

BRAF Gene Mutation Analysis in Colorectal Cancer in South of Iran

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Received: May 1, 2014; Accepted: May 5, 2014

Background: Colorectal carcinoma is the third most commonly diagnosed cancer worldwide and many studies have focused on its molecular basis. BRAF V600E is the single most important mutation of this gene in cancers. It shows variable, but usually low, frequency in colorectal cancer, but is associated with distinct histologic, prognostic and molecular features. It is highly associated with MSI (microsatellite instability), CIMP-H (CpG island methylator phenotype), serrated pathway, poor prognosis and resistance to anti-EGFR (epidermal growth factor) drugs and interestingly absent in Lynch syndrome.

Objectives: In this study, we analyzed BRAF V600E mutation in colorectal cancer in Iranian patients.

Patients and Methods: One hundred patients with colorectal cancer, operated in hospitals affiliated to Shiraz University of medical Sciences (Faghihi and Namazi Hospital) were selected. The cases were chosen from the pathology files of the above mentioned hospitals, during 2011-2013. Genomic DNA were extracted from the paraffin blocks, by the DNP TM kit, amplified using conventional PCR and DNA sequenced using the automated sequencer (Sanger method).

Results: Patients were 21-87 years old (mean = 59.8), 45% female and 55% male. Thirty percent of the cases had right-sided and 70% left-sided cancer, of which 83% were well and 17% moderately differentiated. No BRAF V600E mutation was detected in these 100 cases.

Conclusions: The BRAF mutation is not common in colorectal cancer patients in the South of Iran. Our results are very similar to very few previous studies conducted in Iran, although we suggest further studies with larger numbers of patients to confirm these results.

Keywords: Colorectal Cancer; Gene; Iran

1. Background

Colorectal cancer (CRC) is the third most common cancer in men, the second in women and the fourth-leading cause of cancer death worldwide (1). Pathological staging is one of the most reliable prognostic markers for therapeutic modality selection, but CRC is a heterogeneous disease and, for instance, 10%–20% of patients with stage II cancer develop recurrence (2). Many efforts have been made for CRC molecular classification, in order to properly diagnosis, predict the natural course and prognosis, treat and define the pathogenesis of this disease. Multiple pathways have been identified, including: (1) chromosomal instability (CIN), (2) microsatellite instability (MSI), (3) CpG island methylator phenotype (CIMP), (4) miRNA and (5) serrated pathway (3-7). The BRAF protein is the last discovered member of the RAF family in Mitosis activated protein kinase signaling pathway, which acts with KRAS, as a downstream serine-threonine kinase effector, downstream to epidermal growth factor receptor (EGFR) and promoting cell proliferation (8). The most prevalent mutation in the BRAF gene in all cancers involves transversion of thymidine to adenosine, at the nucleotide position 1799 of exon 15, leading to conver-

sion of GTG codon (valine) to glutamic acid, labeled as V600E, accounting for more than 90% of the detected mutations in this gene (8, 9). The V600E BRAF gene mutation in colorectal cancer is associated with older age, female gender, proximal colon location, poor differentiation, mucinous histology, infiltrating lymphocytes and advanced stage (10, 11). The BRAF mutations occur more frequently in MSI and CIMP-H CRCs and only rarely with MSS CRCs and mutually exclusive with KRAS mutations (10, 12). The BRAF mutation seems to be an independent negative prognostic factor in CRCs (13, 14). The BRAF gene mutation analysis is also one step of a suggested molecular approach for hereditary non-polypoid colorectal cancer (HNPCC) syndrome exclusion by some studies (15, 16). Two monoclonal antibodies, cetuximab and panitumumab, target the epidermal growth factor receptor (EGFR), have been approved for the treatment of CRC (17). Analysis of mutational status of the BRAF, KRAS and PI3KCA, is recommended before initiating these new targeted therapies in patients with metastatic CRC by some studies (17, 18). The BRAF gene mutation has shown a diverse, but usually low, frequency of incidence in different studies.

2. Objectives

In Iran CRC is the third most common cancer in women and fifth in men, with an increased incidence during the recent years, parallel with the lifestyle changes, called westernization (19). There are limited numbers of studies on molecular events, like the *BRAF* mutation in CRC in Iran and this study was designed for the *BRAF* gene mutation analysis status in two large referral centers in South of Iran.

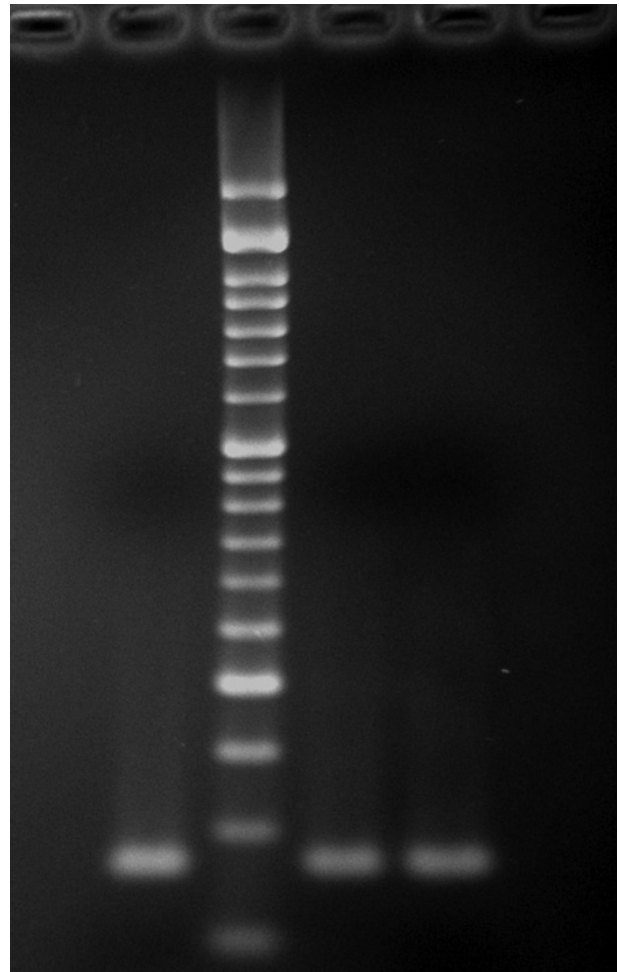
3. Patients and Methods

Total number of 100 formalin fixed paraffin embedded (FFPE) blocks from surgically resected tumors of 100 hospitalized newly diagnosed CRC patients in hospitals affiliated to Shiraz University of Medical Sciences, 2011-2013, were selected. These patients had not been on chemotherapy or radiotherapy and were not known cases of the familial adenomatous polyposis (FAP) syndrome. The related hematoxylin and eosin slides were examined by a pathologist for confirmation of the diagnosis of adenocarcinoma, having more than 50% tumoral tissue and no necrosis.

3.1. DNA Extraction and *BRAF* Mutation Detection

The FFPE blocks were cut into 10 sections with 10 μm thickness by microtome and collected in 1.5 mL Eppendorf tubes. Then the tissues were treated by xylene for deparaffinization as described by manufacture's guidelines. Genomic DNA was extracted using the CINNAGEN DNP™ kit. The isolated DNA was amplified using conventional PCR, which was performed using primers for exon 15 of the *BRAF* gene: 5' AACTCTTCATAATGCTTGCTCTGA 3' (forward) and 3' AGTAACTCAGCAGCATCTCAGG 5' (reverse). The following program was used: initial denaturation at 95°C for 10 minutes, followed by 40 cycles for

Figure 1. Agarose Gel Electrophoresis of PCR (polymerase chain reaction) Products



1: Negative control; 2: 50 bp ladder; 3: patient sample and 4: positive control

T T ¹⁰ C A T C G A G A T T ²⁰ T C A C T G T A G C ³⁰ T A G A C C A A A T ⁴⁰ C A C

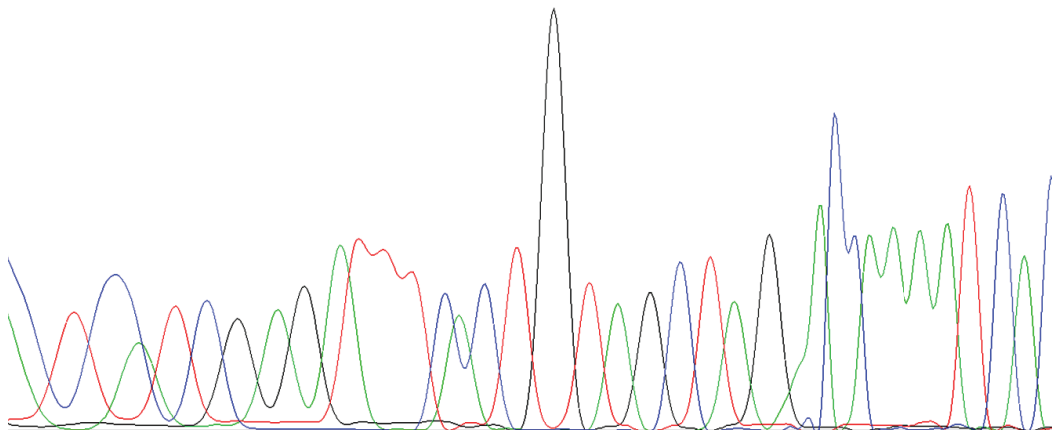


Figure 2. Wild-Type *BRAF* Gene in a Patient Sample

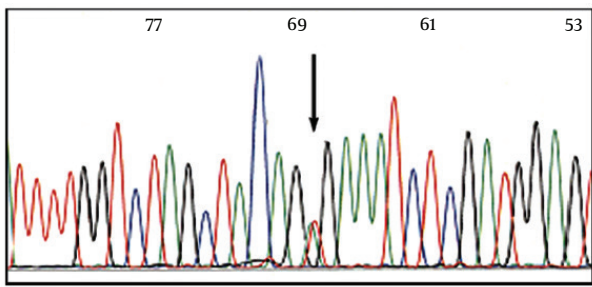


Figure 3. Mutated *BRAF* Gene in Positive Control Specimen

Table 1. Frequency of Important Parameters in Colorectal Cancer Patients ^a

Parameter	Percent, %
Gender	
Female	45
Male	55
Tumor site	
Right side	30
Left side	70
Tumor type	
Conventional	94
Mucinous	6
Tumor grade	
Well differentiated	83
Moderately differentiated	17
Poorly differentiated	0
Tumor stage, TNM	
I	18
II	48
III	30
IV	4

^a Abbreviation: TNM, Tumor node metastasis.

denaturation at 95°C for 45 seconds, annealing at 59°C for 45 seconds and elongation at 72°C for 45 seconds. After the last cycle, a final extension at 72°C for 10 minutes was performed. After ethidium bromide staining, PCR products were visualized on 1.5% agarose gel electrophoresis. DNA bands were distinguished using a 50 bp ladder, prepared by CINNAGEN^R (Figure 1). Afterwards, direct DNA sequencing of the 251 bp PCR-amplified product was performed bi-directionally with an ABI 3730XL automated sequencer (by Sanger method), using the above-mentioned primers. Results were analyzed using Chromas Lite version 2.01 software (Figures 2 and 3). Patients' data were analyzed, using SPSS 17.0 program.

4. Results

We tested 100 sample isolated DNA sequences, from CRC

specimen FFPE blocks to identify the V600E *BRAF* gene mutation frequency. Age range was 21-87 (mean = 59.8 and SD = 15.55). Further data are shown in Table 1.

No V600E *BRAF* gene mutation was detected among the 100 tested isolated DNAs from patients with CRC. No other *BRAF* gene mutations were identified in this 251 bp PCR-amplified product of exon 15.

5. Discussion

In the present study we analyzed the V600E *BRAF* gene mutation, using conventional PCR-amplified isolated DNAs of 100 patients with CRC by direct sequencing (Sanger's method). No V600E *BRAF* gene mutation was detected among the 100 selected patients. Our findings were similar to that of the Naghibalhossaini et al. (20) and compatible with the very low frequency results reported by Brim et al. (21) and Ghaffarpour et al. (22). The reported frequency of V600E *BRAF* gene mutation in CRC ranges between 0 and 24% in different studies (Table 2).

Different V600E *BRAF* gene mutation frequency reports can be due to the environmental and genetic factors in different ethnics, different mutation detection methods and presence of *BRAF* gene mutations other than V600E, in addition to different sampling methods. Based on the results presented in Table 2, it is obvious that in most East and South East Asian countries, like China, Thailand, Taiwan, Malaysia and South Korea this gene mutation has a low frequency and surprisingly other *BRAF* gene mutations have higher prevalence. Brim et al. analyzed 61 Omani (African origin), 53 Iranian (Caucasian origin) and 95 African American patients with CRC and found that 19%, 2% and 10% of them have V600E *BRAF* gene mutation, respectively (21). Hanna et al. investigated 83 Asian, 149 black and 195 white patients and found the V600E *BRAF* gene mutation in 4%, 8% and 17% of them, respectively. They concluded that the V600E *BRAF* gene mutation frequency was significantly lower in Asian patients, in comparison with white patients ($P = 0.004$) (41).

Chaiyapan et al. found no cases of V600E *BRAF* gene mutation in 133 patients with CRC, studied in Thailand (38). Yuen et al. from found four V600E and seven non-V600E *BRAF* gene mutations in 215 studied CRC cases in Hong Kong (42). In Iranian patients, two studies have shown very high frequencies of V600E *BRAF* gene mutation in thyroid papillary carcinoma (43, 44). Three previous studies, in addition to the present one, showed very low frequency of this hot-spot mutation in patients with CRC. Iran is a vast country with different ethnic groups, therefore more studies with higher number of patients from different cities and evaluation of other *BRAF* mutations are essential for more precise estimation of the *BRAF* mutation frequency in CRC and for developing regional management guidelines for patients' care.

Table 2. BRAF Gene Mutation Frequency in Some Unselected Specimens of CRCs^a

Study (Reference)	Country	Year	Number of Patients	Mutated BRAF, No. (%)	Detection Method
Nagasaka (23)	Japan	2004	234	21 (9)	PCR-RFLP
Samowitz (24)	US	2005	911	87 (9.5)	DS
Miranda (25)	Italy	2006	202	7 (3.5)	PCR-RFLP
Wei Qi Li (10)	Australia	2006	275	23 (8.9)	PCR-SSCP
Samowitz (26)	US	2006	1271	123 (9.7)	DS
Maestro (27)	Spain	2007	324	12 (3.7)	Real time-PCR and DS
Lee (28)	South Korea	2008	134	6 (4.5)	PCR-RFLP
Bougatef (29)	Tunis	2008	48	4 (8)	DS
Asaka (30)	Japan	2009	908	41 (4.5)	PCR-RFLP
Vilkin (31)	Israel	2010	85 ^b , 43 ^c	20 (23.5) ^b , 9 (20.9) ^a	DS
Li (32)	China	2011	200	14 (6.5)	PCR-Pyrosequencing
Naghbalhosaini (20)	Iran	2011	110	0 (0)	PCR-RFLP
Luevano-Gonzalez (33)	Mexico	2011	105	0 (0)	Real time PCR
Band (34)	Australia	2012	1052	128 (12.2)	Allele discriminating assay
Sylvester (35)	U.S	2012	418	32 (8)	PCR-DS
Li-liny Hsieh (36)	Taiwan	2012	182	2 (1.2)	HRM and DS
Palomba (37)	Italy-Sardinia island	2012	475	1 (0.3)	PCR-DS
Chaiyapan (38)	Thailand	2013	133	0 (0)	PCR-DS and HRM
Yip WK (39)	Malaysia	2013	43	1 (2.3)	Real time-PCR
Aissi (40)	Tunis	2013	51	1 (2)	DS
Ghaffarpour (22)	Iran	2011	27	1 (3.7)	DS

^a Abbreviations: PCR, polymerase chain reaction; RFLP, Restriction fragment length polymorphism; SSCP, Single strand conformational polymorphism; HRM, High resolution melting.

^b Jewish Patients.

^c Arab Patients.

Authors' Contributions

Farshid Javadi: performing the research and writing the manuscript, Bitá Geramizadeh: idea of the research, performing the research and writing the manuscript and Mitra Mirzai: performing the laboratory tests.

Funding/Support

The present article was financially supported by Dean of Research, Shiraz University of Medical Sciences grants number 2968.

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