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High Frequency of BRAF Proto-oncogene Hot Spot Mutation V600E in Cohort of Colorectal Cancer Patients from Ahvaz City, Southwest Iran

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Abstract: Colorectal cancer (CRC) is one of the most common forms of cancer around the world. Sporadic CRCs are caused by accumulation of mutations in essential genes regulating normal proliferation and differentiation of cells. The proto-oncogene BRAF encoded by the BRAF gene is involved in the RAS/RAF/MAPK pathway of signal transduction during cell growth. Acquired mutations in BRAF have been found at high frequencies in adult patients with papillary thyroid carcinoma and sporadic CRC. One of the predominant hot spot point mutations is T1799A (V600E) mutation among a cohort of CRC patients from Ahvaz city, southwest Iran. The aim of this study was to estimate the frequency of V600E mutation in CRC patients from Ahvaz city, southwest Iran. We analyzed exon 15 of the BRAF gene in isolated DNA from 80 Formalin Fixed Paraffin-embedded (FFPE) CRC tumor tissues using PCR-RFLP method. Data were analyzed using SPSS statistical program. According to our results 37 out of 80 cases (46.25%) were heterozygous for the mutation while the remaining 43 cases (53.75%) had normal homozygous genotype. No homozygous mutant genotype was found. Based on our findings, the frequency of V600E mutation appears to be significantly increased among CRC patients of the studied population but there was no significant relationship between genotypes and age and sex. In conclusion, these findings might prove the effect of V600E mutation on CRC pathogenesis. However, the exact effect of the mutation in CRC progression requires further work.

Key words: Colorectal cancer, adenocarcinoma, BRAF proto-oncogene, V600E, mutation, PCR-RFLP

INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent types of cancers (Rasuck *et al*., 2012). Possibly due to progressive proliferation of cancer cells in the colon, rectum and vermiform appendix. Adenocarcinoma of colon is a malignant tumor starting out as benign adenomas whose polyps may gradually increase in size and develop into cancer. Although CRC can manifest in all ages, it mostly occurs after age 50. CRC mostly starts from the appearance of small mushroom-shaped polyps in the bowel wall which are benign but after sometimes may grow and develop into cancer. Invasive cancers which develop inside the colon wall (stage I and II) are commonly curable by surgery. Available data from England and Wales suggest that more than 90% of diagnosed patients in this stage survive beyond 5 years after diagnosis. If patients are left untreated, polyps would spread in regional lymph nodes (stage III). As reported in England and Wale, near 48% of diagnosed patients in this stage continue their life beyond 5 years after diagnosis. Cancers which metastasize to distance region (stage IV) are not usually curable while approximately 7% of these patients survive beyond 5 years after diagnosis (Woodman *et al*., 2001; Rachet *et al*., 2009). CRC is more widespread in developed countries (Australia, New Zealand, Europe and North America) in compare to its lowest rates in Africa and Asia (Jemal *et al*., 2011; Center *et al*., 2009). Although resulted data from Tanzanian population revealed that the Colorectal Cancer was not uncommon in that area (Chalya *et al*., 2013). According to Kamangar *et al*. (2006), it is reported as the third commonest cancer among women and fourth one among men (Kamangar *et al*., 2006; Rachet *et al*., 2009). More than half of the CRC victims live in developed part of the world (Ferlay *et al*., 2010).

Hereditary Non Polyposis Colorectal Cancer (HNPCC) is the most prevalent type of familial CRC which accounts

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for 0.9-2.5% of all CRCs (Kerber *et al*., 2005). Germ line mutation in DNA Mismatch Repair (MMR) genes may lead to HNPCC especially due to mutations in the MLH1 or MSH2 genes. Most of the cases of this cancer show high levels of DNA microsatellite instability (MSI) (Aaltonen *et al*., 1998). Furthermore, 5-10% of CRCs are due to hypermethylation of the MLH1 gene promoter (Kane *et al*., 1997). Recently, a sporadic type of CRC has been discovered in patients who show defect in BRAF protein (Davies *et al*., 2002).

The BRAF gene encodes the BRAF proto-oncogene which is a Serin/threonine-protein kinase (Sithanandam *et al*., 1990, 1992). The BRAF protein is involved in signal transduction during cell growth. Some acquired mutations in the gene may lead to adult cancers such as adenocarcinoma of colon (Davies *et al*., 2002). Based on some findings, 90% of BRAF mutations leading to CRC is due to T1799A transversion in exon 15 leading to V600E (valine replacement by glutamine) (Rajagopalan *et al*., 2002; Wang *et al*., 2003). As the result, the BRAF protein's structure and function are negatively affected. Later, the mutation was found to play roles in different adult cancers such as Papillary Thyroid Cancer (PTC) and about 30-75% of sporadic MSI-H (high microsatellite instability) CRCs (Wang *et al*., 2003; Deng *et al*., 2004; Kambara *et al*., 2004; Vandrovcova *et al*., 2006).

High prevalence of CRCs in Iranian population as well as other countries encouraged us to test frequency of the commonest mutation in the BRAF gene. The purpose of this study was to estimate the V600E mutation frequency in CRC patients and to determine its association with CRC occurrence. Furthermore, the correlation between sex and age and occurrence of the acquired point mutation V600E were considered.

MATERIALS AND METHODS

Subjects: Formalin Fixed Paraffin Embedded (FFPE) colorectal adenocarcinoma tissues were collected from patients filed at Emam khomeini hospital of Ahvaz, southwest Iran, diagnosed between 2000-2010 during biopsy or surgery. Totally, 80 samples from patients with advanced CRC (metastasis) were included in the study. The mean age of patients was 44.25 years. Thirty six out of the 80 patients were female and the remaining patents were male. Most cases regardless of their gender were in 40-50 age range.

This study was approved by the Review Board of the School of Medicine, Ahvaz Jundishapur University of Medical Sciences.

Genotyping: FFPE blocks were prepared to 10 μm thick sections by microtome and collected in 1.5 mL microtubes. The blocks were treated by xylene to remove the paraffin. Genomic DNA was extracted by QIAamp DNA FFPE Tissue kit (Cat#56404) (Qiagen, Germany).

PCR-RFLP: Isolated DNA was amplified by PCR using the following pair of specific primers for exon 15 of the BRAF gene:

- 5'TCA TGA AGA CCT CAC AGT AAA AAT3' (Forward) and 5'TGG ATC CAG ACA ACT GT T CAA3' (Reverse) (Takahashi *et al*., 2007). The PCR thermal program was as follows: initial denaturation at 95°C for 5 min, followed by 40 cycles for denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and elongation at 72°C for 30 sec. After the last cycle, a final extention at 72°C for 5 min was performed. Amplicons were digested by the restriction enzyme TspRI (New England Biolabs, Ipswich, MA) at 60°C for 16 h. TspRI recognizes and cuts the ACAGTGAAA restriction site in the wild type genotype. However, T1799A point mutation, which replaces T by A, removes the restriction site. Therefore, the 98 bp PCR product will remain intact. In the presence of the normal genotype, two distinct 46 and 52 bp fragments are produced. RFLP products were visualized on 8% Poly Acrylamid Gel Electrophoresis (PAGE) after ethidium bromide staining. DNA bands were distinguished by use of 100 bp ladder (GeneOn GmbH, Germany).

DNA sequencing: DNA sequencing of the PCR product was carried out bi-directionally on an ABI 3130 automated sequencer (Applied Biosystems) (Bioneer, South Korea) using the same primers. The results were using Chromas lite version 2.0 software.

Statistical analysis: The results were represented as the frequency and percentage of each genotype. Based on results, there were 43 patients with normal genotype (A/A) which was equal to 53.75%. Additionally, 37 out of 80 patients carried mutant genotype (A/T) which was 46.25% of all CRC tested patients. We also considered the relationships between genotypes, age and sex. Forty four and 36 out of the 80 patients were female and male, respectively. 37.5% of cases were in 40-50 age range (Table 1). Data were analyzed using SPSS 16.0 computing

Table 1: Age and sex frequencies in CRC patients

	Sex		Age			
	Male	Female	20-30	30-40	40-50	50-60
Frequency	36	44	10	13	30	27

Table 2: Relationships between age or sex and BRAF genotypes

	OR	95%CI	df	χ^2	p-value
Sex and genotype	0.670	0.249-1.787	1	0.65	0.420
Age and genotype	0.786	0.317-1.948	1	0.27	0.603

program and relationships were tested by χ^2-test (Table 2). No significant relationships were observed between sex or age and BRAF genotypes.

RESULTS

We tested 80 isolated DNA samples from FFPE colorectal cancer (CRC) tissues to identify the frequency of V600E mutation among Ahvaz CRC patients. Using the PCR-RFLP leaded to finding mutation in 37 (46.25%) CRC cases. All mutations were in heterozygous state carrying T→A transversion at nucleotide 1796 identified by sequencing. Forty three samples (53.75%) showed normal homozygous genotype. The results were represented as frequency and percentage of each genotype in statistical analysis. As expected, normal homozygous genotypes produced two bands on PAGE include 47 and 52 bps while heterozygotes showed 3 bands: 47, 52 and 100 bp (Fig. 1). Male and female frequencies and age ranges are also shown in Table 2. Based on χ^2 test results regarding testing correlation between sex (OR = 0.67, df = 1, p = 0.42) or age (OR = 0.79, df = 1, p = 0.603) and genotypes, no significant relationships were observed (Table 2). Although most patients were in 40-50 age range, their ages did not influence their BRAF genotype and vice versa.

DISCUSSION

Regarding the high frequency of colorectal cancer (CRC) in Iran and the dramatic role of BRAF mutation in CRC malignancy and progression, it was necessary to estimate the frequency of V600E mutation as the most common point mutation in CRC patients from Ahvaz city, Iran. Due to the present problems in collecting human samples, we were able to conduct our research with approximately small number of tumor samples. Totally we have tested 80 CRC samples, among which 46.25% (37 samples out of 80) showed heterozygous mutant genotype. The results support those of previous studies addressing the BRAF mutations in carcinomas (Davies *et al*., 2002; Deng *et al*., 2004; Benvenuti *et al*., 2007). In the present study, we have obtained the frequency of V600E mutation to be 53.75% in a cohort of CRC patients sampled during 2000-2010. About 91% of CRC with MSI-H (Jensen *et al*., 2008) and 15% of all sporadic CRCs (Deng *et al*., 2004) are reported to be positive for BRAF mutations. Thus, our results corroborated previous studies on BRAF mutation

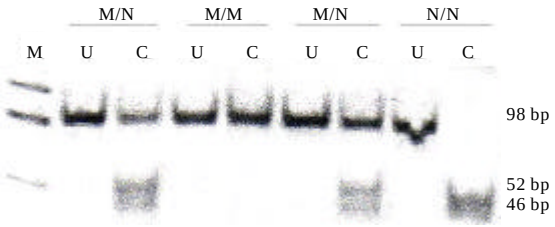

Fig. 1: Position of RFLP bands after cutting by TspRI restriction enzyme to identify V600E BRAF mutation. From left to right, samples no. 2, 6 and 8 are heterozygous while sample no 4 shows homozygous genotype. M: Marker, U: Uncut, C: Cut, M: Mutant, N: Normal, bp: Base pair

frequencies in CRC patients. Although, direct DNA sequencing as a highly sensitive and precise methodology, was applied to confirm our results, several factors including the relatively small sample size, presence of the normal tissue beside the malignant one in FFPE block and presence of most of the tumors in metastasis phase can lead to the high frequency of the mutation in heterozygous state obtained in this study.

No direct and significant relationships were found in our study between age and BRAF genotypes. Misdiagnosis of patients in younger ages with BRAF mutation or poor prognosis of disease might lead to manifestation of complexity encountered in later ages. Based on recent studies, BRAF mutations lead to poor prognosis and late diagnosis of the disease (Yokota *et al*., 2011). Our results would confirm those of recent studies which showed no association between BRAF mutations and the patients' sex and age (Yuen *et al*., 2002).

BRAF genotyping is important for several reasons: different studies have shown that BRAF mutations can affect the therapeutic response to anti epidermal growth factor receptor (anti-EGFR) therapy. Cetuximab (Erbitux) and Panitumumab (Vectibix) are two monoclonal antibodies that inhibit EGFR used in chemotherapy to treat CRC and PTC patients (Meyerhardt and Mayer, 2005). Based on Cappuzzo *et al*. (2008) research, CRC patients with BRAF mutations had poor response to anti-EGFR therapy (Cappuzzo *et al*., 2008; Di Nicolantonio *et al*., 2008). According to another study performed in Italy, BRAF V600E mutation may account for another 12% of resistant cases to anti-EGFR therapy (Benvenuti *et al*., 2007). Other investigations carried out in France (Laurent-Puig *et al*., 2009), Italy (Loupakis *et al*., 2009) and Belgium (De Roock *et al*., 2010) support the result. On the other hand, it has been explained that LV5FU-Oxaliplatin combination seems beneficial as

first-line therapy in advanced CRC with BRAF mutations (De Gramont *et al*., 2000). Finally, some of the investigators believe that BRAF mutations especially V600E transition can be used as a biomarker for selecting patients suitable for anti-EGFR treatment. The importance of the mutation determination in cancer patients might be proved by its effect on response to the treatment.

CONCLUSION

According to our findings, there was a significant relationship between BRAF genotype and disease occurrence, but no significant correlation between genotype and patients' age and sex were observed ($p>0.05$).

While there are various involved genes and mutations which may lead to CRC development and progression, further studies should be performed in the Iranian population to help finding the most prevalent mutation in the context of genetic background and environmental factors.

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