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The correlation between *KRAS* mutations and *H. pylori* in gastric cancer patients

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Abstract: *Helicobacter pylori* (*H. pylori*) is the most important etiologic factor for gastric cancer ,it is one of the most common human pathogens, which colonizes in the mucus layer of the gastric epithelium in more than 50% of the population. It is the only bacterium that classified as a class I carcinogen by the WHO .The clinical outcomes of *H. pylori* infection is determine by host genetic predisposition, bacterial virulence factors, and environmental factors. *H. pylori* can induce chronic inflammation and oxidative stress that provides a permissive environment to DNA damage, this damage can lead to genetic instability and eventually, neoplastic transformation. *KRAS* is one of the most frequently mutated oncogenes in human cancers, in particular in pancreatic, colorectal and lung cancers. However, oncogenic mutations of *KRAS* are infrequent in gastric cancer. Point mutations of the *KRAS* are found predominantly in adenocarcinomas. Codons 12 and 13 are the most frequently detected mutation "hot spots", they make the protein resistant to GTP hydrolysis by GTPases, thereby leading to constitutive *KRAS* activity. The results revealed that no mutations in codons 12 or13 detected in all patient groups, but the sequencing analysis detected other mutations in exon 2. These mutations were found in 7 /20 (35%) patients of HIP-GC group, 5/16(31.25%) patients of HIN-GC group and 4/15(26.7%) patients of the ulcer group.

Keywards: K-ras, H.pyolri, Gastric cancer, Gastric epithelium, Mutation**.**

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Introduction:

Gastric cancer is a major public health issue as the fourth most common malignancy, and the third most common cause of cancer-related death in both sexes worldwide (1), partly, because patients are not diagnosed until late-stage cancer is present and a poor prognosis (2). In Iraq, gastric cancer have low rate (3), and it is the ninth most common cancer(4).

Carcinogenesis of stomach progresses through a sequence of preneoplastic lesions that manifest histologically as atrophic gastritis, intestinal metaplasia, and dysplasia, and it is a multistep and multifactorial process (5). Although a number of risk factors such as the infection of *H. pylori*, salt intake, smoking, alcohol, family history, atrophic gastritis, and intestinal metaplasia are well known, they cannot account for all gastric cancers, since gastric carcinogenesis is a multifactorial process. Indeed, a very wide range of factors environmental, genetic and epigenetic affects the risk of developing gastric cancer (6). The development of gastric cancer is a complex, multistep process involving multiple genetic and epigenetic alterations in oncogenes, tumor suppressor genes, DNA repair genes,

cell cycle regulators, and signaling molecules (7,5).

Helicobacter pylori (H.pylori) is a Gram negative spiral, microaerophilic bacterium (8,9). It is a human pathogen that colonizes the stomach's mucosal lining, and it has colonizing and coevolving in the human gut for more than 50,000 years(10). The infection with *H. pylori* is a significant risk factor for the development of gastric carcinoma (11). In 1994 the WHO classified *H. pylori* as a class I carcinogen (12). The constant infection with *H .pylori* causes the development of gastric cancer as a consequence of chronic inflammation of the gastric mucosa (13). *H .pylori* can develops gastric cancer through various mechanisms one of these mechanisms the oxidative stress. *H. pylori* is known to produce reactive oxygen ROS and nitrogen species RNS , causing in DNA damage and mutation in epithelial cells leading to cancer (14).

KRAS is a tumor suppressor gene, which is located on chromosome 12p12, it is encodes a small GTPase protein with a molecular mass of 21.6 kD (15). *KRAS* protein acts as a binary switch, the active GTP-bound form is ON and inactive GDP-bound form is OFF, to activate several important cellular signaling pathways. When GDP exchange to GTP by GEFs, *KRAS* will be active and able to stimulate downstream pathways which are important in cell growth, differentiation and cell survival (16,17,18). Mutations that convert *KRAS* protooncogenes to oncogenes are typically point mutations causing amino acid changes, particularly at positions 12, 13, and 61. They make the protein resistant to GTP hydrolysis by GTPases, thereby leading to constitutive *KRAS* activity. Somatic Mutations of this gene lead to conformational changes in the *KRAS* protein, which leads to an independent activation of the downstream signaling transduction system, stimulating proliferation, inhibiting apoptosis, and regulating various growth and survival signals (19,20). These mutations are located near to the GTP binding site (21). *KRAS* gene mutations are more frequent in gastric cancer patients infected with *H .pylori* than in cancer-free patients, suggesting that *KRAS* mutation may be involved in the early stage of gastric carcinogenesis (22). This study aims to find the effect of *H. pylori* on the occurrence of *KRAS* mutations in codons 12 and 13 in exon 2.

Materials and Methods:

Samples Collection:

A total of 36 formalin fixed paraffin embedded tissues (FFPE) of gastric cancer patients, in addition to 15 FFPE samples of patients with peptic ulcer as control group for genetic mutations that histopathologically proved free of malignancy, FFPE of patients was attending to the Gastroenterology and Hepatology Diseases Center Teaching / Baghdad between January 2014, to December, 2015 were collected with their clinical and pathological data. All these cases subjected to molecular oncological test for detection The *KRAS* gene mutations in codons 12 and 13. Gastric cancer FFPE samples grouped in two subsets according to the presence of *H. Pylori* , FFPE positive for *H. pylori* and FFPE negative for *H . pylori*. Specialized consultant histopathologist examined the H & E sections of the FFPE blocks samples to determine the definite presence of these bacteria.

DNA extraction:

FFPE sectioned by microtome, serial tissue were sectioned in to 10 µm and 8 sections for each FFPE blocks . Genomic DNA extracted from these sections by using QIAamp DNA FFPE Tissue kit (German) according to the manufacturer's instructions. Gene was detected by using polymerase chain reaction. The DNA quality was evaluated by amplification of a 115-bp fragment of *KRAS* gene, the gene was PCR amplified in each sample using the following primers sequences:

Forward 5' CCTGCTGAAAATGACTGAAT 3'
Reverse 5' TGTTGGATCATATTCGTCCA 3'

All oligonucleotides used in the study were synthesized by Alpha DNA Company (Canada). The *KRAS* gene was amplified using Veriti Thermal Cycler (USA) , genomic DNA was amplified in a final volume of 25 µl (4 µl Genomic DNA , 0.6µl forward primer ,0.6 µl reverse primer , 12.5 µl green master mix (Promega,USA) , 7.3 µl Nuclease free water) using the following conditions: Denaturation at 95°C- 3 minutes, followed by 40 cycles of (denaturation at 95°C -1 minute , annealing at 55°C-1 minute , and extension at 72°C-1 minute) and a final extension was at 72°C for 5 min , then hold at 4°C and End .

Then the amplification products were separated by electrophoresis through 2.5 % agarose gel stained with Ethidium bromide .

Sequence Analysis:

The PCR products of the analyzed *KRAS* gene region were sent to Macrogen Company (U.S.A) for sequencing. The sequencing data was analyzed using the National Center for Biotechnology Information (NCBI) site and Bioedit system.

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 was used to significant compare between means in this study .

Results and Discussion:

Thirty-six FFPE samples of gastric cancer patients enrolled in the molecular study in addition to fifteen FFPE samples of gastric ulcer patients. Out of the 36 patients, 20 (55.6%) were positive to *H. pylori* infection (HIP-GC) and 16(44.4%) were negative to *H. pylori* infection (HIN-GC), and the difference statically was significant ($p< 0.05$)(Table-1). The ratios of gastric cancer patients in both groups of gastric cancer in the current study were similar to the ratios that obtained by (22). It were (56.4%) and (43.5%) for *H . pylori* positive and negative infection gastric cancer groups respectively , while in another study by (23), the ratio of the positive *H. pylori* infection gastric cancer was higher (68.5%) .

the mean age in the *H. pylori* infected positive- gastric cancer (HIP-GC) was 56.60 years and the age mean in the *H. pylori* infected negative-gastric cancer (HIN-GC) was 58.87 years, the difference was not statistically significant .

Depending on gender there is a high percent of male in comparison with female percent it was 25/36 (69.5%) , 11/36 (30.5%) respectively (Table-1), with respect to gastric cancer incidence disease , which is constant with (24), which stated that the ratio of men to women is about 2:1 , The reasons for

such differences are not clear. Environmental or occupation exposures may play a role. Men have historically tended to smoke more than women (25), while estrogens may protect women against the development of gastric cancer , delayed menopause and increased fertility may also lower the risk of gastric cancer in women (26 , 27) . In a previous Iraqi study by (28) , also found that males are affected more than females ,and this finding reflects the results of high exposure to carcinogens in male including smoking, dietary habits and probably higher incidence of *H. pylori* .

Out of 36 patients samples, 14/20 (70%) were male in the HIP-GC group and 11/16 (68.75%) in the HIN-GC group, while 6/20 (30%) were female in the HIP-GC group and 5/16 (31.25%) in the HIN-GC group. Likewise, the difference was not statistically significant between male and female in both groups Table (1).

The results mentioned in Table (2) represents that gastric cancer patients showed different clinicohistopathological characteristics which involves, type of gastric cancer (intestinal and diffuse type), and differentiation grade (well, moderate and poor differentiation adenocarcinoma). It was found that 23 / 36 (64%) of tumors were intestinal type and low percent 13/36 (36%) were diffuse type and the results also showing that the male is the predominant in both tumor types**.** These findings agree with (8), which stated that the predominant type of gastric cancer is the intestinal and this tumor type shows a male predominance (male to female ratio of 2:1).

Table (1): Number of gastric cancer patients according to the gender in both groups of gastric cancer

Gender	HIP-GC	HIN-GC	Total	p - value	Chi-square (χ^2)
Male	14 (70.00%)	11 (68.75%)	25 (67.5%)	0.561	0.673 NS
Female	6 (30.00%)	5 (31.25 %)	11 (30.5%)	0.561	0.673 NS
Total	20(55.61)	16 (44.49)	--	--	--
Chi-square (χ^2)	10.75 **	10.83 **	--	---	---

**** (P<0.01), NS: Non-significant.**

Table (2): Characteristics of gastric cancer patients in the *H. pylori* infection and non-infection groups.

Parameters	HIP-GC	HIN-GC	Total	p - value	Chi-square (χ^2)
Type					
Intestinal	12 (60.00%)	11 (68.75%)	23(64%)	0.044	4.516 *
Diffuse	8 (40.00%)	5 (31.25 %)	13(36%)	0.044	4.516 *
Chi-square (χ^2)	7.25 **	10.83 **	---	---	---
Differentiation grad					
Poorly	10 (50.00%)	5 (31.25%)	15(41.7)	0.0129	8.291 **
Moderately	9 (45.00%)	7 (43.75)	16(44.4)	0.564	0.671 NS
Well	1 (5.00%)	4 (25.00%)	5(13.9)	0.013	8.418 **
Chi-square (χ^2)	11.72 **	7.16 **	36	---	---

***(P<0.05), ** (P<0.01), NS: Non-significant.**

Statistically , significant differences (p<0.05) were seen in the ratio of the intestinal type (68.75%) in the HIN-GC group when compared with HIN-GC

group (60.%) .in contras , the ratio of the diffuse type in the HIP-GC group (40%) was higher than the HIN-GC group (31.25%) ,which is statically significant (Table-1) .

With regard to the differentiation grade, in all gastric cancer patients, it was found that (41.7%) were poor differentiated, (44.4%) were moderately differentiate, while 31.9% were well differentiated in low percent. In the HIP-GC group, (50%) were poor differentiated , which is significantly higher ($p < 0.01$) than the HIN-GC group(31.25%) , while the well differentiated in HIN-GC group (25%) was significantly higher than the HIP-GC group (5%) , and the difference was not statistically significant in the moderately differentiated in both groups (45% in the HIP-GC group vs. 43.75% in the HIN-GC group)(Table-2) . The poorly differentiated was the more frequent ,followed by the moderately differentiated , then the well differentiated in HIP-GC group which is differ than the HIN-GC group in which the more frequent was the moderately differentiated , followed by the poorly differentiated ,then the well differentiated .

With respect to gastric ulcer group in this study, which is consisted of 15 FFPE samples, this group was composed of 8/15 (53.3%) male and 7/15 (46.7%) female, and the mean age was 47.93 years and ranged between (21 - 63) years. The gastric ulcer in this group were occur due to *H . pylori* infection , and it used as control disease group .

The PCR amplified regions of codons 12 and 13 of *KRAS* gene in gastric cancer patients and ulcer patients showed a molecular weight of about 115 bp as seen in Figures (1, 1A, 1B).

Homo sapiens Kirsten rat sarcoma viral oncogene homolog (*KRAS*), RefSeqGene on chromosome 12:

NCBI Reference Sequence: NG_007524.1
10527
CCTGCTGAAAATGACTGAATATAAA
CTTGTGGTAGTTGGAGCTGGTGGCGT
AGGCAAGAGTGCCTTGACGATACAG
CTAATTCAGAATCATTTTGTGGACGA
ATATGATCCAACA 10641
(115 bp region from base 10527 to 10641 for exon 2).

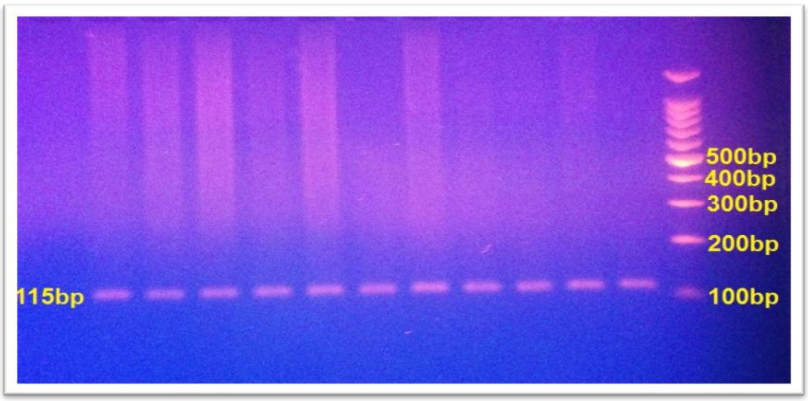

Figure (1): The exon 2 amplified area according to the NCBI KRASRefseq gene NG_007524.1 .
Figure (1A): Gel electrophoresis of codons 12 and 13 of *KRAS* PCR products for gastric cancer patients on 2.5 % agarose at 75volt for 90 minutes stained with Ethidium Bromide (0.5 µg\ml) and visualized under U. V. light . ladder (100 –1000bp).

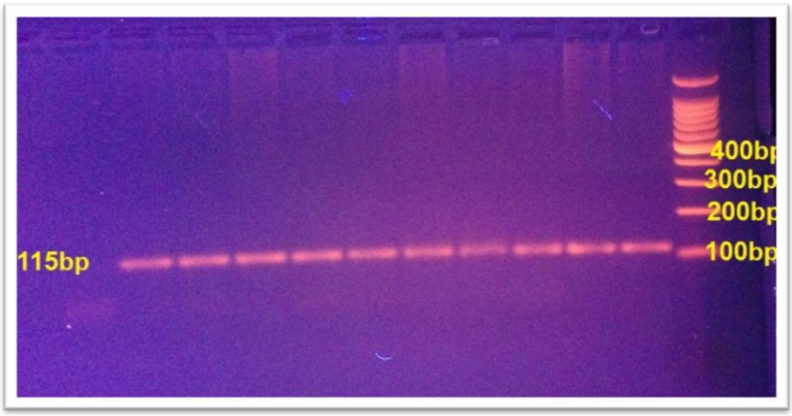

Figure (1B): Gel electrophoresis of codons 12and 13 of *KRAS* PCR products for gastric ulcer patients on 2.5 % agarose at 75volt for 90 minutes stained with ethidium bromide (0.5 µg\ml)and visualized under U. V. light . ladder (100 –1000bp).

The gastric cancer and ulcer patient's PCR products of the *KRAS* gene screened for mutations by sequencing. The sequencing results compared to human reference *KRAS* gene sequences (http: NCBI Reference Sequence). The results revealed that no mutations in codons 12 or13 detected in all patient groups, but the sequencing analysis detected other mutations in exon 2. These mutations were found in 7 /20 (35%) patients of HIP-GC group, 5/16(31.25%) patients of HIN-GC group and 4/15(26.7%) patients of the ulcer group. There were 25 deletions detected in all patient groups, 10/25 (40%) of them were in the HIP-GC group, 7/25(28%) in the HIN-GC group and 8/25(32%) in the ulcer group (Table-3).

The mutations detected in Exon 2 include two types of deletions, Figures (2 and 3). First type g. 10583 del A in codon 16 was found in 4/10 (40%) patients in the HIP-GC group, 5/7 (71.4%) patients in the HIN-GC group, and 4/8 (50%) patients in the ulcer group. The second type g. 10591 del A in codon 19 was found in 6/10 (60%) patients in the HIP-GC group, 2/7 (28.5%) patients in the HIN-GC group, and 4/8 (30.77%) patients in the ulcer group Table (3).

Table (3): Types of *KRAS* mutations in exon 2 that detected in HIP-GC , HIN-GC and ulcer groups.

***KRAS* mutations**	**Accession No. NG_007524.1 From 10527 to 10641**			**HIP-GC**	**HIN-GC**	**ulcer**	**Total**
AAG to A - G	g.10583 delA	Framshift	Chang all the amino acids chain	4(40%)	5 (71.4%)	4(50%)	**13(52%)**
AAC to - AC	g.10591 delA	Framshift	Chang all the amino acids chain	6(60%)	2(28.5%)	4(50%)	**12(48%)**
Total				10(40%)	7(28%)	8(32%)	**25**

```
Query  44     GCTGG-GGCGT-GGCA-GAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGAC  100
              ||||| ||||| |||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  10567  GCTGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGAC  10626
```

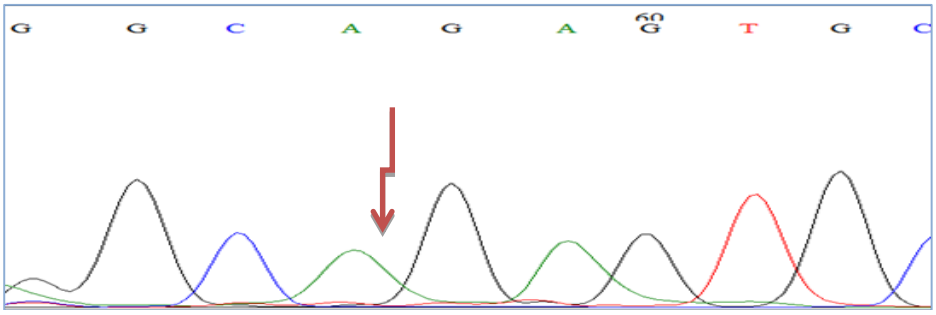

Figure (2): Site g. 10583 del A, nucleotide sequence (Forward) in *KRAS*, Exon 2, codon16.

```
Query  40     CTGT-TCGTCA-GGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAGTTTATATTC  97
              ||||  ||||||  ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10602  CTGTATCGTCAAGGCACTCTTGCCTACGCCACCAGCTCCAACTACCACAAGTTTATATTC  10543
```

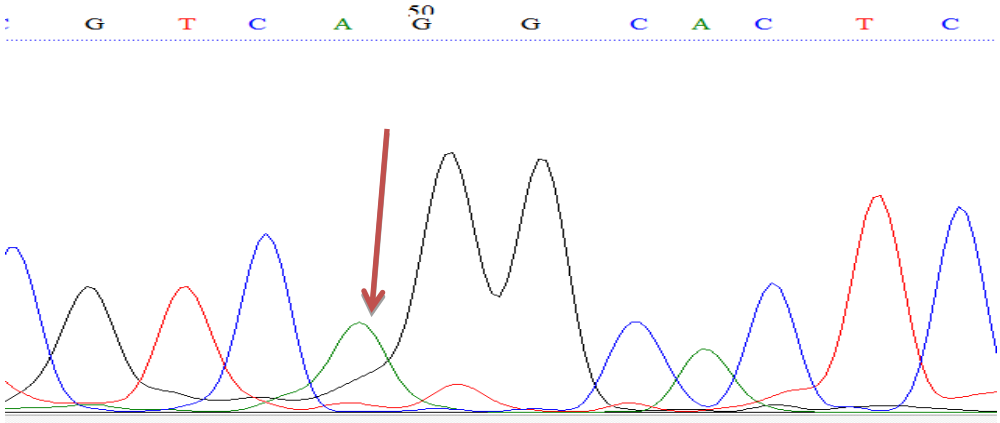

Figure (3): Site g.10591 del A , nucleotide sequence (Reverse) in *KRAS* , Exon 2 , codon 19.

Moreover, the results reveled that nine patients were with compound mutations, three patients in the HIP-GC group, two in the HIN-GC group (13(52%), and four in ulcer group. The more frequent mutation was (g. 10583 del A) followed by (g. 10591 del A)(12) (48%) Table (4).

Table (4): Frequency of mutations detected in all patients groups

Mutations	Frequency
g. 10583 del A	13(52 %)
g. 10591 del A	12 (48 %)
total	25(100%)

According to Table (5), the first type of deletion (g.10583 del A) was higher in the HIN-GC group and the difference was significant ($p < 0.05$) ,while the second type (g.10591 del A) was higher in the HIP-GC group and the differences were highly significant ($p < 0.01$). From the results of the second mutation (g.10591 del A) ,we could notice that there is an effect of *H*

.pylori in the increasing the frequency of this mutations rather than the other mutation (g.10583 del A) which means this type of mutation has a correlation with *H. pylori* infection , we expected that the results will be more clearly when the samples numbers increased.

Table (5): Comparison between KRAS mutations in HIP-GC vs. HIN-GC

Mutations	HIP-GC n=20	HIN-GC n=16	p -value	Chi-square (χ^2)
g.10583 del A	4 (20%)	5 (31.25%)	0.0427	4.365 *
g.10591 del A	6 (30%)	2 (12.5%)	0.0142	6.216 **

*** (P<0.05), ** (P<0.01).**

According to Table(6), the occurrence of *KRAS* mutations was not differ between the HIP-GC group and gastric ulcer group for the first deletion , and likewise the difference statistically was non-significant .while the second type of deletion (g.10591 del A) was slightly higher in the HIP-GC group rather than the ulcer group ($p < 0.05$). The results indicated that the first mutation (g.10591 del A) was more frequent in gastric cancer rather than gastric ulcer which means it has a critical role in the development of gastric ulcers and gastric cancer in the presence of *H. pylori.*

Since the bacteria play a role in the pathogenesis of both ulcer and stomach cancer may be that is the reason why there were no significant differences between the two groups with respect to frequency of the first mutation.

Table (6): Comparison between KRAS mutations in HIP-GC vs. Gastric ulcer

Exon	Mutations	HIP-GC n=20	Gastric ulcer n=15	p -value	Chi-square (χ^2)
2	g.10583 del A	4 (20%)	4(26.7%)	0.683	0.944 NS
	g.10591 del A	6 (30%)	4(26.7%)	0.0462	5.026 *

*** (P<0.05), ** (P<0.01), NS: Non-significant.**

According to Table (7), the first deletion (g.10583 del A) was also higher in the HIN-GC group rather than the ulcer group suggesting that *H .pylori* cannot be considered to have an effect on this type of deletion , however the difference was non-significant . The second mutation (g.10591 del A) was higher in the ulcer group rather than the HIN-GC group which leads to the belief that *H. pylori* have a noteworthy effect on the increase the recurrence of this mutation ,the difference was significant ($p< 0.05$). These findings indicated that the second mutation has a correlation with gastric ulcer development more than gastric cancer in the absence of bacteria. While the last two mutations (g.28545 del A and g.28597 del A) .

Table (7): Comparison between KRAS mutations in HIN-GC vs. Gastric ulcer

Mutations	HIN-GC n=16	Gastric ulcer n=15	p -value	Chi-square (χ^2)
g.10583 del A	5 (31.25%)	4(26.7%)	0.702	0.983 NS
g.10591 del A	2 (12.5%)	4(26.7%)	0.0424	4.936 *

*** (P<0.05), NS: Non-significant.**

With regard to Table (8), there were slightly increase in the ratio of the mutations in the ulcer group compared with gastric cancer group, but these differences were non-significant. These mutations may be having a role in the transformation of ulcer to precancerous lesion so maybe that is the reason why there were no significant differences between the two groups.

Table (8): Comparison between KRAS mutations in Gastric cancer vs. Gastric ulcer

Mutations	Gastric cancer n=36	Gastric ulcer	p -value	Chi-square (χ^2)
g.10583 del A	9 (25%)	4(26.7%)	0.526	0.088 NS
g.10591 del A	8 (22.2%)	4(26.7%)	0.855	0.872 NS

NS: Non-significant.

Through the combined effects of *H. pylori* virulence factors and inflammatory mediators released in response to infection, ROS levels increase in the host epithelial cells and lead to modulation of nucleic acid bases leading to DNA damage (29). Oxidative damage caused by *H. pylori* infection can lead to point mutation in *KRAS* gene which is involved in gastric carcinogenesis (30).

In the current study, we analyzed *KRAS* mutations and compared these mutations between two patients groups of gastric cancer (HIP-GC and HIN-GC) and with patients with gastric ulcer disease, which considered as disease control group. Idyllically, we should take healthy controls instead of disease controls but practically that was not possible because healthy individuals cannot be subjected to endoscopy.

Furthermore, regarding to the type of all *KRAS* mutant gastric cancer investigated in the present study, (66.7%) and (33.3%) were intestinal and diffuse type respectively Figure (4), which is in agreement with previous study that found that almost two : third of *KRAS* mutant gastric cancer were intestinal type gastric cancer (31 , 32).

In the HIP-GC group, the type of the *KRAS* mutant gastric cancer were 5/7 (71.42%) while in the HIN-GC group were 3/5(60%) and the diffuse type were 2/7 (28.6%) in the HIP-GC group while 2/5 (40%) in the HIN-GC group.

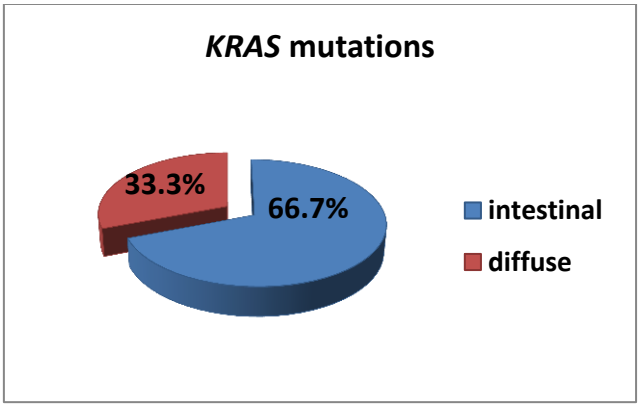

Figure (4): Types of *KRAS* mutant gastric cancer

Hiyama *et al.* (21) were stated that the genetic mechanism of carcinogenesis differs between the differentiated type and the undifferentiated type of gastric cancer and that *KRAS* mutations may be involved in the early stages of gastric carcinogenesis of the differentiated type. Several abnormalities in cellular oncogenes affecting the expression or function of controlling cell growth and differentiation are considered the main cause of cancer.

About 20% of tumors have activating point mutations in *RAS*, the highest incidence was found in KRAS (about 85 %), then NRAS (about 15%), and the lowest incidence was found in *HRAS* (less than 1%) (33). The incidence of mutated *KRAS* genes varies strongly among the different tumor types. *KRAS* point mutations are found predominantly in adenocarcinomas and the highest incidence is found in pancreas adenocarcinoma which harbor a mutated *KRAS* in approximately (90%) of tumors (22). The molecular events in the pathogenesis of gastric cancer are mostly unknown ,variable incidence of *KRAS* mutation in gastric cancer has been reported , Many studies have reported a low incidence of *KRAS* mutations in gastric cancers (21).

Van Grieken *et al.* (32) reported that the frequency of *KRAS* mutations in gastric cancer was (1.5%–5.8%), while in another studies, the frequency of *KRAS* mutations in gastric cancer were about (7–20%) (34, 35), whereas, studies on gastric carcinomas of Asian patients have shown that *RAS* gene mutations can occur in up to (35%) of cases (36). The frequency of *KRAS* mutations in gastric cancer patients of both groups in our study was 12/36 (33.3%) .

Some investigators found no evidence for mutation in codon 12 of *KRAS* gene in gastric cancer which are consistent with the finding in the present study (22).

A previous study found that , the age did not affect the *KRAS* mutation frequency , suggesting that *KRAS* mutation does not play a role in patient age (37) which is in agreement with the finding in the present study which showed that *KRAS* mutations represents in different ages in gastric cancer patients and their ages was ranged between (40 - 95) years .

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