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Assessment the Immunohistochemical Expression of Wild BRAF and Mutant BRAF^{V600E} in Iraqi Patients with Colorectal Carcinoma

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Abstract:
In the current study hundred ten of Iraqi patients with colorectal tumors were studied to evaluate the expression of wild BRAF and BRAF^{V600E} using Tissue microarray-Immunohistochemisty (TMA-IHC) technique. Of them, Ninety cases had colorectal carcinoma, and twenty had benign tumors. A group of twenty cases of non-specific colitis and other twenty colonic biopsies with no significant pathology were also studied. Results of the study demonstrated that BRAF expression was positive in 86.7% of carcinoma cases, and there was significant differences (P=0.038) in the expression of BRAF within tumor stages. Whereas BRAF^{V600E} expression was negative in groups of adenoma, colitis and cases with no significant pathology compared to carcinoma group in which 13.3% showed positive expression. We found significant correlation between the expression of BRAF^{V600E} and the age (P=0.047), right sided colon tumor (P=0.041), and mucinous type carcinoma (P=0.021). And there was significant differences between wild BRAF and mutant BRAF^{V600E} in age, female gender, right-sided colon tumor, tumor grade, and type of tumor (p=0.0001).
Keywords: BRAF, mutant BRAF, CRC, prognostic markers in CRC.

1. Introduction:

Among the various types of cancers, colorectal cancer (CRC) is a serious and common health problem worldwide. This cancer is the third most common visceral malignancy with nearly 1.4 million new cases diagnosed in 2012[1]. Despite advances in surgical techniques and therapeutic interventions during the past few decades, CRC remains a major health problem worldwide due to therapy resistance [2, 3]. Studies aiming at optimizing the diagnostic process and treatment of this disease are increasing, which has probably caused CRC to be one of the most-studied and best characterized processes of tumorigenesis. Through more biological knowledge of tumorigenesis in CRC, more emphasis on early detection and development of new and improved treatment regimens [4, 5]. Because CRC is a major cause of cancer related deaths worldwide, a lot of researches have been focused on the discovery and development of biomarkers to improve the diagnostic process and to predict treatment outcomes. Up till now only a few biomarkers are recommended by expert panels. Discovery of additional prognostic markers might permit the development of guidelines for better management of CRC in order to improve overall survival [6]. Of them, the v-raf murine sarcoma viral oncogenes homolog B1 (BRAF), a member of the RAF family of serine/threonine protein kinases that displays the best binding to RAS and has the highest phosphorylating activity [7]. Active RAS induces conformational changes in RAF that allows its recruitment to the cell membrane, promoting changes in the phosphorylation status and triggering its kinase activity [8]. Different genetic aberrations of *BRAF* have been reported in various malignancies [9, 10]. Mutations in BRAF and RAS generally occur in a mutually exclusive fashion, suggesting that aberrant regulation of the RAS/ BRAF/MEK/ERK pathway may be the pathogenesis of these tumor types, which can be achieved at different levels of the pathway [11]. The predominant mutation in the BRAF gene involves a thymidine to adenosine transversion at nucleotide 1799(T1799A), accounting for greater than 90% of the observed mutations in BRAF [12]. *BRAF* gene mutation testing has emerged as an important tool for diagnosis, prognosis, treatment, and predicting patient outcome in response to targeted therapy for multiple cancer types. V600E is the most common mutation for the BRAF [13]. Until recently, the detection of BRAF mutations was performed with Sanger sequencing or PCR-based assays. These methods require representative amount of malignant cells and extraction of the DNA. Immunohistochemical detection of BRAF with a mutation specific antibody was first described in metastatic melanoma and papillary thyroid carcinoma [14]. The advantage of

immunohistochemistry (IHC) lies in the minimal amount of the needed tissue and the availability of this technique in most pathological laboratories [15, 16].

2. Materials & Methods:

A total of 150 cases of colonic biopsies were collected. Among these, 90 cases colorectal cancer, 20 cases benign lesions, 20 cases non-specific colitis and 20 cases reveal no significant pathology. Clinical information regarding patient's age, tumor size, grade, and pathological stage was obtained from the available histological reports. Hematoxylin and Eosin (H&E) stained sections were re-examined by two pathologists. All the preparations for tissue microarray (TMA) and immunohistochemistry (IHC) were performed in Pathology Unit - Southern General Hospital (SGH), University of Glasgow, United Kingdom.

2.1 Construction of tissue microarrays (TMAs)

Tissue cores of 0.6 mm in size were obtained from three paraffin-blocks in this cohort. Five tumor tissue cores (0.6mm in diameter) were taken from each paraffin block with Beecher automated tissue arrayer (Beecher Instruments, Sun Prairie, Wisconsin, USA). The cores were placed in a new recipient paraffin block that ultimately contained 325 tissue cores. The information of all TMAs cases were put in data sheet which called TMA map. This TMA map consists of a simple excel sheet, which served as a guideline to blocks arrangement and sequence in which they arrayed. Thus the TMA map was contained the exact location for each core in TMAs slide or block and another map (TMA code map) contained the code number for each core to each block. TMA block was cut at a thickness of 5µm on a microtome cutter (Leica RM2235). Sections were then placed on Salinized coated slides, (DAKO, UK) and heated at 58°C for 24 hours.

2.2 TMA- Immunohistochemistry:

IHC was applied on TMA sections. Staining was carried out for wild and mutant BRAF. Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol (100%, 90%, 80%, 70%). Antigen was retrieved using citrate buffer (prepared by dissolving 2.1 gm of citric acid powder into 1 litre of dH2O, adjust pH to 6) for 10 min in pressure cooker. Slides were incubated in peroxidase – blocking solution (Dako, ready- to- use) for 20 minutes. Non-specific binding of antibodies was blocked by the addition of 2.5% normal horse serum, from (ImmPRESS™ ,Vector , USA).

Primary antibodies were diluted (1:100) for wild BRAF and (1:50) for $BRAF^{V600E}$ using antibody diluant (ready-to-use, Code no. ADS-125, Spring Bioscience,USA), and incubated overnight at 4°C. Rat monoclonal antibodies kit (**Spring Bioscience**, USA) was used to detect primary antibodies for wild BRAF. Whereas mouse monoclonal antibodies kit (**Spring Bioscience**, USA) was used to detect primary antibodies for $BRAF^{V600E}$. Secondary antibodies for wild BRAF (Anti Rat Ig. peroxidase , Cat. No. MP-7404, ImmPRESS™ Vector, USA), and for $BRAF^{V600E}$ (Anti mouse Ig. peroxidase , Cat. No. MP-7402, ImmPRESS™ Vector, USA) were applied to the slides and incubated for 30 minutes at room temperature in a humidified chamber. The colorimetric detection of reaction was achieved by the Diaminobenzidine (DAB) Peroxidase Substrate method. Then, sections were counterstained with haematoxylin, dehydrated, and mounted. Tissue microarray slides scanned by using of digital image scanning analysis computer,(NDP, U10074-01, UK).

2.3 Scoring system of IHC in TMAs:

For BRAF and BRAF ^{V600E}, the positive expression was observed as a diffuse brown cytoplasmic staining. Any cytoplasmic staining was recorded as positive expression, irrespective to the intensity of staining according to Nicolas *et al.* 2015. The score was done taking into account the percentage of positively staining tumor cells with no relation to signal intensity: tumors were assessed as BRAF negative if >90% of the cancer cells were unstained (<10% of positive cells) and as BRAF positive if more than 10% of cells were immune stained [17].

Statistical analyses

Statistical analysis was carried out using (SPSS V. 20). The association between wild and mutant BRAF, and patients' clinico-pathological features was assessed by chi -square test and Fisher exact test when the Chi square test was not fit. Statistical tests were approved by assuming a null hypothesis of no difference between variables, a probability was considered statistically significant when P values ≤ 0.05.

3. Results & Discussion:

3.1 TMA Immunohistochemical expression of BRAF:

In current study, wild BRAF was positive in 70% of colonic adenoma, and in 86.7% of colorectal carcinoma (Figures 1 & 2).

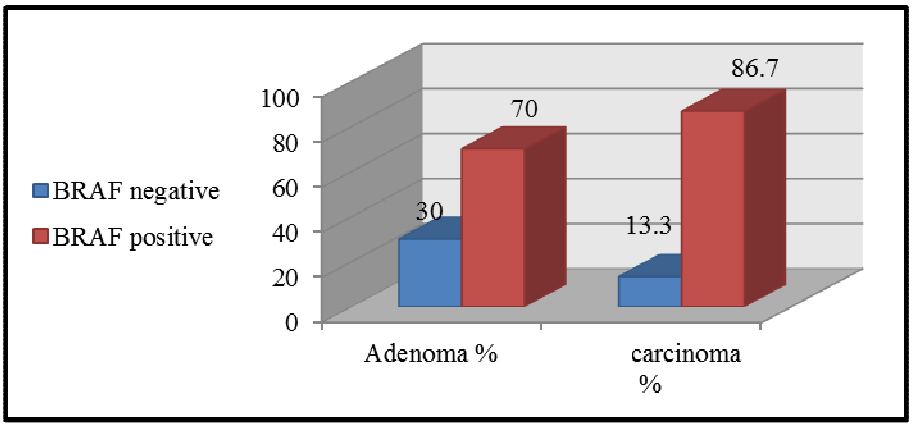
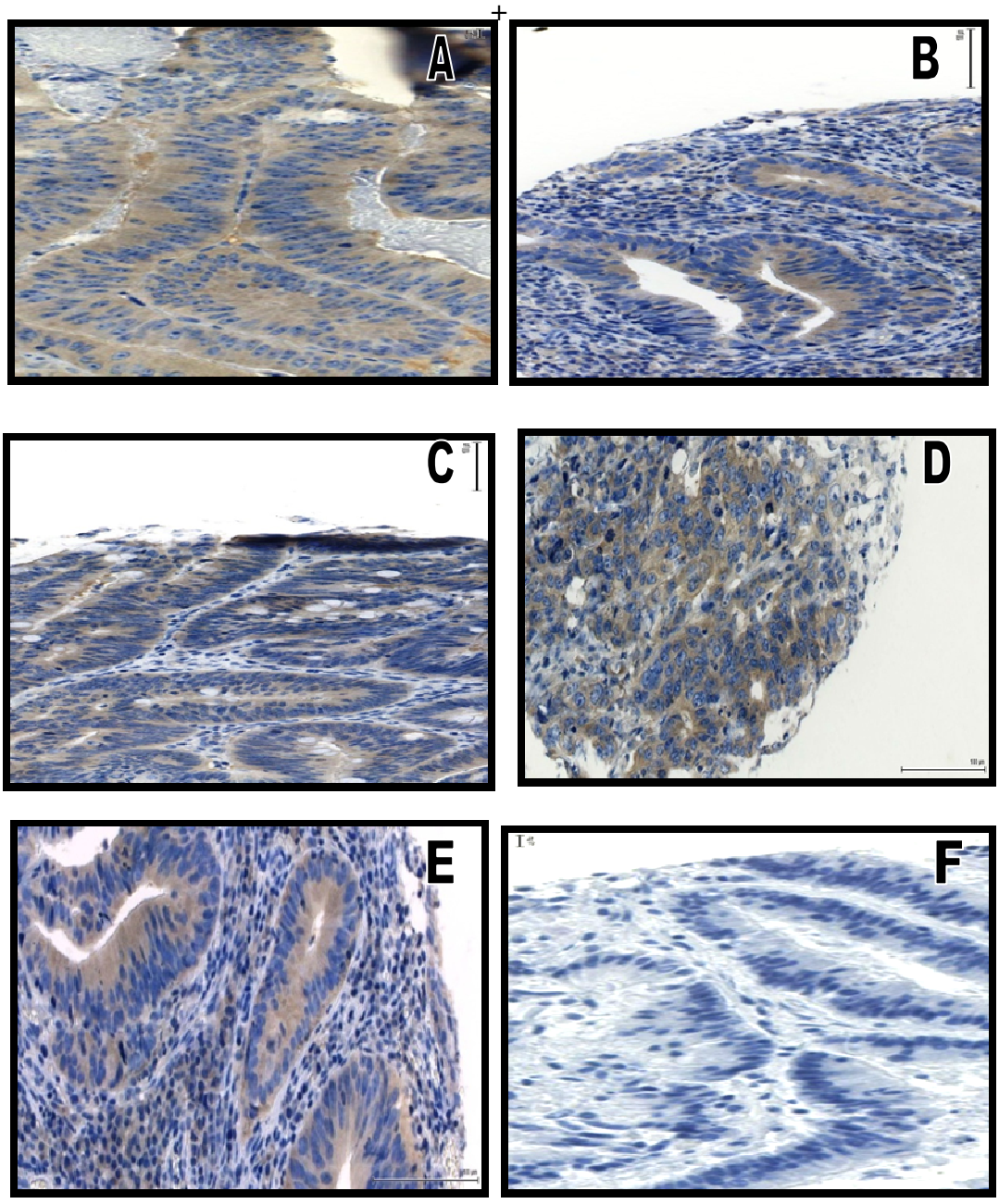


Figure (1) Expression of BRAF in Colonic Adenoma and Colorectal Carcinoma.



Figure(2)Immunohistochemical Assessment Of BRAF A: Villous Adenoma Showing Positive Expression Of BRAF(Hematoxylin & DAB(20X) , B: Tubular Adenoma Showing Positive Cytoplasmic Expression Of BRAF, (Hematoxylin & DAB(30X) C:Tubulovillous Adenoma Showing Positive Cytoplasmic Expression Of BRAF, (Hematoxylin & DAB(30X) D: Moderatelly Differentiated Adenocarcinoma Showing Diffuse Cytoplasmic Positive Expression Of BRAF(Hematoxylin & DAB(20X) E: Well Differentiated Adenocarcinoma Showing Diffuse Cytoplasmic Positive Expression Of BRAF(Hematoxylin & DAB(20X) F: Well Differentiated Adenocarcinoma Showing Negative Expression Of BRAF, (Hematoxylin & DAB(30X)

In colitis group, 25% of cases were positive BRAF expression (Figure 3 &5) whereas 40% of positive cases were distinguished in cases without significant pathology (Figure 4 & 5).

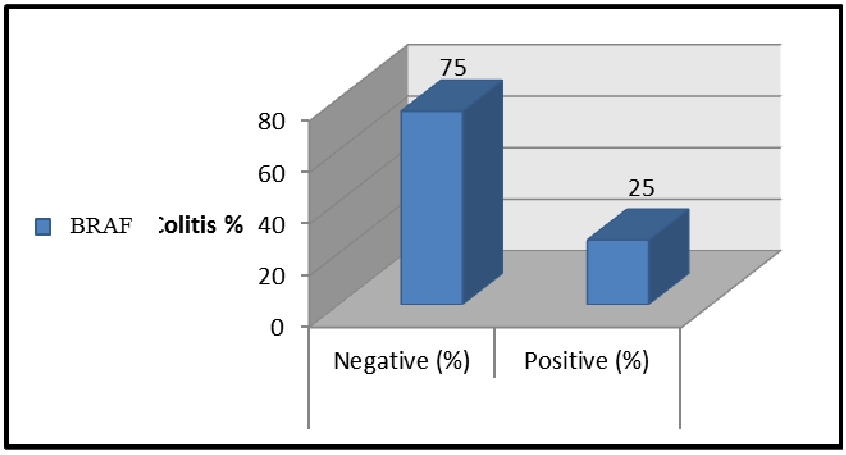


Figure (3) Expression of BRAF within Colitis Group.

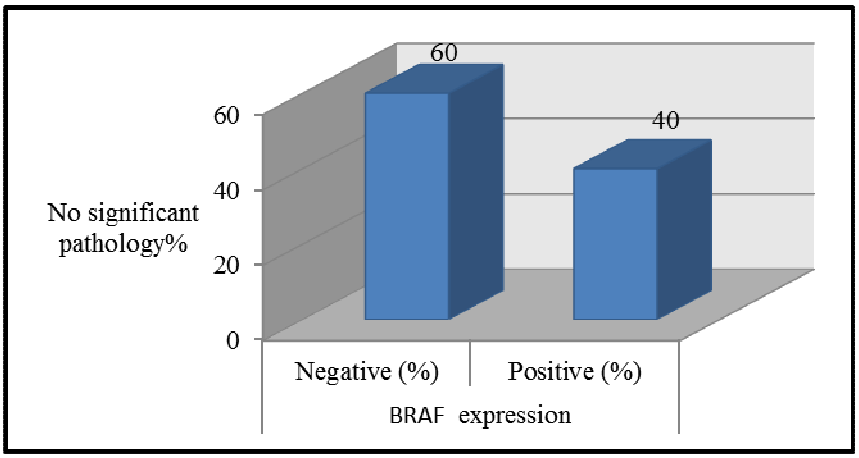
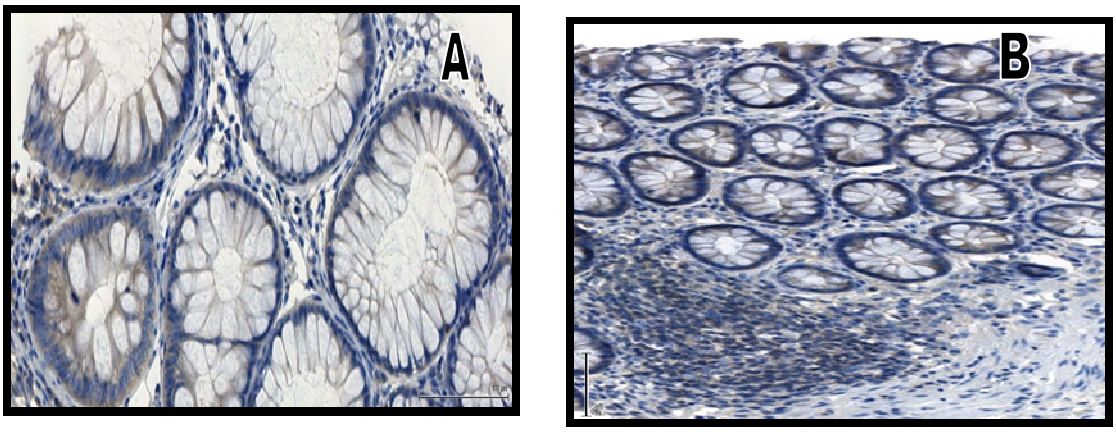


Figure (4) Expression of BRAF within Cases of No Significant Pathology



Figure(5)Immunohistochemical Assessment Of BRAF (A: Non Specific Colitis Tissue Showing Positive Expression Of BRAF(Hematoxylin & DAB(20X) , B: Normal Colon Tissue Showing Positive Cytoplasmic Expression Of BRAF, (Hematoxylin & DAB(30X)

3.2Association of BRAF expression with clinicopathological features:

3.2.1 Association of BRAF expression with age:

Results revealed that there were no significant differences in BRAF expression within age groups (Table1).

Table (1) Association of BRAF Expression with Age

Groups	Age (years)	No. of cases n	%	Negative n	%	Positive n	%	Fisher exact test	*P value
Carcinoma	**≤ 55**	43	47.7	5	5.5	38	42.2	0.2	0.527
	> 55	47	52.3	7	7.8	40	44.5		
Adenoma	**≤ 55**	14	70	4	20	10	50	0.4	1.00
	> 55	6	30	2	10	4	20		
Colitis	**≤ 55**	16	80	11	55	5	25	0.3	0.530
	> 55	4	20	4	20	0	0		
No significant pathology	**≤ 55**	17	85	9	45	8	40	0.2	0.242
	> 55	3	15	3	15	0	0		

(*P< 0.05)

3.2.2 Association of BRAF expression with gender:

Results found out that no significant differences in BRAF expression between males and females in malignant cases, but there was a significant difference (P=0.032) in the expression of this marker between males and females in adenoma cases, only (2\18)of males showed positive BRAF expression, in contrast, all females showed positive expression of this marker (Table 2).

Table (2) Association of BRAF Expression with Gender

Groups	Gender	No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Carcinoma	**Male**	53	58.8	43	47.7	10	11.1	7.1	0.116
	Female	37	41.2	35	39	2	2.2		
Adenoma	**Male**	18	90	2	10	16	80	3.2	0.032 *
	Female	2	10	2	10	---	---		
Colitis	**Male**	12	60	---	---	12	60	1.0	0.147
	Female	8	40	2	10	6	30		
No significant pathology	**Male**	14	70	5	25	9	45	0.3	0.642
	Female	6	30	3	15	3	15		

(*P< 0.05)

3.2.3Association of BRAF expression with tumor site:

This study showed no significant differences in BRAF expression and tumor location in adenoma and carcinoma groups (Table 3).

Table (3) Association of BRAF Expression with Tumor Site.

Groups	Tumor location	No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Carcinoma	Rt.Colon	28	31	23	25.5	5	5.5	0.1	0.307
	Lt.Colon	62	69	55	61.1	7	7.8		
Adenoma	Rt.Colon	4	20	4	20	---	---	0.2	0.267
	Lt.Colon	16	80	10	50	6	30		

(*P< 0.05)

3.2.4Association of BRAF expression with tumor type, grade & stage:

Results showed that there were significant differences (P=0.038) in the expression of BRAF within tumor stages, the largest proportion of positive expression of BRAF was in cases within stage C (C1=15.5%, C2=22.2%), but there were no significant correlations between the expression of this marker and tumor type or grade (Table 4).

Table (4) Association of BRAF Expression with Tumor Type, Stage and Grade.

Carcinoma group		No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Histological type	Adenocarcinoma	81	90	72	80	9	10	6.5	0.077
	Mucinous	9	10	6	6.7	3	3.3		
Histological grade	Well diff.	29	32.3	26	28.9	3	3.3	2.4	0.283
	Moderately diff.	25	27.7	23	25.5	2	2.2		
	Poorly diff.	36	40	29	32.3	7	7.8		
Dukes stage	A	15	16.7	14	15.5	1	1.1	2.0	0.038 *
	B 1	15	16.7	14	15.5	1	1.1		
	B 2	19	21.1	16	17.8	3	3.3		
	C 1	21	23.3	14	15.5	7	7.8		
	C 2	20	22.2	20	22.2	0	0		

(*P< 0.05)

3.3 TMA Immunohistochemical expression of BRAF^{V600E}:

BRAF^{V600E}was scored the same way of BRAF, taking into account the percentage of positive staining cells, regardless to the intensity of staining [17]. Results of BRAF^{V600E} expression in groups of adenoma, colitis and cases with no significant pathology, showed negative expressions when stained with this marker in comparison to carcinoma group in which 13.3% were positive expression (Figures 6 & 7).

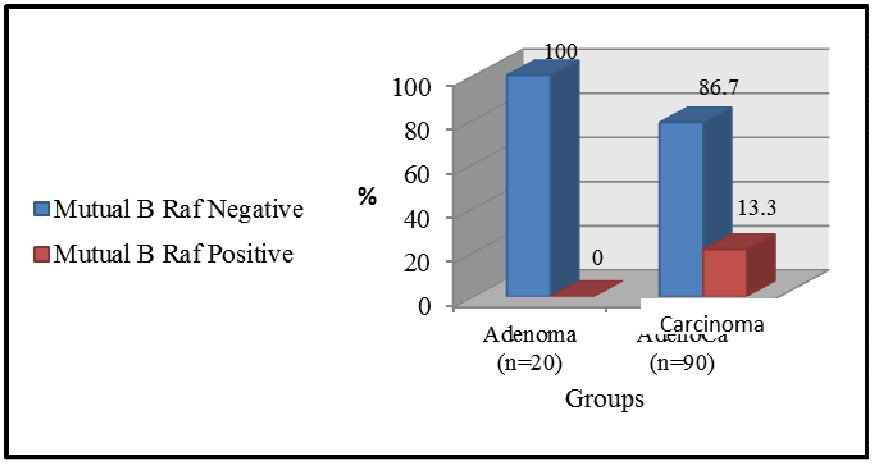


Figure (6) Expression of BRAF^{V600E} in Colonic Adenoma and Colorectal Carcinoma

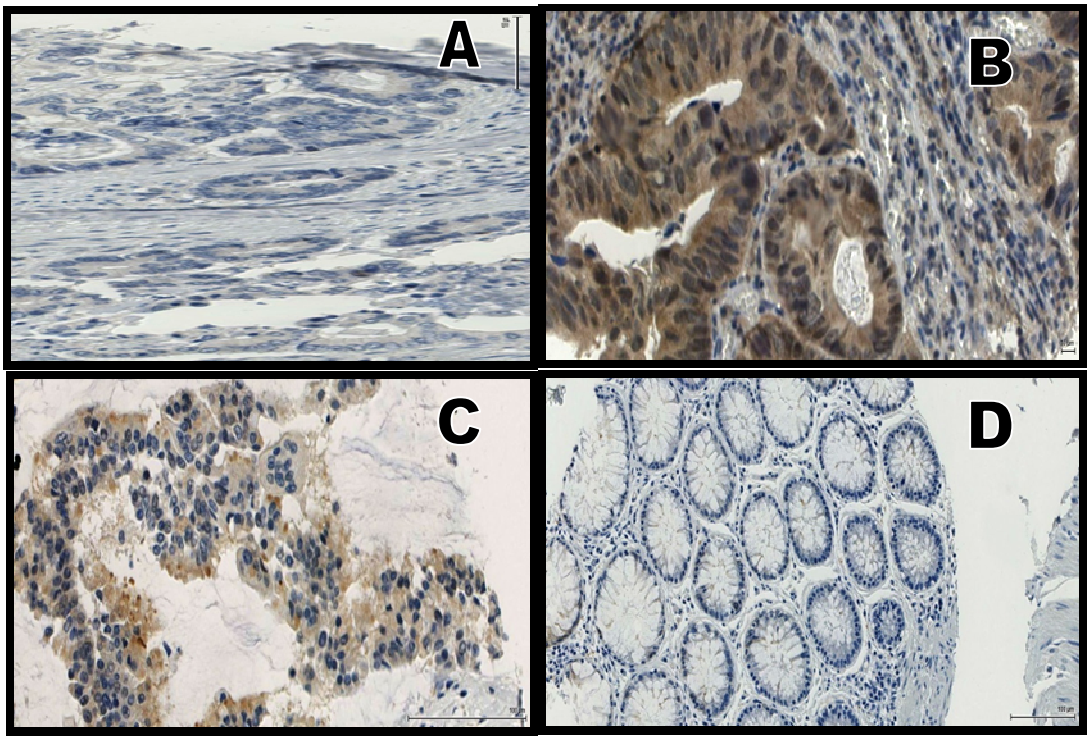


Figure (7) Immunohistochemical Assessment Of $BRAF^{V600E}$ A: Well Differentiated Adenocarcinoma Showing Negative Expression Of $BRAF^{V600E}$, (Hematoxylin & DAB, 20X) B: Well Differentiated Adenocarcinoma Showing Positive Cytoplasmic Expression Of $BRAF^{V600E}$,(Hematoxylin & DAB, 30X) C: Poorly Differentiated Adenocarcinoma Showing Positive Expression Of $BRAF^{V600E}$, (Hematoxylin & DAB, 20X) D: Normal Colon Tissue Showing Negative Expression Of $BRAF^{V600E}$, (Hematoxylin & DAB, 20X).

3.4 Association of $BRAF^{V600E}$ expression with clinicopathological features:

3.4.1 Association of $BRAF^{V600E}$ expression with age:

Results showed significant correlation (P=0.047) between the expression of $BRAF^{V600E}$ and the age in carcinoma group, that is, all $BRAF^{V600E}$ positive expression was detected in age group >55(Table 5).

Table (5) Association of $BRAF^{V600E}$ Expression with Age.

Groups	Age (years)	No. of cases n	%	Negative n	%	Positive n	%	Fisher exact test	*P value
Carcinoma	**≤ 55**	43	47.7	43	47.7	0	0	2.6	0.047 *
	> 55	47	52.3	35	38.9	12	13.3		
Adenoma	**≤ 55**	14	70	14	70	0	0	1.00	1.00
	> 55	6	30	6	30	0	0		
Colitis	**≤ 55**	16	80	16	80	0	0	1.00	1.00
	> 55	4	20	4	20	0	0		
No significant pathology	**≤ 55**	17	85	17	85	0	0	1.00	1.00
	> 55	3	15	3	15	0	0		

(*P< 0.05)

3.4.2Association of BRAF^{V600E} expression with gender:

No significant differences were found in the expression of BRAF^{V600E} between male and female in the all studied groups (Table 6).

Table (6) Association between the expression of BRAF^{V600E} and gender.

Groups	Gender	No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Carcinoma	Male	53	58.8	5	5.6	48	53.3	0.2	0.638
	Female	37	41.2	7	7.7	30	33.3		
adenoma	Male	18	90	---	---	18	90	1.0	1.00
	Female	2	10	---	---	2	10		
Colitis	Male	12	60	---	---	12	60	1.0	1.00
	Female	8	40	---	---	8	40		
No significant pathology	Male	14	70	---	---	14	70	1.0	1.00
	Female	6	30	---	---	6	30		

(*P< 0.05)

3.4.3 Association of BRAF^{V600E} expression with tumor site:

A significant differences (p=0.041) were found in the expression of BRAF^{V600E} between right and left colon, since the positive expression was more in right colon tumors. (Table7).

Table (7) Association between the Expression of BRAF^{V600E} and Tumor Site.

Groups	Tumor site	No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Carcinoma	Rt.Colon	28	31.1	8	8.9	20	22.2	3.2	0.041*
	Lt.Colon	62	68.9	4	4.4	58	64.5		
Adenoma	Rt.Colon	4	20	---	---	4	20	1.0	1.00
	Lt.Colon	16	80	---	---	16	80		

(*P< 0.05)

3.4.4 Association of BRAF^{V600E} expression with tumor type, stage & grade:

Results showed no significant differences in the expression of BRAF^{V600E} with stages and grades of the tumor in carcinoma group, but there were significant differences (P=0.021) in BRAF^{V600E} expression between tumor types, that is, all mucinous cases showed positive expression of BRAF^{V600E} (Table 8).

Table (8) Association between the Expression of BRAF^{V600E} with Stage & Grade.

Carcinoma group		No. of cases n	%	Positive n	%	Negative n	%	Fisher exact test	*P value
Histological type	Adenocarcinoma	81	90	3	3.3	78	86.7	1.2	0.021 *
	Mucinous ca.	9	10	9	10	0	0		
Histological grade	Well diff.	29	32.3	1	1.1	28	31.1	4.1	0.915
	Moderately diff.	25	27.7	1	1.1	24	26.7		
	Poorly diff.	36	40	10	11.1	26	28.9		
Dukes stage	A	15	16.6	0	0	15	16.6	2.6	0.942
	B 1	15	16.6	1	1.1	14	15.6		
	B 2	19	21.1	1	1.1	18	20		
	C 1	21	23.4	4	4.4	17	18.9		
	C 2	20	22.3	6	6.7	14	15.6		

(*P< 0.05)

3.5 Association of wild BRAF & mutant BRAF^{V600E}with clinico pathological features:

Differences in clinical characteristics between wild BRAF and mutant BRAF^{V600E} tumors are displayed in (Table 9). Results showed significant differences in age between wild and mutant BRAF, since the positive expression of mutant BRAF^{V600E} was 100% in cases >55 years old, in comparison to 51.3%, for wild BRAF (P=0.001). On the subject of the gender, BRAF^{V600E} was significantly more likely to occur in females in comparison with wild BRAF (58.3% v 44.9%, P=0.0001). As well, the association between BRAF^{V600E} and right-sided tumors was

statistically significant, overall, 66.7% of BRAF mutant tumors were right-sided compared to 29.5% for wild-type tumors (p=0.0001).

Although the stage C2 showed larger proportion of $BRAF^{V600E}$ expression compared to wild BRAF (50%v 25.6%), no statistically significant differences were found between wild and mutant BRAF concerning tumor stage (P=0.155), whereas tumor grade displayed high significant differences(p=0.0001) between wild and mutant BRAF as the poorly differentiated grade showed highest proportion in $BRAF^{V600E}$ against wild BRAF(83.3% v 37.2%).

Finally, highly significant differences(P=0.0001) were detected between wild, mutant BRAF and tumor type, since $BRAF^{V600E}$ expression was detected in 75% of mucinous CRC cases in comparison with wild BRAF which was highly associated with adenocarcinoma cases,(92.3%) of them showed positive expression of wild BRAF.

Table (9) Differences between Clinicopathological Features in Wild BRAF & $BRAF^{V600E}$

Clinicopathological features		**Wild BRAF**		**$BRAF^{V600E}$**		**P value**
		n	**%**	**%**	**n**	
Age	>55	40	51.3	100	1	***P= 0.001**
	≤55	38	48.7	0		
Gender	Male	43	55.1	41.7	5	****P=0.0001**
	Female	35	44.9	58.3	7	
Site	Rt. colon	23	29.5	66.7	8	****P=0.0001**
	Lt. colon	55	70.5	33.3	4	
Stage	A	14	17.9	0	0	**P=0.155**
	B1	14	17.9	8.3	1	
	B2	16	20.5	8.3	1	
	C1	14	17.9	33.3	4	
	C2	20	25.6	50	6	
Grade	Well diff.	26	33.3	8.3	1	****P =0.0001**
	Mod. diff.	23	29.5	8.3	1	

	Poorly diff.	37.2 29	83.3 10	
Tumor type	Mucinous ca.	7.7 6	75 9	****P =0.0001**
	Adeno ca.	92.3 72	25 3	

(*P≤ 0.001) (**P≤ 0.0001)

BRAF mutations have become an important research topic in cancer biology since the original observation by Davies *et al*. in 2002, who revealed that high frequency of *BRAF* mutation is a common phenomenon in multiple types of cancers[18]. In the current study, the positive expression of wild BRAF was detected in 86.7% of malignant cells, 70% of colonic adenoma, and 25% of colitis cases and in 40% of cases with no significant pathology, whereas positive expression of mutant $BRAF^{V600E}$ was observed only in 13.3% of CRC cases versus totally negative expression in other groups of the study.

Our results regarding the immunodetection of wild and mutant BRAF expression in CRC group are close to what was reported by Kalady *et al.* in 2012 who mentioned that only 12% of the samples harbored BRAF mutation versus (88%) that harbored wild BRAF [19], and Ben *et al.* (2014) who reported that (11%) patients had BRAF mutant tumors and (89%) had BRAF wild-type tumors [20]. In contrast, Nicolas *et al*. in 2015 found in their study on CRC patients that mutant BRAF expression was present in (66%) of patients, while it was absent in the remaining (34%) of non-mutated patients who have wild type BRAF[17], while Roth *et al*. in 2010 detected 7.9% of BRAF mutation in their study on patients with stage II to III colon cancer[21], and Naguib *et al.* in 2010 conducted that $BRAF^{V600E}$ mutation was found in 15.6% of CRC in their study population[22] as well as Phipps *et al.* in 2012 who reported that $BRAF^{V600E}$ mutation was observed in (12%) of their study population[23]. Chen *et al.* in 2014 found (10.8%) of $BRAF^{V600E}$in CRC patients, and mentioned in their study of estimation the effect of $BRAF^{V600E}$ mutation on the clinicopathological characteristics of CRC, that the highest $BRAF^{V600E}$ mutation rate was (21.8%) in a study in the United States reported by Shaukat *et al.(*2010), whereas the lowest mutation rate was(5.0%) in a study completed in Israel by Rozek *et al.* (2010). They concluded that the differences in BRAF expressions may be attributed to the different ethnicities of the study populations [24, 25, 26].

Numerous studies investigated the role of *BRAF* mutation in cancer development and progression. In a point of view, $BRAF^{V600E}$ mutation, as the most prevalent *BRAF* mutation, changes the inactive conformation of BRAF kinase to a very active state [27]. This simple point mutation leads to a constitutive activation of whole MAPK pathway, which mediates the cell surface growth signals to transcriptional activity of cell cycle regulatory genes [28]. $BRAF^{V600E}$ mutation, which is virtually absent in hereditary colorectal cancer, is often present in sporadic CRC that has a CpG island hypermethylation phenotype (CIMP-high), resulting in hypermethylation of promoter regions. BRAF mutated CIMP-high CRC is frequently MSI-H, as the MLH1 promoter has been methylated, resulting in an MLH1-deficient tumor [29].

Several publications have reported the possibility of immunodetection of wild and mutated proteins of BRAF using a specific monoclonal antibody directed against those proteins**;** Yu *et al.* in 2009 generated monoclonal antibodies specific to the more frequently mutated $BRAF^{V600E}$ mutation which only reacts with the protein product of the $BRAF^{V600E}$ and not with protein associated with other mutations of BRAF[30], as well as Capper *et al.* (2011) who developed a novel mouse monoclonal mutation-specific anti-$BRAF^{V600E}$ antibody, which is suitable for immunohistochemistry on routinely processed formalin fixed paraffin embedded tissue that can differentiate $BRAF^{V600E}$ and wild type protein[14]. They recommended that using monoclonal antibody may substantially facilitate molecular analysis of $BRAF^{V600E}$ status for diagnostic, prognostic, and predictive purposes. Koperek and Kornauth in 2012 concluded that IHC for $BRAF^{V600E}$ is more sensitive and specific than Sanger sequencing in the routine diagnostic setting and may represent the new gold standard for detection of $BRAF^{V600E}$ mutation in papillary thyroid carcinoma [31]. Nicolas *et al*. in 2015 confirmed the use of immunohistochemistry in detecting $BRAF^{V600E}$ mutation protein as an alternative to molecular testing in CRC, they reported that immunohistochemical evaluation could also be useful in cases where the quality of the material is not suitable for molecular investigation as it is possible to assess diagnosis of malignant tumors only by the presence of scattered cells in the specimen compared with molecular techniques which require at least 5% of tumoral cells in the tested sample[17].

The current result about the expression of wild and mutant BRAF expression in tumor free tissues (no significant pathology cases), match with Capper *et al.* in 2011 who reported that normal tissue surrounding the tumor was

$BRAF^{V600E}$ negative, and the investigation of the wild type cases using antibody of wild BRAF revealed equally large amounts of total BRAF protein as in $BRAF^{V600E}$ mutated cases[14].

Conversely, the present results concerning the expression of wild and mutant BRAF in cases of colonic adenoma, agree with Rosenberg *et al.* in 2007who reported that $BRAF^{V600E}$ mutation was identified in (10\16)of serrated adenomas compared with(1\33) of conventional adenoma(P= 0.001)[32], and with Spring *et al.* in 2006 who concluded in their study on patients with adenomas (tubular & tubulovillous adenomas), that BRAF mutation was rare in adenomas since they detected the mutation in 1/248 (0.4%)of cases [33], as well, Carr *et al.* in 2009 mentioned that BRAF mutation was detected in 2/42 (5%) of conventional adenomas [34]. Kambara *et al.* in 2004found that none of (28) of conventional adenomas cases had BRAF mutation compared to other types (sessile serrated adenoma (75%), mixed polyps (89%), hyperplastic polyps (19%), serrated adenomas (20%), and conventional adenomas (0%) (p<0.001) [35]. Sclafani *et al.* in 2013 reported in their meta-analysis study that approximately (6–85%)of hyperplastic polyps,(20–100%) of mixed polyps, and(20–100%) of serrated adenomas have been reported to harbor a BRAF mutations compared to only(0–5%)of conventional adenoma[36]. Nevertheless, it was suggested by some studies that $BRAF^{V600E}$ mutation was detected in much more frequency in adenomas, of them; Beach *et al.* in 2005 detected BRAF mutation in 30% of tubular adenomas[37]. Lee *et al.* in 2005mentioned that mutant BRAF expression was found in 27 (77.1%) of serrated adenomas and (7\13) of hyperplastic polyps, recommending that BRAF mutations are early and a critical event in serrated adenomas, and most serrated adenomas in both sides of colon may progress from micro-vesicular hyperplastic polyps via BRAF mutations[38]. On the other hand, Chan *et al.* in 2003 reported that BRAF mutations were detected in 18 /50 (36%) hyperplastic polyps, 2/10 (20%) admixed hyperplastic polyp/adenomas, and 9 / 9 (100%) serrated adenomas. they concluded that acquisition of a BRAF mutation appears to be associated with the progression of hyperplastic polyp to serrated adenoma, and suggested that the high incidence of BRAF mutations in hyperplastic polyps and serrated adenomas is consistent with the concept that the group of CRC carrying BRAF mutations may harbor most that have progressed through the hyperplastic polyp - serrated adenoma-carcinoma pathway[39]. In accordance with the theory of BRAF mutation as an early event in colorectal tumourigenesis, a recent systematic review of published data demonstrated a high concordance rate (98%) of BRAF mutations between primary and metastatic tumors [40].

In the present study, wild BRAF did not associated with age, gender, tumor type, grade and location. Surprisingly, all $BRAF^{V600E}$expression was detected in patients above 55, with significant differences (P=0.047), and this is compatible with Roth *et al*. in 2010 who confirmed that BRAF mutation was highly significant associated with older age[21] and with Chen *et al*. in 2014 who demonstrated in their analysis that (18.6%) of patients were 60 years or older, were $BRAF^{V600E}$ mutation positive, compared with (6.7%) of patients younger than 60 years old, detecting a significant association between $BRAF^{V600E}$ mutation and age 60 years or older[24]. As well, Tie *et al.* in 2011 reported that the prevalence of $BRAF^{V600E}$ was considerably higher(P=0.04) in older(age > 70)[41], as well as, Lubomierski *et al.* in 2005 reported that the median age of patients with $BRAF^{V600E}$ was older compared with the median age of patients without this mutation (P = 0.001; 78 vs. 49 years)[42]. Berg *et al*. in 2010 recorded in their work that BRAF mutation in patients <50 was 7% while in those >70 was 19%, recommending that BRAF mutation increased with patient's age[43]. However, the association of BRAF mutations with age and gender is controversial and has not been demonstrated in other studies [36, 44].

The current Results showed that $BRAF^{V600E}$expression was significantly related (p=0.041) to right sided colon, this is in agreement with Fariña-Sarasqueta *et al*. in 2010 who mentioned that $BRAF^{V600E}$ mutation was significantly associated with right-side location (*P*< 0.001)[44], and with Roth *et al*. in 2010 who reported that *BRAF* mutations were highly significantly more frequent in right-sided colon tumors($P < 10^{-4}$)[21]. As well, Zlobec *et al*. in 2010 concluded that BRAF gene mutation is an adverse prognostic factor in right-sided colon cancer patients independent of MSI status and, moreover, the molecular analysis for BRAF may be a useful biomarker for identifying patients with right-sided colon cancer with poor outcome who may benefit from a more individualized course of therapy[35]. Tie *et al*. in 2011 recorded that right-sided tumor location (p< 0.0001) were independently associated with $BRAF^{V600E}$ [41]. And Chen *et al*. in 2014 observed that (21.6%) of patients with tumors in the proximal colon were $BRAF^{V600E}$ mutation positive, compared with (4.8%) of patients with distal colon or rectal tumors, they detected a significant association between $BRAF^{V600E}$ mutation and proximal colon tumor location[24]. Also, Sclafani *et al*. in 2013concluded that BRAF mutations have been found to occur significantly more frequently in tumors arising from the right colon than left colon with a percentage of (57–89% vs 11–43%)[36]. In contrast, Yuen *et al*. in 2002 found (45) tumors in the right colon and (170) in the left colon [46]. The explanation of being BRAF mutation tumors more prevalent in right side than left side may be return to a higher frequency of MSI which is a poor prognostic factor in CRC, has been reported to be more

prevalent in right side compared to left side of colon[47, 48]. Other hand, Benedix *et al*. in 2012 suggested that these differences between right and left sided colon are more related to the anatomical site of the cancer origin rather than simple right and left categorization [49]. Otherwise, despite a number of descriptive studies on the prevalence of BRAF mutation and its correlation with clinicopathological characteristics, there has been no comprehensive comparison on the effect of BRAF mutation on patient's survival in separate groups of right and left colon cancers [28].

Also in this work, a significant association was detected between *BRAF*^{V600E} expression and tumor type (P=0.021), since all mucinous cases which account for (10%) of total CRC cases in this study, showed positive expression of this marker. This finding agreed with what was reported by Yoshitake *et al*. in 2007, who recorded in their study of evaluation the status of BRAF and other markers to clarify their association with tumorigenesis of colorectal mucinous carcinoma, that all mucinous colorectal carcinoma cases harbor BRAF mutation, recommending that BRAF mutation plays an important role in the tumorigenesis of colorectal mucinous carcinoma [50]. Chen *et al*. in 2014 detected a rate (19.4%) of patients with mucinous histology were BRAF^{V600E} mutation positive, whereas (8.1%) of patients with non-mucinous histology were BRAF^{V600E} mutation positive, observing a significant association between BRAF^{V600E} mutation and mucinous histology [24]. Other work by Tanaka *et al*. in 2006 observed BRAF mutation in (46%) of mucinous CRC versus (16%) of non- mucinous CRC ($p < 0.01$) [51]. Li WQ *et al*. in 2006 explained in their work that BRAF^{V600E} mutation is 5-10folds more frequent in tumors with high grade and mucinous appearance ($P< 0.002$ for each)[52], and Fariña-Sarasqueta *et al*. in 2010 agreed that the BRAF^{V600E} mutation confers a worse prognosis to stage II and stage III colon cancer patients independently of disease stage and therapy[44], other study by Ogino *et al*. in 2012 found that BRAF mutations were more frequent in the mucinous (27%) than non-mucinous carcinoma (8.6%) ($P<0.001$)[9].

Other finding in this study revealed that wild BRAF is associated with tumor stage since it was expressed mainly in cases of stage C, while no significant association was noticed in *mutant BRAF*^{V600E} expression among tumor stages, and this is in accordance with Roth *et al*. in 2010 who explained that BRAF^{V600E} expression were not significantly different according to tumor stage[21], compared with Tie *et al*. in 2011 who suggested that BRAF expression is associated with poorer outcomes in CRC patients[41]. Other works by Gavin *et al*. in 2012, de Roock *et al*. in 2010 and Oikonomou *et al*. in 2014, who referred in their adjuvant studies in patients with stage II/III colon cancer and in metastatic disease, that BRAF^{V600E} mutation is associated with worse clinical outcome, and agreed that immunodetection of BRAF^{V600E} mutation could provide prognostic information[53, 54, 55]. Yuan *et al*. in 2013 stated in their study that BRAF mutation is a predictive biomarker of poor prognosis in metastatic CRC patients treated by anti-EGFR monoclonal antibodies, especially in KRAS wild-type patients [56]. However, Phipps *et al*. in 2012 concluded that the prevalence of BRAF mutations in CRC differs by patient and tumor characteristics and suggest that the association between BRAF status and CRC survival may differ by some of these factors [23].

With the exception of tumor stage which displayed insignificant differences, the results of this study revealed significant differences in the clinicopathological features concerning age, gender, tumor site, grade and type between wild and mutant BRAF, which indicate that mutant BRAF is associated with bad prognosis in CRC patients since it is significantly correlated with high grade and mucinous type of cancer. And this is match with Clancy *et al. in 2013 who concluded that BRAF* mutation appears to be associated with distinct, unfavorable clinicopathological characteristics in CRC[57], and with Chen *et al*. in 2014who demonstrated that the BRAF^{V600E} mutation in CRC was associated with advanced TNM stage, poor differentiation, mucinous histology, female gender, older age, proximal colon, concluding that BRAF^{V600E} mutation was significantly correlated with adverse pathological features of CRC and distinct clinical characteristics. They suggested that BRAF^{V600E}mutation could be used to supplement standard clinical and pathological staging for the better management of individual CRC patients, and could be considered as a poor prognostic marker for CRC as well as could alert physicians to patients that may be at increased risk of carrying a BRAF^{V600E} mutant tumor as the focus for screening [24]. As well, Ardekani *et al*. in 2012 found that BRAF mutation increases the risk of mortality in CRC patients for more than two folds, and concluded that BRAF mutation is an absolute risk factor for patient's survival in CRC since the risk of mortality in CRC patients harboring BRAF mutation is more than 2 folds higher than those with wild BRAF. They recommended that the key regulatory role of BRAF mutation in MAPK pathway is to block signaling pathway for cancer treatment[28]. However, the response rate of CRC patients harboring BRAF mutation to BRAF inhibitor treatments is much lower than other types of cancers [21].

In the present study, $BRAF^{V600E}$ mutation was significantly associated with several clinical and pathological factors. Therefore, we infer that $BRAF^{V600E}$ mutations may play an important role in tumor development and the subsequent prognosis.

4. Conclusions:

There were significant differences in the expression of BRAF in patients with Dukes' stage A, B and C colorectal cancer. $BRAF^{V600E}$ mutation was positive only in carcinoma group and associated significantly with different clinical and pathological factors. Therefore, we infer that $BRAF^{V600E}$ mutations may be a promising tool for early detection of micrometastatic circulating tumor cells in CRC patients. And there were significant differences between wild BRAF and mutant $BRAF^{V600E}$ in relation to patients' age, female sex, right-sided colon tumor, tumor type and grade. Those results suggest that both markers are useful tool for determining which patients are at high risk for recurrence and poor prognosis.

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