The International Journal of Biological Markers, Vol. 24 no. 1, pp. 17-21


# Mutational analysis of the *BRAF* gene in transitional cell carcinoma of the bladder

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**ABSTRACT:** ***Purpose:*** **Mutational activation of the MAP kinase pathway is frequently found in many types of cancer. Recently, activating mutations in the *BRAF* gene, an important activator of this pathway, have been described in several tumor types including melanoma, colorectal and papillary thyroid cancer. The most frequent mutation in exon 15 (V600E) as well as several other mutations within exons 11 and 15 result in constitutive activation of the oncoprotein.**
***Materials and methods:*** **Our study aimed to investigate *BRAF* mutations in 30 human bladder tumors and their adjacent normal tissues. The V600E mutation was screened by PCR/RFLP and exons 11, 14 and 15 of *BRAF* including intron-exon boundaries were sequenced.**
***Results:*** **We detected two tumor specimens bearing two different mutations, both of which were found in exon 15. One sample showed the T1799A (V600E) and the other the G1798T (V600L) mutation. The first specimen was stage pT1a and grade II, whereas the second was stage pT2b and grade III. No mutations within the coding region of exons 11, 14, 15 and the intron-exon junctions for the remaining samples were found.**
***Conclusions:*** **Our results suggest that involvement of *BRAF* mutations in the development of transitional cell carcinoma of the bladder is infrequent. (Int J Biol Markers 2009; 24: 17-21)**

**Key words: *BRAF* mutations, Transitional cell carcinoma**



## INTRODUCTION

Bladder cancer (BCa) is the fourth most common malignancy in men (7%) and the twelfth most common in women (2.5%) and accounts for 3% of all deaths in men in the United States. In 2008, it is estimated that 68,810 new patients will be diagnosed with bladder cancer, with 14,100 of these patients succumbing to the disease (1). Transitional cell carcinomas (TCCs) of the bladder comprise nearly 90% of primary bladder tumors and develop via 2 distinct but somewhat overlapping pathways, the papillary and non-papillary. BCas (~80%) consist of superficial exophytic papillary lesions that originate from urothelial hyperplasia. These typically low-grade papillary tumors may recur, but they rarely invade the bladder wall or metastasize. The remaining 15-20% of tumors represent high-grade solid non-papillary BCas arising from high-grade intraurothelial neoplasia. These tumors aggressively invade the bladder wall and have a high propensity for distant metastasis (2).

The MAP kinase pathway is a significant signaling pathway that regulates cellular processes. Dysregulation of this pathway caused by mutations in different mediators is frequently found in many types of cancer (3). Somatic activating mutations of the *BRAF* gene have been reported in melanomas (>60%) and other cancer types, predominantly the V600E substitution in exon 15 (4). The most commonly reported mutation was a T®A transversion at nucleotide 1799 (V600E) observed in 80% of the malignant melanomas studied; this mutation was previously named T1796A (V599E) (4). Functional analysis revealed that the transversion was the only detected mutation that caused constitutive activation of BRAF kinase activity, independently of RAS activation, by converting BRAF into a dominant transforming protein (4). Additionally, *BRAF* mutations such as the V600E were described in *KRAS*-negative colon carcinomas, suggesting that *BRAF/KRAS*-activating mutations are alternative genetic events in colon cancer (5-7).

The incidence of *BRAF* mutations in TCC of the bladder is not well established. Davies et al screened the entire *BRAF* coding region in 10 bladder cancer cell lines, but no mutation was detected (4). Moreover, the number of published reports regarding the *BRAF* mutation status in TCCs is limited. Our study therefore aimed to screen *BRAF* exons 11, 14 and 15 for the presence of mutations in TCC of the bladder.

0393-6155/017-05$25.00/0

## MATERIALS AND METHODS

### *Study design and clinicopathological data*

Paired tumor and normal tissue samples from a consecutive series of 30 patients with newly diagnosed BCas undergoing transurethral tumor resection at the Department of Urology of the Asklipieio General Hospital in Athens were prospectively studied for *BRAF* gene mutations by restriction fragment length polymorphism (RFLP) analysis and direct sequencing. The patients studied were of advanced age (mean age ± SD of the patient population was 72.2±10.6 years). The majority (26/30, 87%) were smokers or former smokers, while 19 (63%) were characterized by some level of occupational exposure to agents associated with BCa such as paints and chemicals (Tab. I).

Tumor specimens were classified and graded by the same pathologist. Histological grading was performed using both the 1973 World Health Organization (WHO) and the 2004 WHO/International Society of Urologic Pathology (ISUP) classifications (8).

Tumor stage was assessed according to the 2002 American Joint Committee on Cancer staging system (9). Written informed consent was obtained from the patients included in this study. The study protocol was approved by the Ethics Committee of the University of Crete. Eligibility criteria included electively resected primary BCas and the availability of DNA from normal and tumor tissue for biomolecular analyses. Exclusion criteria were a history of previous neoplasms and chemotherapy or radiation therapy prior to surgery.

**TABLE I -** CLINICOPATHOLOGICAL CHARACTERISTICS OF THE PATIENTS

| | |
|---|---|
| Subjects (N) | 30* |
| Sex | |
| Male | 27 |
| Female | 3 |
| Age (years) | |
| Mean | 72.2 |
| Range | 44-86 |
| Smoking† | |
| NS | 4 |
| FS | 8 |
| S | 18 |
| Occupational exposure‡ | |
| Yes | 19 |
| No | 11 |
| Stage | |
| pTa | 1 |
| pT1 | 12 |
| pT1a | 4 |
| pT1b | 1 |
| pT2 | 2 |
| pT2a | 1 |
| pT2b | 5 |
| pT2+in situ | 3 |
| pT3a | 1 |
| Grade (WHO 1973) | |
| I | 0 |
| II | 10 |
| III | 20 |
| Grade (WHO/ISUP 2004) | |
| Low | 8 |
| High | 22 |

* 30 TCCs and 30 adjacent normal tissue specimens
† NS, non-smoker; FS, former smoker; S, smoker
‡ Exposure to chemicals, paints, pesticides, petroleum or ink

Tissue samples were obtained at surgery from the tumor and the 3 selected grossly normal sites (cold cup biopsies) were posterior wall, trigone, and area adjacent to the tumor. Parts of the resected normal samples were sent for histopathological analysis. Tumor and normal tissues were frozen immediately in liquid nitrogen, transported, and stored at -80˚C until DNA extraction.

Patients with non-muscle-invasive BCas were followed-up with periodical cystoscopic examinations and intravesical treatment as indicated. Patients with invasive BCas were offered radical cystectomy with or without systemic chemotherapy. After a mean follow-up of 24±3 months, 8 (26.6%) patients had recurrent tumors. In Ta/T1 tumors the frequency of recurrence was 29.4% (5/17) compared with 23% (3/13) of T2-T3 tumors. In patients with non-muscle-invasive BCas, the progression rate was 11.1% and 22.2% for grade II and III tumors, respectively. All recurrences were proven by biopsy.

### *DNA extraction, oligonucleotide primers and PCR*

Genomic DNA was extracted using proteinase K, followed by phenol extraction and ethanol precipitation according to standard procedures, as previously described (10). We amplified *BRAF* exons 11, 14 and 15 by polymerase chain reaction (PCR) using the primers and thermal conditions as referred to in Zaravinos et al (11). All primers were located in the introns flanking the coding exons of the gene to include sequences from the intron-exon boundaries in the amplified products and the final sequencing traces. PCR products were purified with the QIAquick PCR purification kit (QIAGEN Inc.) and stored at -20˚C for further RFLP and sequencing analysis.

### *RFLP analysis*

RFLP analysis involved 10-µL aliquots of the amplification products being digested for 16 hours at 65˚C with 10 U of *TspR* I restriction enzyme (New England BioLabs, Beverly, MA, USA) in a 30-µL reaction volume. After replacing the enzyme with deionized water, the same reaction was used as a negative control. RFLP products were analyzed on a 3% agarose gel and photographed on a UV light transilluminator.

### *Direct DNA sequencing*

Direct DNA sequencing was used to identify and/or verify mutations within exons 11, 14 and 15 of the *BRAF*

gene. Exons 11 (G-loop) and 15 (activation segment) have been detected in malignant melanoma and other cancer types, resulting in the permanent activity of *BRAF* (4). Since exon 14 is adjacent to exon 15, thereby encoding an amino-acid sequence close to the protein's activation segment, we also included it in our study. The primers used for the forward reading of the reactions were: *BRAF* exon 11, 5′-TGTTTGGCTTGACTTGAC-3′; *BRAF* exon 14, 5′-AGATTTCGAGGCCAGAGTCC-3′; and *BRAF* exon 15, 5′-CCCTGAGATGCTGCTGAGTT-3′. The sequencing reactions were carried out using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) in a 10-µL volume containing purified PCR product and the sequencing primer. The temperature conditions set for the sequencing reactions were 96˚C for 2 minutes followed by 25 cycles at 96˚C for 30 seconds, 54˚C for 10 seconds, and 60˚C for 4 minutes. The reaction products were precipitated with 2-propanol, washed with 75% ethanol, re-suspended in 25 µL water, and loaded onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Sequencing data were analyzed using sequence analysis software (Sequence Analysis 3.7; Applied Biosystems).

*Statistical analysis*

Statistical analyses were performed with SPSS 11.5 (SPSS, Chicago, IL, USA). Statistical significance was set at the 95% level ($p<0.05$).

## RESULTS

All 30 TCCs were successfully screened for the presence of *BRAF* mutations within exons 11, 14 and 15. The V600E mutation (nucleotide change T1799A in exon 15) was found in 1 TCC specimen, whereas sequencing analysis also detected a V600L (nucleotide change G1798T in exon 15) in another TCC sample (Fig. 1). The first specimen was stage pT1a and grade II, the second was stage pT2b and grade III. No mutations within the coding region of exons 11, 14, 15 and the intron-exon junctions for the remaining samples were found. As only 2 mutations were present, no statistically significant result could be attained.

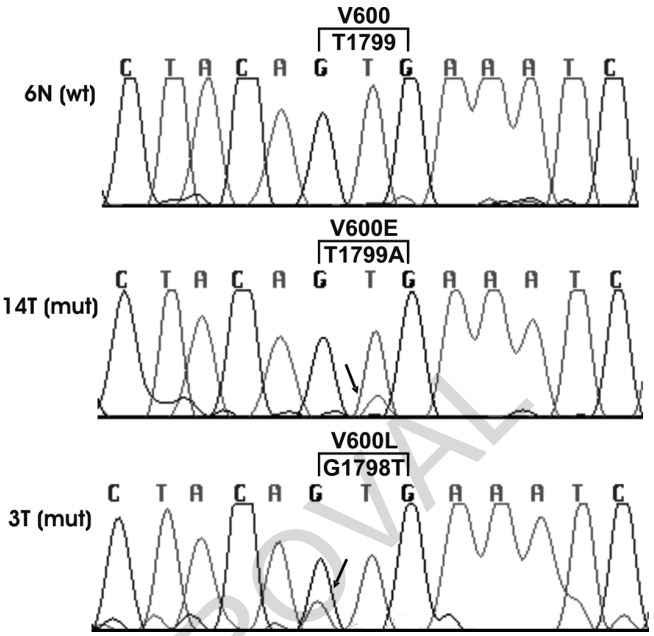


**Fig. 1 -** *No mutations within exons 11, 14, 15 and the intron-exon junctions were detected. However, direct sequencing for DNA samples 14T and 3T (middle and lower panel, respectively) gave 2 different heterozygous mutations: a T→A substitution at nucleotide 1799 (V600E) and a G→T substitution at nucleotide 1798 (V600L). The upper panel shows the wt BRAF exon 15 sequence from a normal tissue sample (6N).*

## DISCUSSION

Many fundamental cellular processes including differentiation, proliferation, survival, motility, and transformation are regulated by the RAS-RAF-MEK-ERK pathway, an evolutionary conserved signaling module (12). The RAF proteins are highly conserved serine/threonine protein kinases that activate mitogen-activated protein kinase kinase (MEK), which in turn activates the mitogen-activated protein kinase (MAPK) pathway (13). Inappropriate and/or continuous activation of this pathway results in a potent promitogenic force with ensuing abnormal proliferation and differentiation in many human cancer types (14). Activating somatic mutations in the serine/threonine kinase BRAF were reported in a large proportion of malignant melanomas and papillary thyroid cancers, as well as in a small fraction of other cancers and cancer cell lines including colorectal carcinomas, ovarian neoplasms, non-small cell lung cancers and various sarcomas (4,15-18). Besides acting as an MAPK pathway activator, RAF was shown to be involved in the oncogenic signaling of protein kinase C epsilon (31, 32). Therefore, more than one oncogenic pathway might be coordinated by mutant, constitutively-activated BRAF.

Stoehr et al previously screened 121 paraffin-embedded urothelial carcinomas of the urinary bladder and 27 tumors from the upper urinary tract without detecting any *BRAF* mutations (19). The majority of TCCs studied did not reveal any mutations, indicating that the most frequent *BRAF* alterations described in several tumor types appear not to play an important role in urothelial carcinogenesis.

Regarding similar studies of the urinary tract, Cho et al reported an incidence of 10.2% of *BRAF* codon 600 mutations in prostate adenocarcinomas (20). Their results are not in accordance with those of Burger et al (21),

who had previously reported a lack of *BRAF* mutations in 79 prostatic adenocarcinomas. Moreover, activating *BRAF* missense mutations have been identified in 9% of non-seminomas but not in seminomas (22). It has been postulated that *BRAF* mutations do not play a role in the development of renal cell tumors (23), in testicular germ cell tumors (24), or in cercival, endometrial and ovarian carcinomas (25). Recently, de Jong et al, in a multidisciplinary approach to TCam-2 cells, confirmed that this is a seminoma cell line bearing the V600E *BRAF* mutation (26).

Interestingly, we detected 2 bladder cancer specimens bearing 2 different mutations, both within exon 15. One sample had a T1799A (V600E) and the other a G1798T (V600L) mutation. The first specimen was stage pT1a and grade II, the second was stage pT2b and grade III.

The majority of studies thus far have focused on the V600E mutation, which—although most frequently reported on—still appears only in a limited region of the *BRAF* gene. Bearing in mind therefore that other regions of the gene reveal a mutation spectrum in urothelial cancers, we also screened exons 11 and 14. However, no mutation was found, corresponding with the results of comprehensive analyses that have sequenced the entire *BRAF* coding region and failed to show mutations outside exons 11 and 15 (7). Thus, *BRAF* mutations appear to be infrequent in TCC.

In contrast, a significant proportion of TCCs (10-30%) harbor mutations of H-*RAS*, another significant activator of the MAP kinase pathway, in codon 12 (27, 28, 30). This indicates a low frequency of mutational activation of the MAP kinase pathway in urothelial carcinogenesis, and appears to be consistent with the concept that *BRAF* and *RAS* mutations are mutually exclusive events, suggesting a linear functional relationship for these components in the complex signaling pathways (4, 29).

In summary, the present study demonstrates that involvement of *BRAF* mutations in the development of TTC of the urinary bladder is infrequent.

**Abbreviations**

TCC, transitional cell carcinoma
BCa, bladder cancer
PCR/RFLP, polymerase chain reaction/restriction fragment length polymorphism

**Conflict of interest: ????**

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Received: December 1, 2008
Accepted: February 4, 2009