.

A peptide nucleic acid (PNA) clamp qPCR assay was developed to detect not only the V600E mutation but also other mutations in the BRAF gene. This method is based on the use of a PNA probe that inhibits the amplification of the wild-type allele while enabling the extension of mutant alleles. In this study, a 3-bp deletion mutation (c.1799_1801delTGA) was identified in a subject with a PTC by PNA clamp qPCR, in contrast with the results obtained by AS-PCR, which did not detect the mutation.

Case report

A 69-yr-old female patient was transferred from the local clinic to Soonchunhyang University Bucheon Hospital (Bucheon, Korea) in order to evaluate thyroid masses detected with thyroid ultrasonography. The largest mass was isoechoic, and measured ~0.9x1.2 cm, with a benign nature among multiple masses in both lobes. A smaller nodule sized mass of 0.7x1.0 cm located in the lower pole of the left lobe was hypoechoic, ill-defined and solid, and suspected to be malignant. Fine-needle aspiration cytology and BRAF mutational analysis were performed on the suspicious lesion.

A thyroid aspiration sample was obtained from the patient after obtaining informed consent. Genomic DNA was isolated from the sample using the QIAamp DNA Mini kit (Qiagen, Chatsworth, CA, USA). AS-PCR was performed to detect mutant alleles in the BRAF gene using the Anyplex BRAF V600E Real-time Detection kit (Seegene, Seoul,
In order to investigate the possibility of other BRAF mutations, PNA clamp qPCR was performed using the PNA Clamp BRAF Mutation Detection kit (Panagene Inc., Daejeon, Korea) according to the manufacturer's instructions. PCR was performed in a total volume of 20 µl containing 50 ng DNA and 15 µl PCR master mix. PCR cycling was performed at 95°C for 15 min followed by 15 cycles at 95°C for 15 sec and 60°C for 1 min, and 35 cycles at 95°C for 30 sec and 60°C for 32 sec. The fluorescent dyes, 6-carboxyfluorescein (FAM) probe was designed to hybridize completely to the V600E allele of the BRAF gene. The Quasar 670 probe (Seegene) was used for hybridization with an internal control. The threshold cycle (Ct) was automatically calculated from PCR amplification plots, in which fluorescence was plotted against the number of cycles. If the Ct of the FAM probe was <33 and the Ct of Quasar 670 was <30, the BRAF mutation was determined to be positive, according to the instructions. A Ct value >33, characteristic of the wild-type sequence, was calculated in this study.

Exon 15 was analyzed since the majority of BRAF mutations, including V600E, occur within exon 15 of the BRAF gene. The amplification product (5 µl) was treated with 2 units shrimp alkaline phosphatase and 10 units exonuclease I (USB Corp., Cleveland, OH, USA). Direct sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction kit on an ABI Prism 3130 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were analyzed and compared with a reference sequence (NM_004333.4). A 3-bp deletion mutation in coding nucleotides 1799 to 1801 (c.1799_1801delTGA) within the BRAF mutational hotspot region was identified (Fig. 1). The conversion of codons 600 (GTG) and 601 (AAA) into a single codon (GAA) resulted in the insertion of a glutamic acid into the activation segment of the B-raf protein (p.V600_K601delinsE).

A total thyroidectomy with dissection of cervical lymph nodes was performed on the patient for PTC. Pathological findings of the nodule revealed classical PTC. The gross surface of the lesions were ill-defined with a hard consistency and measured 1.2x0.9 cm in the lower pole of the left lobe. No metastasis was detected in the four resected lymph nodes. The study was approved by the ethics committee of the Institutional Review Board of Soonchunhyang University Bucheon Hospital (Bucheon, Korea).

**Discussion**

In this study, a rare deletion mutation that was undetectable by AS-PCR was identified. The 3-bp deletion mutation (c.1799_1801delTGA) detected in this study has been reported in other studies concerning thyroid cancer and other malignancies (8,9). Rare BRAF mutations in PTC have been summarized in a previous study (10) and on an online database (www.sanger.ac.uk/genetics/CGP/cosmic). Jung et al reported four cases with unusual BRAF mutations (excluding V600E) in 1,041 patients with PTC (11). In the present study, another case of a BRAF deletion mutation in PTC was identified. In this case, the pathology of the carcinoma was of the classic papillary type.

Numerous laboratories perform screenings specifically for BRAF V600E, which is the most common mutation in PTC, using AS-PCR or other amplification methods. However, these initial screenings may consider a deletion or other mutations in exon 15 of the BRAF gene (excluding the thymidine to adenine substitution at nucleotide position 1799) as the wild-type BRAF gene, as described in this study. Therefore, the use of other analytical methods, which are capable of detecting a variety of atypical mutations in the BRAF gene, is important for
the evaluation of the biological characteristics, pathological features and clinical behaviors of tumors harboring \textit{BRAF} mutations. There are a number of moderate to highly sensitive PCR methods for mutant amplification that block normal allele amplification, including PCR clamping mediated by PNA or locked nucleic acid (LNA), amplification refractory mutation system PCR and thermostable restriction endonuclease-mediated selective PCR (12). PCR clamping by PNA is a simple and sensitive mutant amplification technique, based on inhibition of the amplification of the normal allele due to the strong binding of the PNA probe to the wild-type allele. If a \textit{BRAF} mutant is present, the binding capacity of the PNA probe for the mutant allele is weaker than for the wild-type allele, as nucleotides of the mutant allele do not match the PNA probe. Consequentially, a \textit{BRAF} mutation may be selectively amplified; therefore, this method is useful for detecting mutant alleles, even if they are present at low concentrations in the tissue. This analytical technique is easier to design, compared with previous methods, and may be applicable to the clinical and diagnostic screening of PTC. The mutant amplification efficiency of PNA‑clamp qPCR is comparable to that of PCR combined with sequencing when the reaction conditions are optimized (13). In the present study, the sequencing method successfully detected an atypical \textit{BRAF} deletion mutation.

In conclusion, PNA-clamp qPCR was used to detect a rare 3-bp deletion in coding nucleotides 1799 to 1801 in the \textit{BRAF} gene in a PTC patient. In cases where PTC is highly suspected, but no mutation is detected using AS‑PCR specific for V600E, the use of PNA clamp qPCR (which is complementary to the sequencing method) to detect other mutations in the \textit{BRAF} gene is recommended.

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