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Genetic variation in *BRAF* gene among Iraqi colorectal cancer patients.

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Abstract: The RAS/RAF/MEK/MAP kinase pathway is essential to intracellular signaling transduction regulating cell proliferation, differentiation and death. *BRAF* gene encodes a serine /threonine kinase and plays an important role in the mitogen-activated protein kinase signaling pathway. Frequent*BRAF* mutations were reported recently in a variety of human malignancies, including colorectal cancer (CRC).This study was designed to investigate the *BRAF* mutations in exon 15 in Iraqi colorectal cancer patients. Ninety samples of fixed formalin paraffin embedded tissue (FFPE) were enrolled in this study and divided into three groups according to histopathology report (16 apparently healthy, 37 suffering colorectal cancer adenocarcinoma and 37 benign tumors).DNA from the FFPE samples were extract and the *BRAF* gene was screened for the presence of mutations using PCR technique and direct sequencing. The results revealed that there are no *BRAF* gene mutations in exon 15 in Iraqi colorectal cancer patients. These results were confirmed previous articles regarding low rate of *BRAF* gene mutation in Asia and south Iran. The results of colorectal cancer in Iraqi patients may be indicating the possibility of CRC patients treatment with monoclonal antibodies. **Conclusion:** Despite the limited study sample our data suggest that *BRAF* mutations might be less frequently than other genes in the RAF family in Iraqi CRC patients. Further researches involving large patient series will be necessary to confirm these findings and to asses possible ethinic/environmtal and lifestyle influences on *BRAF* mutagenesis.

Key words: Malignancies, Colorectal cancer,exon15 *BRAF*, mutagenesis.

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Introduction:

Cancer is a disease in which a group of abnormal cells grow uncontrollably by defective genes that transform the normal cells into cancer cells (1). These cancer cells result in uncontrolled growth and proliferation which no longer and escape from the programmed death process (apoptosis) resulting in cells that keep dividing to form cancer (2). Colorectal cancer (CRC) is the most common cancer worldwide; it is the third most internal malignancy leading causes of cancer death (3).

CRC is a result of complex interactions between epigenetic, genetic and environmental factors, these factors cause changes in the fine pathways of the normal cell growth and proliferation, but the genetic factors are one of the important factors during which alterations in the genes are involved in coding of proteins result in the failure of normal gene functions. The development of CRC like other cancers occurs through genetic deviations in multistep processes that led to inactivation of tumor suppresser genes and activation of proto-oncogenes by mutation (4).

In Iraq, Colorectal cancer is one of the most first leading cancer in males and females patients. CRC is the seventh commonest cancer by primary site in 2009, according to the gender CRC is the sixth commonest cancer in females and the fifth in males .The high incidence rate for CRC patients in 2009 by gender and according to the specific incidence age was in females from 60-64 years old while in the males at age 65-69 years old (5).

BRAF proto-oncogene, serine/ threonine kinase is a member of RAF kinase family of growth signal transduction protein kinase this protein includes isoforms: ARAF, BRAF and CRAF while each of these isoforms plays a role in the RAS-RAF pathways ARAF is not functionally redundant with CRAF and cannot substitute for CRAF downstream of RAS. ARAF binds to and is activated by BRAF and that ARAF also forms complexes with CRAF. Critically, ARAF seems to stabilize BRAF: CRAF complexes in cells treated with RAF inhibitors and thereby regulate cell signaling in a subtle manner to ensure signaling efficiency.

The *BRAF* gene also plays a role in the regulating of MAP kinase/ERK signaling pathway which affects cell division, differentiation and secretion (6). *BRAF* gene encodes a serine/thereonine protein kinase that functions downstream of RAS in the RAS-RAF MEK-ERK signaling pathway which was able to stimulate angiogenesis through changes in expression of genes directly involved in the formation of new blood vessels ,also the gene known as a mitogen activates protein kinase that is important in the regulating cellular responses to extracellular signals including epidermal growth factor (7).The aim of this study was to detect frequency of mutations in *BRAF* gene.

Materials and methods:

Tissue Samples:

In this study a total number of 90 samples of formalin fixed paraffin embedded tissue (FFPE) collected from the hospital of Gastrenology and Hepatology in the medical city in Baghdad. According to the information's were obtained in the histopathology report from the hospital the samples have been divided into three groups. (n=16) apparently healthy control, (Benign, n=37) and (Malignant, n=37).

Deparaffinisaion of sections:

Each eppendorf contain 25mg of FFBE incubated in water bath for 30 minutes at 55-60 $^{\circ}$C. After incubation an amount of 1400µl of xylene homogenized by vortex then incubated in the water bath for 10 minutes. Centrifugation and the xylene were discarding. Repeated step 2 three times with using also the micropestle in each step.

An amount of ethanol absolute 1400µl was added homogenized by vortex and left for 10 minutes at room temperature. Centrifugation at 13500 rpm for 1minute and then the ethanol was discarded. An amount of 70% ethanol 1400µl was added homogenized by vortex and left for 15 minutes at room temperature. Centrifugation at 13500 rpm for 2 minutes then the

ethanol 70% was discarded and the tissue is ready to use for DNA protocol according to the kit.

DNA Extraction:

Genomic DNA was extracted from 5-8µm-thick paraffin sections containing a portion tumor tissue using the gSYNC^{TM} DNA Extraction Kit, Geneaid, Taiwan. After extraction of genomic DNA and after, gel electrophoresis was done to ensure the presence of DNA.

PCR and Sequencing:

More than 220 bp of DNA fragment of the exon 15 of *BRAF* gene was target to amplify using forward primer, (5′TGCTTGCTCTGATAGGAAAATG′3) and reverse primer, (5′AGCATCTCAGGGCCAAAAAT3′). Each 25µl PCR reaction mixture for *BRAF* gene amplifications contained 9.5µl of genomic DNA, 12.5 µl of master mix and 1.5 µM of each primer PCR; amplications were performed in an applied biosystem 96 thermocycle. Amplications reaction was done using a 5-min initial denaturation at95°C, followed by 35 cycles of 30sec at 94°C, and annealing at 55°C for 30sec, extension at 72°C of 30 min and 5min final extension at 72°C.PCR products separated in 1.5%agarose gel after staining with ethidium bromide. 25 PCR product was send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using genious software.

Statistical Analysis:

The Statistical Analysis System-SAS (2012) program was used to effect of difference factors in study parameters. LSD test (ANOVA) was used to significant compare between means variablesin this study (8).

Results and Discussion:

In this study 90 patients of FFPE were examined for mutations in exon fifteen of the *BRAF* oncogene. The samples of FFPE include sixteen patients were apparently healthy, thirty seven suffering with colorectal cancer adenocarcinoma and thirty seven with benign tumors in colon and rectal. The average age of colorectal patients were 37-53 this average includes 48.89 %(44 cases) males and 51.11 %(46 cases) females. Most of the patients of CRC were found in the colon than in rectal as 56.67%, 43.33% respectively. The most frequent stage with colorectal patients were stage III-B 48.65% then 27.03% in stage III-A and the lowest percentage was 10.81observed in stage II-B for 4 cases, also there were a high significant increase between the stages in this study ($P< 0.0001$) as shown in table (1) this may be due to lack of health education which is the preeminent explanation and a considerable proportion of the population unwilling toparticipate in colon cancer screening program (9). Amplified exon fifteen of *BRAF* gene by PCR technique and screened for presence of mutations in the CRC patients. The result showed that the product size was 228bp (figures -1).

Table (1): Characteristic of total samples in this study

Samples	Total No.	Percentage (%)	P -value
FFPE	**90 Sample**		
Gender			
Male	44	48.89	0.1073 NS
Female	46	51.11	
Age			
< 50	37	41.11	0.0097 **
> 50	53	58.89	
Site of tumor			
Colon	51	56.67	0.0327 *
Rectal	39	43.33	
Type of tumor			
Control	16	17.78	0.0001 **
Benign	37	41.11	
Malignant	37	41.11	
Stages of tumor:			
Stage II-A	5	13.51	0.0001 **
Stage III-A	10	27.03	
Stage III-B	18	48.65	
Stage II-B	4	10.81	
Tumor size cm^2	**11.98 ± 0.72**	**-**	**-**
*** (P<0.05), ** (P<0.01).**			

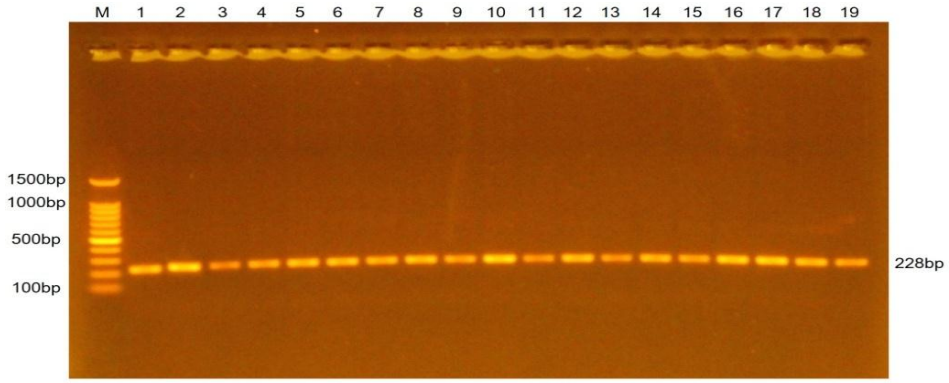

Figure (1): PCR products for exon 15 of *BRAF* gene for DNA samples of colorectal cancer on 1.5 % agarose gel.

Table (2) mentions the distribution of thirty seven CRC patient and most of the patients of CRC located in the right site of the colon 86.67% of 13 cases while in the left 13.33% for 2 cases. The results differ from (9) that indicate the left colonic cancers are more likely than right colon cancers to cause partial or complete intestinal obstruction because the left colonic lumen is narrower and the stool in the left colon tends to be better formed because of re-absorption of water in the proximal colon. While, (11) found there was no difference depend on location in their study which conduct in north of Iraq. Our results is similar with the study of *BRAF* mutations in Chinese colorectal cancer patients, *BRAF* mutations were more common on the right side of the colon than the left side of the colon and rectal (12). Also this result is similar to find in previous study (13).

The severity of the tumor was shown to be moderately differentiated adenocarcinoma 81.08% (30 cases), villous differentiated adenocarcinoma 10.81% (4 cases), well differentiated

adenocarcinoma 8.11% (3 cases) and poorly differentiated adenocarcinoma was zero and there were a high significant increasing (P< 0.0001) between the types of differentiation. In respect to tumor size the results show high significant correlation between the CRC incidence and tumorsize. The mean tumor size was 11.98 ± 0.72 that may due to diagnosis at high advance staging of tumor.

Table (2) .The distribution of patients with colorectal cancer

Characterization	Total No. (%)	P–value
Factors	**37(90)**	
Age (year)		
< 50	10 (27.03%)	0.0001 **
> 50	27 (72.97%)	
Gender		
Male	14 (37.84%)	0.0002 **
Female	23 (62.16%)	
Site of colon		
Left	2 (13.33%)	0.0001 **
Right	13 (86.67%)	
Differentiation		
Moderately	30 (81.08%)	
Well	3 (8.11%)	0.0001 **
Poorly	0 (0.00%)	
Villous	4 (10.81%)	
**** (P<0.01).**		

The average ages of the patients in CRC were (10-27) years old. According to the age our data shows that younger ages caused by colorectal cancer, this is similar with the American Cancer Society that indicates timely evaluation of symptoms consistent with colorectal cancer in adults younger than age 50 is especially important due to the increase in colorectal cancer incidence in this age group in recent years (14). According to the gender the females have the highest incidence of CRC 62.16% (23 cases) while the males have 37.84% for 4 cases. This differs with (15) that show a high incidence rate of colorectal cancer in males than females 14/26(53.9%) and 12/26(46.1%) respectively this may due to intake of smoking and alcohol make men more prone to colorectal cancer than women in ,addition to that most of female patients are shy the consult physician although they may suffer from this disease. Also our results differ with(16) there is a high percent of male in comparison with women percent it was 34/58(58.62%) , 24/58(41.37%) respectively, showing that there is significant increase(P<0.05), in male over the female with respect to CRC incidence disease. In Taiwan CRC patients study focus on cases for the clinicopathologic and genomic, there were 785 (66.9%) males and 388 (33.1%) females in these sporadic CRC (17). While our data shows a significant increase (P<0.01) in females over the male which is similar with western population-based studies reported that *BRAF*-mutant CRC significantly more likely in females to occur in females (12). To investigate the mutation status of colorectal patients for *BRAF*, direct sequencing was performed after amplified exon fifteen for *BRAF* gene in CRC patient of all cases, the sequence results were compared with NCBI data base. In present articles highlighted of *BRAF* gene mutations which act essential role in the MAPK

(mitogen activates protein kinase) signal transuding pathway, it concerned in the malignant transformation of colorectal precursor. *BRAF* gene is also of interest as being a prospective prognostic and predictive sign in patients with colorectal malignancy (18). The results of the direct sequencing (figure-2) indicated that *BRAF* gene mutations were not detected in any of FFPE colorectal cancer patients. This data is similar with Asian studies (1.1% to 5.8) which are lower compared with several western countries (19, 20, 21). This difference in mutation may be due to the attributable to different sample selections, ethnicities and geographical distribution (13).It is obvious that the in most East and south East Asian countries ,like China, Thailand ,Taiwan ,Malaysia and South Korea have a low frequency and surprisingly other BRAF gene mutations have higher prevalence(21). Our data conform to previous articles regarding low rates of *BRAF* gene mutations from 242 showed that BRAF gene mutations were not present in any of the metastases colorectal cancer tissues which analyzed in Iranian population (22). Also Saudi Arabia 2.5% (19/757) (23) and Turkish population 2% (1/50) (24).In Asian countries previous studies show that a total of eighty five colorectal cancer patients were enrolled in study of Molaei and his colleagues (2016), their results showed no V600E mutations in *BRAF* mutations in stage I and II of Colorectal cancer patients (21).Other article referred no *BRAF* mutations were found in stage III colon cancer(24).BRAF mutations in Taiwan (0%) (26) and Japan in low rate (4.7%)(26). In other studies disgreemant with our results which confirm that *BRAF* mutations are frequent in colorectal cancer patients (27, 28). The *BRAF* mutations are associated with the presence of defective MMR (mismatch repair gene) because of the presence of a germ- line mutation in either *hMLH1* or *hMSH2* (27).

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