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Study of BRAF V600 Mutations in Libyan Colorectal Cancer Patients

Hajir A Alasaifer[1], Enas A Sadawe[1], Asia AM Almakhlufi[1], Salah M Bensaber[1], Abdul M Gbaj[1]* Abdulla M Bashein[2] and Anton Hermann[3]

[1]*Department of Medicinal Chemistry, University of Tripoli, Austria*

[2]*Department of Biochemistry, University of Tripoli, Austria*

[3]*Department of Biosciences, University of Salzburg, Austria*

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***Corresponding author:** Abdul M Gbaj, Associate Professor of Genetics and Biochemistry, Department of Medicinal Chemistry, University of Tripoli, Austria

Abstract

The detection of somatic mutations in tumors is essential for the understanding of cancer development and targeting therapy. The screening for BRAF V600E mutation is employed in clinical practice in Libya for its prognostic and potentially predictive role in patients with metastatic colorectal carcinoma (mCRC). Using of BRAF mutant DNA and wild type DNA targets, we found that the sensitivity of allelic discrimination-Real Time PCR was applicable. The Real Time PCR assay displayed increased analytical sensitivity in detecting the BRAF V600E mutation. The association of BRAF mutations with clinical and pathological features was assessed using Real Time PCR assay. Qiagen Real Time PCR Platform was utilised using a set of primers. forward 5'-GAC.CTC.ACA.GTA.AAA.ATA.GGT.G 3', reverse 5'-TCC.AGA.CAA.CTG.TTC.AAA.CTG.A. 3'. Our study indicates that Real Time PCR-based assays is convenient to detect the BRAF V600E mutation in CRC and that BRAF mutations screening should not be restricted to selected patients on the basis of the clinical-pathological characteristics.

Keywords: Metastatic; Colorectal; Carcinoma; Somatic mutations; Tumors; Cancer development; Targeting therapy; Mutant DNA; DNA targets; Allelic discrimination; Mutation; Screening; Clinical-pathological characteristics

Abbreviations: mCRC: Metastatic Colorectal Carcinoma; V: Valine; E: Glutamic Acid; APC: Adenomatous Polyposis Coli; SNPs: Single Nucleotide Polymorphisms; FFPE: Formalin-Fixed Paraffin-Embedded; CRC: Colorectal Cancer; HRM: High Resolution Melt Analysis; HNPCC: Hereditary Non-Polyposis Colorectal Cancer

Introduction

BRAF is a member of Raf kinase family proteins, it has a molecular weight of 75-100 kDa, and it is the main activator of MEK kinase in Ras-Raf-MEK-ERK pathway [1,2]. It is a serine/threonine kinase that is part of cellular signaling pathways, comprising the MAP kinase signaling system, and is participating in differentiation and cell division. More than thirty mutations of the BRAF gene associated with human cancers were determined. In ninety percent of the cases, thymine is changed with adenine at nucleotide 1799 [3-5]. This directs to an amino acid substitution at codon 600 whereby Valine (V) is replaced by Glutamic acid (E) in the activation segment. This mutation has been found in many tumors. The BRAF V600E mutation (1799T>A nucleotide change) which is characterizing up to 80% of all BRAF mutations and is found in various neoplasms, as follows; colorectal carcinoma (5-22%), malignant melanoma (40-70%), glioma (11%), thyroid papillary carcinoma (36-53%), lung adenocarcinoma (4%), ovary serous carcinoma (30%), and hairy cell leukemia (100%) [6]. In addition, BRAF V600E mutation could be acquired mutation and it is found in many cancers, including colorectal cancer, non-Hodgkin lymphoma, papillary thyroid carcinoma, malignant melanoma, hairy cell Leukemia, non-small cell lung carcinoma, and adenocarcinoma of lung [7-9].

The classic method for detecting BRAF mutation is the Sanger sequencing method, however the test is expensive, and it requires expensive equipment. A study in Tunisia found that a sporadic colorectal tumorigenesis is caused by alterations in the BRAF pathways and it found six novel mutations using polymerase chain reaction sequencing in the mutation cluster region of the APC gene (mutations in the APC gene may result in colorectal cancer) [10]. The clinicopathological analyses showed an alliance between point mutations and the earliest occurrence of sporadic colorectal cancer [10]. The findings of this study confirm the heterogeneity of adenomatous polyposis coli (APC) gene alteration and expose a particular profile of this pathology between Tunisian patients that confirms the epidemiological data for this neighbour country.

Real time PCR is very convenient technique for analysis of single nucleotide polymorphisms (SNPs) and has been progressively more used for this principle since the advent of real-time PCR and as whole genome sequences have become accessible [11]. It needs methods that are sensitive, rapid, inexpensive and specific, and numerous real-time methods have evolved which accomplish these requirements [12]. Additionally, real-time PCR is a technique that is readily open to automation and no post-PCR handling is needed. Different formats have been used including hybridization probes with melting curve analysis, molecular beacons, hydrolysis probes and scorpion primers [13-15]. SNP detection by real-time PCR has established applications in diagnosis of many human disease, clinical microbiology and drug development, pharmacogenetics, and has used instead of techniques like sequencing, restriction enzyme digestion and single strand conformation polymorphism [16,17]. The main aim of this study is to explore the colorectal tumorigenesis which is caused by alterations in BRAF pathways and to analyze the occurrence of these genetic alterations in relation to metastatic colorectal cancer (mCRC) in Libyan patients.

Material and Method

Samples collection and preparation

Samples from 76 different patients were obtained from block archive of the National Cancer Institute Sabratha, Libya. All samples were collected from patients who had been diagnosed with colorectal cancer (CRC) between 2011 and 2016. In order to select only the neoplastic tissue, all Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimens were reviewed by a pathologist. Clinical and pathological information was obtained from clinical files. FFPE sections were heated in the with 200 µL 0.5% tween 20 solution and centrifuged. After cooling, wax discs were removed and samples were digested using 200 µL protein kinase (ABIO pure) overnight at 37 ^{0}C. This is a retrospective clinical validation study; consequently, no consent was required from the Internal Review Board to analyze clinical patient data under the Libyan Law for human medical research (LMO). Data were coded so that they were not appreciable to the individual patient, according to national ethical procedures ('Code for Proper Secondary Use of Human Tissue', Libyan Federation of Medical Scientific Societies).

DNA extraction

DNA extraction was conducted using phenol-chloroform method [18], where the samples were washed twice with an equivalent volume of phenol then twice with an equivalent volume of chloroform, after each wash, the samples were centrifuged for 15 minutes at 13,200 rpm and the supernatant was recovered after each wash, after that DNA was precipitated using 200 µL 100% ethanol solution of 0.03M sodium acetate. The mix was placed at -80 ^{0}C for 30 minutes then centrifuged for 30 minutes at 13,200 rpm and the supernatant was discarded. The samples were further washed with 70% ethanol then air dried. DNA samples were suspended in 50 µL nuclease free water and stored at -20 ^{0}C.

Quantitative PCR high-resolution melting (qPCR-HRM) curve analysis

Real time PCR was performed according to the manufacturer's instruction (Qiagen, Rotor-Gene Q Platform, Germany) using the following set of primers. forward 5'-GAC.CTC.ACA.GTA.AAA.ATA.GGT.G 3', reverse 5'-TCC.AGA.CAA.CTG.TTC.AAA.CTG.A 3' where the melting point of both primers were 58 ^{0}C and the final concentration was 0.2 picomole. The reaction volume was 25 µl, with 2 µl (45 ng/ µl) of input DNA. Rotor gene Q appliance (Qiagen) was used for the analysis according to the following protocol, the amplification was conducted for 45 cycles as following, denaturation at 95 ^{0}C for 10 sec, annealing for 10 sec at 55 ^{0}C, elongation for 40 sec at 72 ^{0}C.Then an HRM analysis was conducted between 70-90 ^{0}C with an interval of 1 sec.

Result and Discussion

The result of HRM (High resolution melt analysis) and the sequence of the relevant segment of BRAF gene of three sample is listed in Table 1. Table 1 shows the thermal denaturation profiles comparing wild and mutated BRAF targets. The PCR showed a sensitivity of 100% for BRAF V600E of the homo and hetero mutated samples indicating the BRAF-V600E (c.1799T > A) which is in agreement with the BRAF-V600-ddPCR results reported by Bisschop et al. [19].

Table 1 indicates that duplexes containing mismatches are considerably destabilized compared with their correctly paired parent the extent being dependent on the base composition and sequence of the oligonucleotide as well as on the type and location of the mismatch. These results indicate that the ΔG contribution of a single T/A mismatch and the position of the mismatch are crucial to duplex stability. The ΔG contribution of a single T/A mismatch to duplex stability was studied by Hatim et al. [20] who found that the ΔG is dependent on the neighboring base pairs and ranges from +1.16 kcal/mol (for the context TGA/AAT) to -0.78 kcal/mol (for the context GGC/CAG). Hatim et al. [20] also showed that the nearest neighbor model is applicable to internal G/T mismatches in DNA. In their study of G/T mismatches, the most stable trimer sequence containing a G/T mismatch was -1.05 kcal/mol for CGC/GTG and the least stable was +1.05 kcal/mol for AGA/TTT.

Table 1: Characteristics of BRAF wild and mutated targets.

Sample Code	Mutated/Wild Type	SNP type	Tm (C^o)	Relevant sequence
2008/14E	Wild type	None	77.79±0.02	CTAGCTACAGTGAAATCTCGA
1234/14	Homo-mutated	V600E and Silent mutation	72.29±0.15	CTAGCTACAGAGAAGTCTCGA
7/14AI	Hetero-mutated	V600E	76.38±0.12	CTAGCTACAGAGAAATCTCGA

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On average, when the closing Watson-Crick pair on the 5' side of the mismatch is an A/T or a G/C pair, G/A mismatches are more stable than G/T mismatches by about 0.40 and 0.30 kcal/mol, respectively [20,21]. When the 5' closing pair is a T/A or a C/G, then G/T mismatches are more stable than G/A mismatches by 0.54 and 0.75 kcal/mol, respectively. Evidently, the different hydrogen-bonding and stacking in G/T and G/A mismatches results in different thermodynamic trends. BRAF V600E mutation testing has demonstrated utility in helping select CRC patients who are considering monoclonal antibody therapy as wild-type BRAF is required for response to anti-EGFR antibodies [22] and improve diagnostic accuracy in thyroid FNA samples [12,13]. In addition, BRAF V600E mutation is connected with sporadic microsatellite instable CRC, but not hereditary non-polyposis colorectal cancer (HNPCC) syndrome [23-25]. Consequently, the presence of BRAF V600E mutation is an elimination criterion for HNPCC genetic testing [23-25]. BRAF V600E mutation testing can also help facilitate clinical studies of BRAF-targeted therapies [24]. The improved understanding of the role BRAF mutations in cancer diagnosis, prognosis and treatment has increased the need for BRAF mutation testing [26,27].

Conclusion

We have used a sensitive, specific and low-cost PCR assay to detect BRAF V600E mutation. The PCR assay can be easily implemented by many molecular laboratories for BRAF V600E mutation testing. Our method using the designed primers provides one of the most sensitive methods for BRAF V600E gene mutation detection. The principal of our study design can be potentially adapted to detect other low abundance point mutations such as tumors with rich background stroma and post-treatment tumor samples.

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