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Molecular Study of *Nras* Gene Among Iraqi Colorectal Cancer Patients.

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ABSTRACT
Mutations in genes such as *NRAS* has become an important part of colorectal carcinoma evaluation. *NRAS* mutations occur in 3-5% of the colorectal cancer. The role of *NRAS* is differently from *KRAS* and *BRAF* mutation it plays as prognostic and predictive markers in metastatic colorectal cancer (mCRC) which has been investigated to a lesser extent. The aim of this study was to assess *NRAS* mutations frequencies in Iraqi colorectal cancer patients and find a correlation between these mutations with their gene expression. A total of 90 samples of FFPE (fixed formalin paraffin embedded tissue) consecutive stage 0-IV CRC tumor samples were analyzed for *NRAS* (exon 2 and 3) using direct sequencing and reverse transcriptase –polymerase chain reation (RT-PCR).The results of CRC mutations in *NRAS* gene(exon 2 and 3) is high in females than males. Sequencing in *NRAS* gene exon 2 and 3 shows a substitution mutation in different types of tumors in Iraqi colorectal cancer patients; also there is a high significant increasing between the substitutions mutations in exon 2 with it gene expression in early stages in CRC patients($P<0.01$) . **Conclusion**: Our results showed that frequency of *NRAS* mutations in Iraqi colorectal cancer is rare, which is very close to other studies from different geographic areas of the world. Further molecular studies involving large patient series will be necessary to confirm these findings and to asses possible ethinic/environmtal and lifestyle influences on *NRAS* mutagenesis and to confirm the possible positive prognoses association with *NRAS* mutations and it gene expression.

Keywords: *NRAS*, carcinoma, mCRC , RT-PCR,FFPE, mutagenesis

INTRODUCTION

Colorectal cancer (CRC) is one of the most frequent cancers worldwide. Each year approximately one million new patients are diagnosed with CRC, and metastatic disease develops in 50% of these patients (1). CRC is the third most commonly diagnosed cancer in males and the second most common cancer in females, worldwide (2)

Colorectal cancer incidence has been increasing in the Arab countries such as Kuwait and Saudi Arabia (3; 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).

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 (6).

NRAS gene was discovered by a small team of researchers led by Robin Weiss at the Institute of Cancer Research in London. It was the third *RAS* gene to be discovered, and was named *NRAS*, for its initial identification in human neuroblastoma cells (7; 8).

The *NRAS* gene provides instructions for making a protein called N-Ras that is involved primarily in regulating cell division. Through a process known as signal transduction, the protein relays signals from outside the cell to the cell's nucleus. These signals instruct the cell to grow and divide (proliferate) or to mature and take on specialized functions (differentiate). The *NRAS* gene belongs to a class of genes known as oncogenes. When mutated, oncogenes have the potential to cause normal cells to become cancerous. The *NRAS* gene is in the Ras family of oncogenes, which also includes two other genes: *HRAS* and *KRAS* (9).

The proteins produced from these three genes are GTPases. These proteins play important roles in cell division, cell differentiation, and the self-destruction of cells (apoptosis). The RAS proto-oncogenes (*HRAS, KRAS* and *NRAS*) encode a family of GDP/GTP-regulated switches that convey extracellular signal to regulate the growth and survival properties of cell (10).

Ras traffics from cytosol to plasma membrane, where it resides and communicates external signals to the nucleus. Certain point mutations within the *Ras* gene lock the protein into a constitutively active state, and leads to aberrant cell signaling even in the absence of external signals; such a deregulated Ras signaling imminently leads to cancer instigation. Mutations in *NRAS* gene become one of the important part of the colorectal cancer evaluation (11).

Mutations of *NRAS* gene located mainly in exon 2 and 3 and the mutations in this gene had a higher risk to develop distant after surgery and had shorter metastasis-free intervals for future studies because in unique carcinogenic mechanism and biological characterstics (12).

This study aimed to detect the frequency of mutations in

exon2 and 3 of *NRAS* gene in Iraqi colorectal cancer patient and the coloration between these mutations and their gene expression.

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 25ng of FFBE incubated in water bath for 30 minutes at 55-60 °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 1 minute 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 and RNA protocol according to the manufacture instructions of the kit.

DNA Extraction:

Genomic DNA was extracted from 5-8µm-thick paraffin sections containing a portion tumor tissue using the gSYNC™ 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 2 and 3 of *NRAS* gene was target to amplify using forward primer exon2, (5′-GATGTGGCTCGCCAAT TAAC-3′) and reverse primer exon2, (5′-CACTGGGGCTCACCTCTATG-3′), forward primer exon3(5′-CACCCCCAGGATTCTTACAG-3′) and reverse primer exon3 (5′CCCCATAAAGATTCAGAACACA-3′) . Each 25µl PCR reaction mixture for *NRAS* 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 for30sec, extension at 72°C of 30min and 5min final extension at 72°C table (1).PCR products separated in 1.5% agarose gel after staining with ethidium bromide. 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.

Table (1) PCR program profile

Steps	C°	m:s	Cycle
Initial Denaturation	95	10.00	1
Denaturation	95	00.20	45
Annealing	58	00:20	
Extension	72	00:30	
Melt on Green Melt from 72°C to 95°C at 0.3°C/s			

RNA extraction:-

RNA was extracted from the FFPE samples by using AccuZol™ kit (Bioneer Company) .A total RNA 10pg(18µl) was reversely transcribed to a complementary DNA(cDNA) by using AccuPower®RocketScrript™RT Premix kit (Bioneer).The procedure was carried out in a reaction volume of 20µl according to the manufacture with modifications, PCR program for cDNA synthesis program (table 2), as follow primer anneling 30°C for 10 minutes,cDNA synthesis 42°C for 30 minutes, heat inactivation 95 °C for 5 minutes.

Table (2) PCR program for cDNA synthesis

Step	Temperature	Time
Primer annealing	30 ºC	10 minutes
cDNA synthesis	42ºC	30 minutes
Heat inactivation	95ºC	5 minutes

Quantitative Real Time PCR (qRT-PCR):

The expression level of *NRAS* gene was estimated by Two step qRT-PCR to comform the expression of target gene, quantitative real time qRT-PCR SYBER Green assay was performed using a syber green master Mix(GoTag qPCR Master Mix,Promega,USA), In 10µl reaction volume in table(3). The cycling protocol was programmed according to the thermal profile shown in table (4).

Table (3): Components of the reaction of qRT- PCR

Master mix components	Stock/ Unit	Volume of 1sample
qPCR Master Mix	2 x	5
MgCl2		0.25
Forward primer	10µ M	0.5
Reverse primer	10 µM	0.5
Nuclease Free Water		2.75
cDNA	10 ng/ µl	1
Total volume		10
Aliquot per single rxn		9µl of Master mix per tube and add 1µl of template

Table (4) Quantitative Real Time PCR program:

Steps	C°	m:s	Cycle
Initial Denaturation	95	10.00	1
Denaturation	95	00.20	45
Annealing	58	00:20	
Extension	72	00:30	
Melt on Green			Melt from 72°C to 95°C at 0.3°C/s

Primer used for Quantitative Real Time PCR:

Primers used for quantitative real time PCR are listed in table (5). The primers was designed according to the National Center Biotechnology Information (NCBI) (13), and stored lypholized at (-20°C).

Table (5): Primer sequencing

Primer Name(*NRAS*)	Seq
Forward primer	5`-GAAGGCTTCCTCTGTGTATTT-3`
Reverse primer	5`-GGTACTGGCGTATTTCTCTTAC-3`
GAPDH-F(H.K)	5`-AGAAGGCTGGGGCTCATTTG-3`
GAPDH-R(H.K)	5`-AGGGGCCATCCACAGTCTTC-3`

Gene Expression Calculation:

Relative quantification:-

Folding $=2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT$ Treated - ΔCT Control

ΔCT =CT gene - CT House Keeping gene.

Statistical Analysis:

The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage and Least significant difference –LSD test (ANOVA) was used to significant compare between means variables in this study (14).

RESULTS

In this study ,90 FFPE tissue blocks were retrieved .The prevalence of CRC was higher in females(51.11%,46/90) than males (48.89%,44/90).There was a high significant in ages of the patients in our data (P< 0.01). Also the majority of the tumor site was in the colon than in rectal 56.67%, 43.33% respectively. Types of tumors including the control, malignant and benign have a significant increasing (P<0.01), also the stages in mCRC patients in this study have a high significant (P<0.01) and the most frequent stage was stage III-B (48.65%, 18/90) then stage III-A (27.03%, 10/90). While the lowest stage was stage II- B (10.81%, 4/90).The mean size of tumors were 11.98 ± 0.72 table (6).

Table (6). Characteristic of total samples in this study.

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

Table (7) represents the characterization of 37 FFPE tissue blocks with colorectal cancer. These characteristics which involves, localization (colon and rectum), colon site (left and right) and differentiation grade (well, moderate and poor differentiation). There were a significant increasing in ages (P<0.01) which is greater than 50 and less than 50. The highest incidence of CRC were in the patients which are greater than 50 (72.97%, 27/37) while less than 50 was (27.03%, 10/37).The females have the most incidence in CRC than males (62.16%, 23/37), (37.84%, 14/37) respectively. Most of the tumors were located in the right side of the colon (86.67%, 13/37) while in the left (13.33%, 2/37).Regarding the histological subtypes, 81.08% (30/37) of tumors were moderately adenocarcinomas and 10, 81% (4/37) were villous adenocarcinomas, 8.11% (3/37) were well adenocarcinomas and poorly adenocarcinomas were 0%.

Table 7.The distribution of patients with colorectal cancer

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

There were no significant with the female colorectal cancer patients according to the site of tumor (colon/rectal) 51.11%, 48.89% respectively while there were significant in males CRC sites between the colon and rectal (P<0.01).The average age of males in colorectal cancer were 41.00± 2.83 while the females were 54.00± 2.83 table (8).

Table 8. The distribution of samples according to the age, gender and site (colon/rectal).

Gender	Age Average	No. Age % Average	Site colon/rectal		P -value
Male (No. = 45)	41.00 ± 2.05	45.55%	28 (62.22%)	17 (37.78%)	0.0002 **
Female (No. = 45)	54.00 ± 2.83	50.00%	23 (51.11%)	22 (48.89%)	0.2783 NS
** (P<0.01), NS: Non-Significant.					

PCR and direct sequencing targeted for *NRAS* gene exon 2 and 3 Figure (1&2). The distribution of polymorphism and allele frequency in *NRAS* gene exon 2 (table 9) shows a highly significant substitution mutation in the site rs753953315 among control ,malignant and benign tumors in the nucleotide GG and AG there were an increased in malignant tumors and the control FFPE patients .

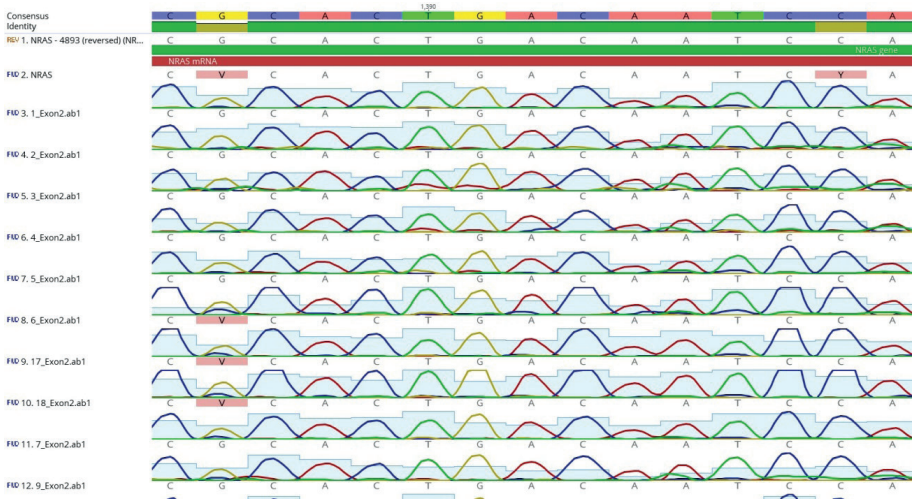


Figure 1.the sequencing of *NRAS* gene (exon 2) when alignment with orginal sequencing.

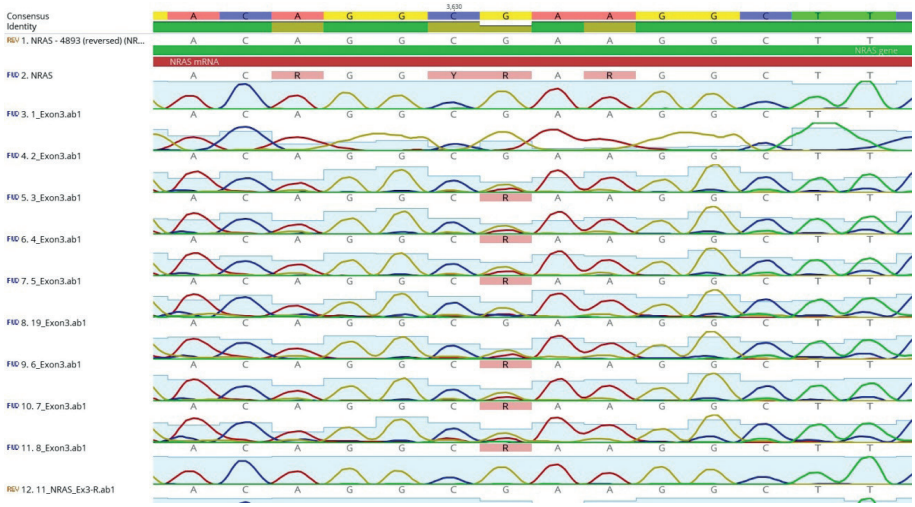


Figure 2.the sequencing of *NRAS* gene (exon 3) when alignment with orginal sequencing.

Table (9) . Distribution of Polymorphism and allele frequency in *NRAS*/Exon 2 gene (Mutation rs753953315-GG).

Polymorphism (rs753953315)	Control No. (%)	Malignant No. (%)	Benign No. (%)	Chi-square	P-value
GG	4 (100%)	6 (60.00%)	5 (50.00%)	12.563 **	0.0001
AG	0 (0.00%)	4 (40.00%)	5 (50.00%)	12.563 **	0.0001
Total	4	10	10	---	---
Chi-square (P-value)	15.00 ** (0.0001)	8.25 ** (0.0067)	0.00 NS (1.00)	---	---
Allele frequency					
G	1	0.80	0.50	---	0.0001
A	0	0.20	0.50	---	0.0001
** (P<0.01).					

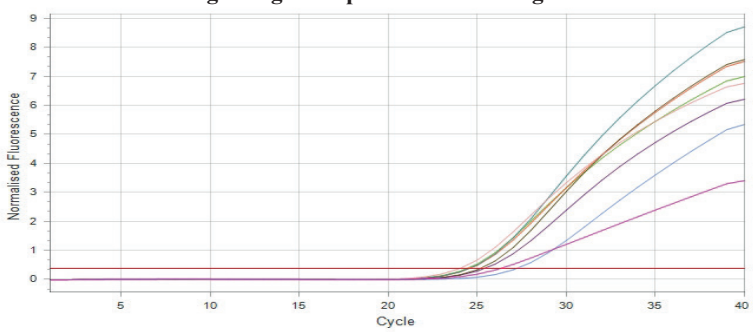
The allele frequency in exon 3 confirmed that there is a significant increase in malignancy patients that changed the nucleotide in the site CC to CT. The types of tumors in our data have a significant rate which were higher in control and benign than malignant patients (50.00%), (50.00%), (40.00%) respectively table (10).

Table 10. Distribution of Polymorphism and allele frequency in *NRAS*/Exon 3 gene (Mutation rs772805532-CC).

Polymorphism (rs772805532)	Control No. (%)	Malignant No. (%)	Benign No. (%)	Chi-square	P-value
CC	2 (50.00%)	4 (40.00%)	5 (50.00%)	4.391 *	0.0407
CT	2 (50.00%)	6 (60.00%)	5 (50.00%)	4.391 *	0.0407
Total	4	10	10	---	---
Chi-square (P-value)	0.00 NS (1.00)	8.25 ** (0.0067)	0.00 NS (1.00)	---	---
Allele frequency					
C	0.50	0.70	0.50	---	0.0219
T	0.50	0.30	0.50	---	0.0219
* (P<0.05), ** (P<0.01).					

Real time PCR assay were validated and performed. We quantified the gene expression of 90 FFPE tissues samples including three groups control; malignant and benign (figure 3).

Figure3.gene expression of *NRAS* gene.



Comparing the effect of site, gender and age groups with the folding of *NRAS* gene table (11). The results shows there were no significant effects between them in the CRC patients while there is a significant effect observed in the stage with the folding of *NRAS* gene (P<0.05) and the most stage which have a high mean was stage II-B as shown in table(12).

Table 11. Effect of Site, Gender and Age groups in Folding of *NRAS* gene.

Factors	Level	Mean ± SE of Folding	P-value
Site	Colon	6.53 ± 2.83	0.6537 NS
	Rectal	6.54 ± 2.87	
Gender	Male	7.82 ± 4.05	0.2661 NS
	Female	5.85 ± 2.20	
Age group (year)	< 50	7.12 ± 2.95	0.5702 NS
	> 50	6.07 ± 2.75	
NS: Non-Significant.			

Table 12. Effect of stage in Folding of *NRAS* gene.

Stage	Mean ± SE of Folding
II-A	7.54 ± 3.63 ab
II-B	12.83 ± 0.00 a
III-B	5.35 ± 2.98 b
LSD value	6.0351 *
P-value	0.0469
* (P<0.05). Means having with the different letters in same column differed significantly	

Malignant group in CRCs patients have mean 7.11± 2.72 comparing between the control and benign groups in folding of *NRAS* gene which shows a significant effect in different groups in our data table (13).

Table 13. Compare between difference groups in Folding *NRAS* of gene.

Group	No.	Mean ± SE of Folding
Control	5	2.25 ± 1.38 b
Malignant	8	7.11 ± 2.72 a
Benign	7	8.96 ± 4.57 a
LSD value	---	4.081 *
P-value	---	
* (P<0.05). Means having with the different letters in same column differed significantly		

Table 14 shows the relationship between rs753953315 (GG) *NRAS* exon 2 and the genotype and the folding of this gene which observed a frequent significant between them .in contrast in exon 3 were non-significant relationship between the folding, genotype and rs772805532 (CC) in the NRAS gene table (15).

Table14. Relationship between rs753953315 (GG) *NRAS* exon 2 genotype and Folding of gene.

Genotype of rs753953315(GG) *NRAS* exon 2	No	Mean ± SE of folding
GG	7	15.16 ± 3.81
AG	8	1.681 ± 0.41
LSD value	--	7.937 **
P-value	---	0.0088
** (P<0.01).		

Table15. Relationship between rs772805532 (CC) *NRAS* exon 3 genotype and Folding of gene.

Genotype of rs772805532(CC) *NRAS* exon3	No	Mean ± SE of folding
CC	4	10.47 ± 4.97
CT	11	7.06 ± 2.97
LSD value	---	8.954 NS
P-value	---	0.422
NS: Non-Significant.		

DISCUSSION

We conduct this study to examine the frequency of *NRAS* mutation in relation with the gene expression of this gene in Iraqi colorectal cancer patients. The RAS pathway plays an important role in the development of various cancers (15, 16, and 17).frequent activating mutations in the *KRAS* oncogene have been identified in colorectal cancer (18).Nevertheless, there are only a few reports on *NRAS* mutations in colorectal cancer and none of these studies correlated RAS mutations with other molecular events (19, 20).Our characteristic of total FFPE samples in this study showed a high significant of tumor types, Ages ($P< 0.01$).

Also there was a significant increase ($P<0.5$) with the site of tumors (colon/rectal) 56.67% in colon while rectal 43.33%.Stage IIIB was the most frequent stage in our study 48.65% and there was a high significant rate between different stages ($P< 0.01$) .This result showed an agreement with (21, 22) that find 55 % of CRC patients were presented to hospitals at advanced stage, while 38% were at less advanced stages and only 6.89 % were presented at an early stage. Also the staging distributions of CRC patients in this study were different to the staging distribution in previous Iraqi study, in which it was found that 31.3% of CRC patients were presented to hospitals at a very advanced stage, while 51.3% were at less advanced stages and only 17% were presented at an early stage. Our study showed statistically significant associations, between *NRAS* mutations and the clincopathoplogical features such as age tumor site, tumor stages and types except the gender. These results found a disagreement with Iranian, Indian and Arabic studies which reported that their results showed no significant difference between factors such as age, sex tumor location, stage of disease with *NRAS* mutations (23, 24, and 25).

The cases of thirty seven CRC patients observed a high significant increasing in age, site of colon and grade differentiation. There were similar results published in Al-Samak, study (2010), With regard to the CRC differentiation grade, it was found that 2/58(3.44%) of histological sections of CRC patients were poor differentiated in low percent, while there was high significant increase($P\leq0.01$), 43/58(74.13%) moderately differentiate and 9/58(15.51%) were well differentiated. Low rate 4/58(6.89%) of villous tumor also included in current study. Our data revealed that 62.16% of females have CRC while males 37.84% which disagree with the study of the largest sets of CRC in Asia, 1,173 CRC tumors were collected from the Taiwan population. To focus on sporadic CRC cases for the clinicopathologic and genomic, There were 785 (66.9%) males and 388 (33.1%) females in these sporadic CRC (26). Our results showed 86.67% of CRC patients found in the right side of colon while 13.33% the left – side which disagree with (26) that mention tumors were found in right-side colon in 294 patients (25.1%), left-side colon in 478 patients (40.8%), and in the rectum in 401 patients (34.2%). Also our data found an agreement with (27; 28) who are reported a higher frequency of *KRAS* mutation in right-sided tumors (37%, 38%) compared to left-sided. The results differ from (29) 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.

According to the results in table (9) and (10) there were a substitute mutation in *NRAS* gene exon 2 rs753953315 (GG) with malignant and control patients while in exon 3 rs 772805532(CC) there were a high significant increase only in the malignancy patients .our results agree with (30-37) that mention the overall frequency of *NRAS* mutations in CRC has ranged between 2-7 (4%) and the difference in frequencies is secondary to ethnic variations. Also some studies have reported the incidence of *NRAS* in wild type *KRAS* and BRAF, while some others searched *NRAS* mutation frequency in both mutant and wild type *KRAS* and *BRAF* CRCs. In wild type *KRAS* and *BRAF*, *NRAS* mutation rate has been 2.4 to 4.7%. Our data was close to two studies from Iran previously showed frequencies of 0 and 2% (38, 39) also our results was close to the reports from the other parts of the world but we report a litter higher mutation rate .this difference could probably be due to differences in geographic regions as well as ethnic population (40-42), (24), (31), (33-35) we agree with (23) which mention that frequency of *NRAS* mutation in colorectal cancer was rare. Also a previous study in Japan reveled that *NRAS* mutations were observed in 35(2.7%) of 1,304 patients, of which 20(1.5%) patients revealed a mutation in exon 2 and others (1.2%) in exon 3which have an agreement with our results (40).

The correlation between the stages of the FFPE tissues and the gene expression of *NRAS* gene (folding) indicate that the most significant increase was in stage II-B which showed that *NRAS* mutations are detected in early ages of CRC and tend to occur more frequently in stage IV cancers than in stage 0-III cancers. Therefore, *NRAS* mutation appear to be acquired at early and advanced stages of CRC (41).There was a correlation between the gene expression of *NRAS* gene exon 2 and the substitution mutations in this gene (GG→AG) which have a high significant ($P<0.01$) ,this may be due to the early stages of colorectal cancer have an hyper activation in the ras/raf pathway the function of the pathway is to transducer signals from the extracellular milieu to the cell nucleus where specific genes are activated for cell growth, division and differentiation .Also the pathway is involved in cell cycle regulation, wound healing and tissue repair, integrin signaling and cell migration(42-44). Deregulation of this pathway is a common event in cancer as RAS is the most frequently mutated oncogene in human cancer (45-46). Mutation activation in the ras oncogen cause amplified and over expression or upstream activation in of the pathway each of this potential cellular alteration will produce increased activation of Ras effectors, thereby promoting development tumors (47).

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