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The Immunohistochemically Estimation of CD63 in Iraqi Patients with Gastric Cancer

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Abstract:

CD63 is -one of the tetraspanin family proteins, which are regarded as: hallmark exosomal markers because it is absent from other types of vesicles. It is expressed in the cell membrane of cancer cells, and cytoplasm of stromal cells. Objective: To assess CD63 expression in gastric cancer (GC) patients, and detected if it could be used as a predictive marker. Furthermore, the current study aimed to find the correlation between CD63 expression and clinicopathological parameters as: gender, age, invasion depth, histopathological type, involvement of lymph nodes, grade and stages of GC (TNM). The current study is a retrospective study in the period time from (2018 to-2020); 50 randomly patients formalin-fixed paraffin embedded blocks (FFPE) of stomach tissue (10 cases normal tissue without GC as control, and 40 patients with GC) with its reports and diagnosis were collected from Pathology Department of the Gastroenterology and Hepatology Teaching Hospital and some private hospitals. The histological sections were stained by hematoxylin and eosin stain (H&E), and immunohistochemistry (IHC) stain for CD63. Statistical analysis accomplished by SPSS system at ($P \leq 0.05$). This study indicated that there were significant differences between control group, and patients group in the expression of CD63, also there was a significant correlation between CD63 expression, and histopathological subtype, invasion depth, involvement of lymph node, and stages in patients, whereas there was a non- significant association between the age, grade, and gender of patients, and the expression of CD63.This result indicates that CD63 could be a good prospective marker in Iraqi cancer patients.

Keywords: CD63, Clinicopathological parameters, FFPE, Gastric cancer, Immunohistochemistry.

Introduction:

Gastric carcinoma (GC) comprises a universal health issue. It is a disease with high aggressive and heterogeneous nature. It is one of the most prevalent reasons of cancer related death and takes advantage of an important encumbrance on international health sponsor [1]. In Iraq malignant neoplasm's represented the second leading cause of death. Gastric cancer trend demonstrated an instant rise after 2007; it is the fifth of eighteenth cancers in Iraq [2]. GC is the third main reason of cancer-related mortality in the world, leading round about 783,000 deaths in 2018, and over 1,000,000 new cases of gastric carcinoma per year[3]. The Lauren classification is the most communal classification of GC. It includes three main subtypes: intestinal, diffuse, and mix[4] which they differ in many properties, such as: clinical characterize, genetics, morphology, epidemiology and development features [5]. Exosome is an extracellular vesicle excreted mostly by many eukaryotic cells. It can be utilized for tumor patients as a prognostic marker and/or grading basis. It is contributed in intercellular communication. It has an important function in controlling plentiful processes during cancer progression such as: tumor growth, metastasis, and angiogenesis, because of it contains proteins, DNA, mRNA, circular RNA, microRNA, long noncoding RNA, etc. [6]. Moreover, it is an

intermediate signal transduction incident that has a main role in the cellular processes regulation such as adhesion motility and differentiation. Exosomes may have a major possibility to use as biomarkers for the premature diagnosis, the prediction of prognosis, and the valuation of the treatment impact in gastric cancer [7]. CD63 which is also called lysosome-associated membrane glycoprotein 3, melanoma-associated antigen ME491 or melanoma-associated antigen MLA1 is a glycoprotein cell surface [8], and one of the tetraspanin family proteins (TM4SF), which is regarded as: hallmark exosome marker because it is absent from other types of vesicles [9], and extremely reinforced on the membranes of intraluminal vesicle (ILV) [10,11] has been correlated with human tumor progression in non-small cell lung cancer, prostate cancer, breast cancer, astrocytomas, pancreatic cancer, and melanoma [12,13]. CD63 magnitude in gastric cancer patients has not been elucidated. Cancer cells and stromal cells exosomes derived could be a major role in the intracellular communications include in the development of cancers [14]. Objective of the current study: To estimate CD63 expression in Iraqi patients with gastric cancer, and detected if it could be used as a predictive marker. Furthermore, the current study aimed to find the correlation between CD63expression, and clinicopathological parameters as: gender, age, invasion depth, histopathological type, involvement of lymph nodes, grade, and stages of GC.

Materials and Methods:

Samples collection

The current research is a retrospective study from January 2018 to December 2020. The total numbers of samples were 50 cases. Forty samples of gastric cancer patients formalin-fixed paraffin embedded blocks (FFPE) have been obtained randomly from surgically resected specimens in Gastroenterology and hepatology Teaching Hospital, Medical City/ Baghdad/Iraq with its reports (no chemotherapy received by these patients) after getting the official agreement from Iraqi Ministry of Health, and Department of Medical City/ Baghdad/ Iraq. Each report contains clinic-pathological parameters (age, gender, histopathological type of tumor, grade of tumor, depth invasion of tumor, lymph nodes involvement, tumor stage/ TNM), which were diagnosed by pathological doctors of hospital. Ten samples of normal stomach tissue have been selected randomly from patients undergo (Sleeve gastrectomy) by private hospitals. These patients' cases were classified depending on Lauren classification [4].

Staining of immunohistochemistry (IHC) & hematoxylin and eosin (H&E)

Each FFPE were cut in 5 µm in thickness before staining, mounted in positive charged slides for IHC, and normal slides for H&E stained. Some of cutting sections for control, and patients groups were stained by routine staining sequent steps depending on Suvarna *et al*. [15]. CD63-antibody (Recombinant Rabbit Monoclonal antibody) (Code No. SY21-02; Dilution1/100; Thermo Fisher, USA) was used for IHC, and protocol of the manufacture company applied to accomplish this staining. Sections were incubated in serum blocking solution, and then slides were incubated with primary antibody (CD63), then with biotinylated link secondary antibody (Abcam, USA). After that slides were incubated with streptavidin-enzyme conjugate (Thermo Fisher, USA), then with substrate-chromogen (DAB) mixture (Abcam, USA). Finally, slides stained with hematoxylin, and mounted with aqueous mounting solution. After each of these steps slides were excess washed with PBS buffer.

Negative and positive control

The positive control of this antibody is lung tissue cancer as in Thermo Fisher Company, in which the cell membrane of these cells was visualized by chromogenic stain (brown color) (Fig.1, B), whereas a negative control is a section from lung tissue cancer without adding primary antibody, so cell membrane was not stained with brown color (Fig.1A). The staining of lung tissue cancer slides was accomplished by using Thermo Fisher company protocol.

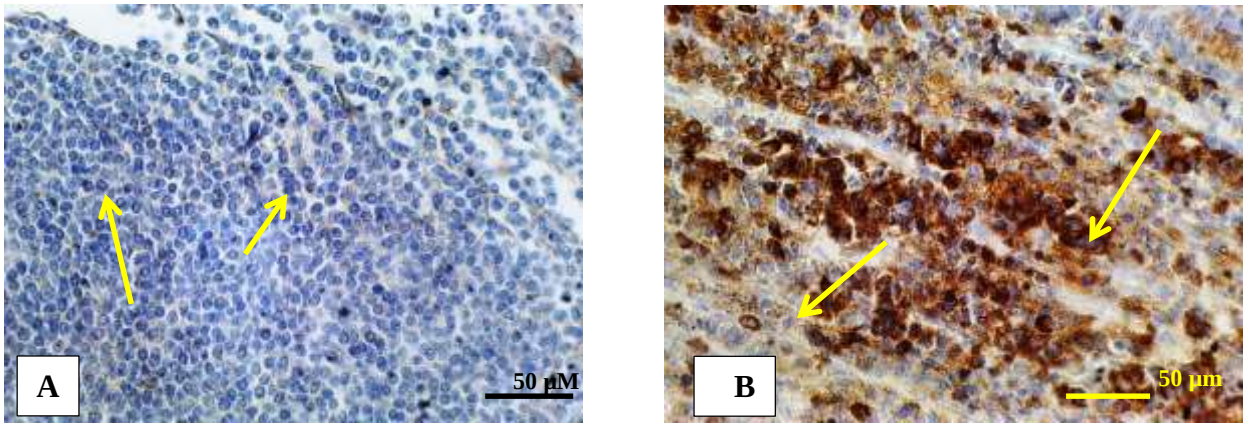

Figure 1. Cross section in lung cancer tissue, IHC, (A. negative control, membranes of cells were not stained in brown color, yellow arrow), (B. positive control, membranes of cells stained in brown color, yellow arrow), scale bar 50 µm, 40X.

Scoring system

Colored cancer cells were calculated by selecting four areas, and total selected cancer cells were not less than 100 cells, at magnification power (10X and 40X). Colored cancer cells were calculated in hall slide, and cells with brown color in cell membrane consider being (positive), and cancer cells that were not colored in cell membrane being (negative). The percentage of colored cancer cells were divided as: Stained cells% (score 0: 0%, score +1: 10%, score +2: 20-30%, score +3: 40-100%) [14].

Statistical analysis

Collected data analysis was accomplished by (statistical package for social science/SPSS version - 24 software/ IBM): Chi square (X2) used to estimate the correlation between clinic-pathological correlations with expressions of the marker, and compered with control group. P-values valuable at the accepted level of significance in ≤ 0.05 was considered significant.

Results:

The results of 50 cases for this study referred to the fact that males' total numbers in control group were 2 (20%) statuses, while females were 8 (80%) statuses, with male: female (M: F) ratio 1:4 (the majority of statuses from females). In patients with gastric cancer group, the number of males was 24 (60%) cases, while females number was 16 (40%) cases, and 3:2 in (M: F) ratio (the majority cases from males). Age was divided into two age groups: equal or less than 50 years, and more than 50 years. Control group age ranged between (19-50) years for 10 (100%) statuses, which were within the age group equal or less than 50 years with mean age (33.9 ± 10.027), and the age of patients with gastric cancer which ranged from (19-83) years with mean (55.325 ± 15.423). Patients number of equal or less than 50 years was 16 (40%) cases with mean (39.875 ± 9.493), and the number of patients in more than 50 years was 24 (60%) cases with mean age (65.625 ± 8.234). Histopathological subtype distributed in: intestinal type: patients' numbers were recorded 23 (57.5%) cases. Diffuse type: 15 (37.5%) cases of the total number of patients, whereas mixed type: patients were registered 2 (5%) cases. The most cases for grade were moderately differentiated, which were registered 25 (62.5%) cases, while poorly differentiated were 15 (37.5%) cases of the total number of patients. Also, the current study showed that gastric cancer invasion (subserosa) found in about 15 (37.5%) cases. In invasion (serosa) total patients were 18 (45%) cases, while (muscularis propria) invasion were 7 (17.5%) cases in total patients. The total number of patients that diagnosed with lymph node involvement was about 32 (80%) cases, whereas patients without lymph node involvement total number was 8 (20%) cases. Invasive depth and lymph node metastasis (TNM) were used to distribute gastric cancer patients into advanced stages (III & IV), and early stage (II). Advanced stages (III & IV) total patient numbers were 27 (67.5%) cases. The total number of patients was about 12 (30%) cases in stage III, whereas IV stage total patient number was 15 (37.5%) cases. The total number of patients in early stage (II) was 13 (32.5%) Tab. 1

Table 1. Study groups distribution depends on clinicopathological parameters

Clinicopathological Parameters	Findings		Frequency (%)
Gender	Control	Male	2 (20%)
		Female	8 (80%)
	Patients	Male	24 (60%)
		Female	16 (40%)
Age	≤ 50	Control	10 (100%) with mean (33.9±10.027)
		Patients	16 (40%) with mean (39.875±9.493)
	> 50	Control	–
		Patients	24 (60%) with mean (65.625±8.234)
Histopathological types	Patients	Intestinal	23 (57.5%)
		Diffuse	15 (37.5%)
		Mix	2 (5%)
Grade of tumor	Patients	Moderately	25 (62.5%)
		Poorly	15 (37.5%)
Invasion depth of tumor	Patients	pT2	7 (17.5%)
		pT3& pT4	33 (82.5%)
Involvement of lymph node	Patients	N0	8 (20%)
		N1,2&3	32 (80%)
Stage of GC (TNM)	Patients	II	13 (32.5%)
		III&IV	27 (67.5%)

The IHC expression of CD63

This study shows that CD63 expressed in high level in patients with gastric cancer only reached 40 (100%), whereas control groups were not expressed in any status for this marker. In patients with gastric cancer (score 0) was not expressed CD63 in any case, while number of patients expressed CD63 at (score +1) were 16 (40%) cases, 10 (25%) cases in (score +2), and 14 (35%) cases at (score +3). This difference in expression level between two groups leads to statistically significant association at P ≤ 0.05, P= 0.0001 as shown in Tab.2, and (Figs.2, 3, 4, 5).

Table 2. The expression of (CD63) in control and patients with gastric cancer.

Expression of CD36		Patients with gastric cancer	Control	P- value
Negative	Score 0	0 (0%)	10 (100%)	P=0.0001*
Positive	Score +1	16 (40%)	0 (0%)	
	Score +2	10 (25%)	0 (0%)	
	Score +3	14 (35%)	0 (0%)	
Total number/ number (ratio)		40 (100%)	10 (100%)	

*Chi square P-value is significant (P ≤ 0.05)

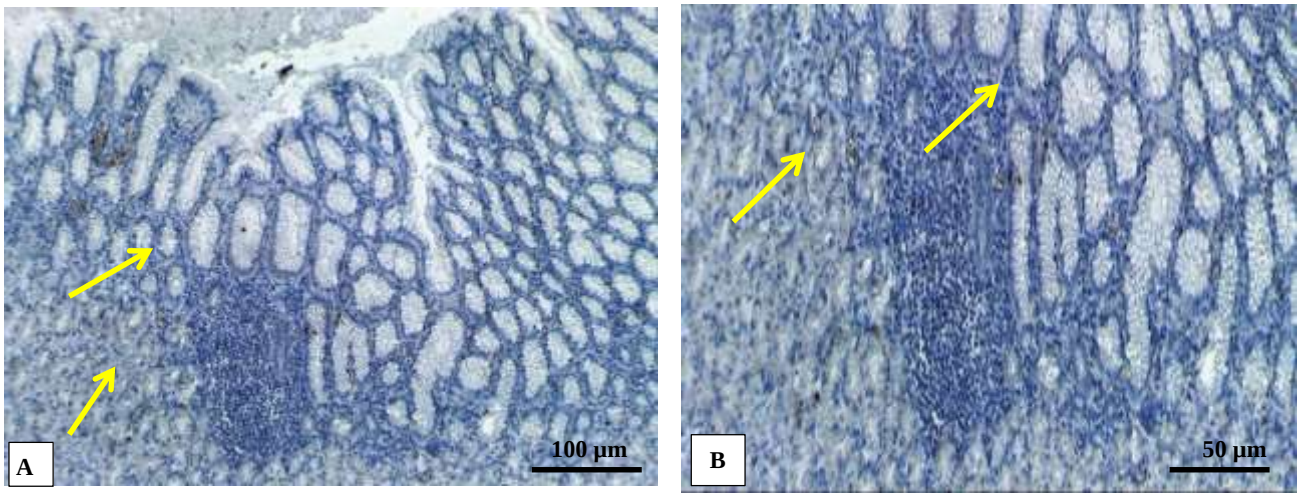

Figure 2. Cross section of control stomach tissue illustrated the negative expression of (CD63) at score 0, no cell membrane stained in brown color (arrow), IHC, (A) scale bar 100 µm, 10X, (B) scale bar 50 µm, 40X.

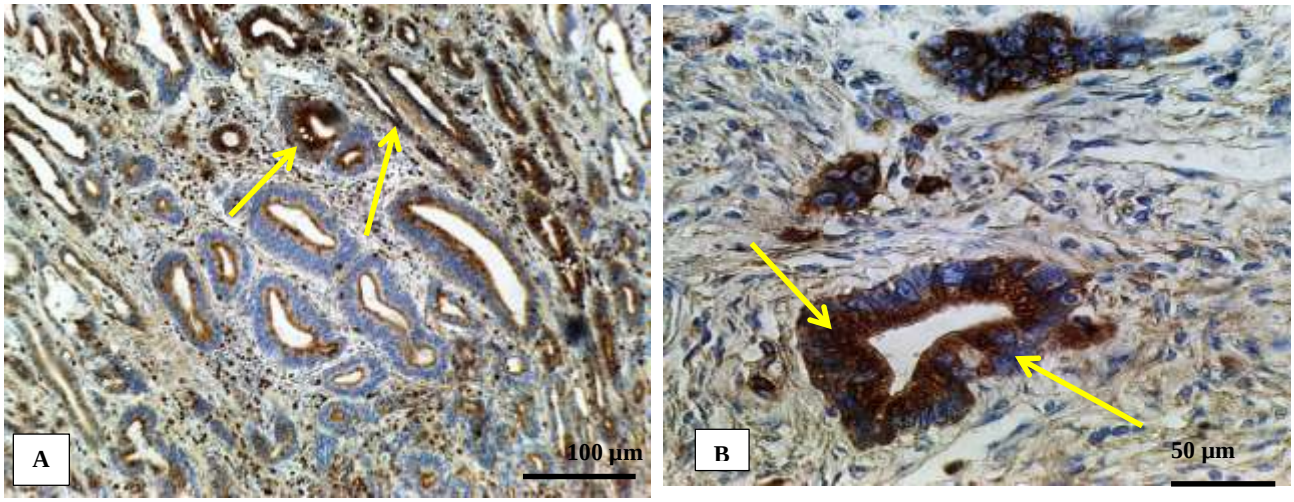

Figure 3. Cross section in gastric cancer tissue, intestinal subtype, moderately differentiated in human stomach wall illustrated gastric cancer tissue positive expression of (CD63) (arrow) at Score +1 (10% of cell membrane stained in brown color), weak stain, IHC, with (A) scale bar 100µm, 10X, (B) scale bar 50µm, 40X.

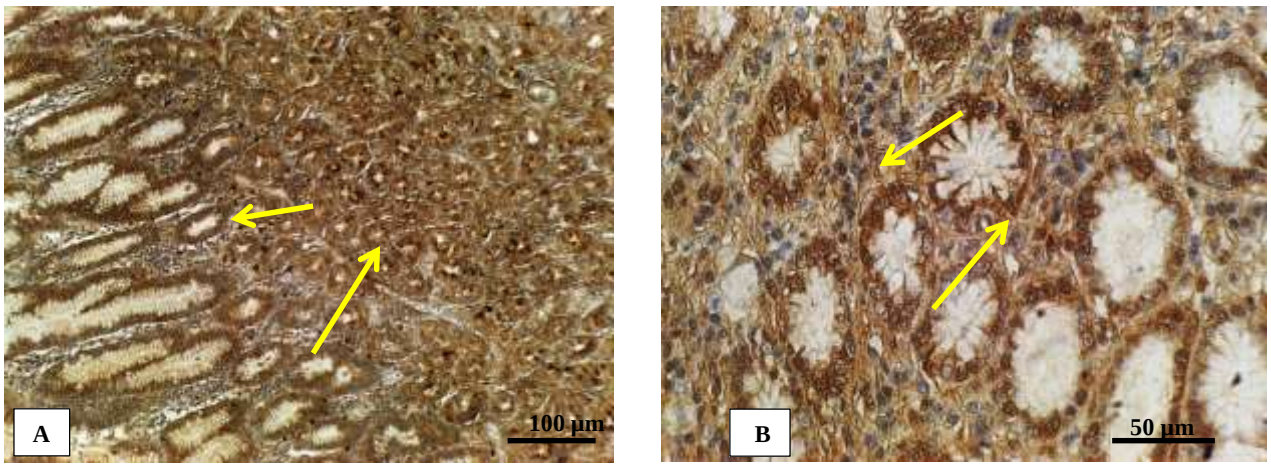

Figure 4. Cross section in gastric cancer tissue, intestinal subtype, moderately differentiated in human stomach wall illustrated gastric cancer tissue positive expression of (CD63) (arrow) at Score +2 (20-30% of cell membrane stained in brown color), moderate stain, IHC, with (A) scale bar 100µm, 10X, (B) scale bar 50µm, 40X.

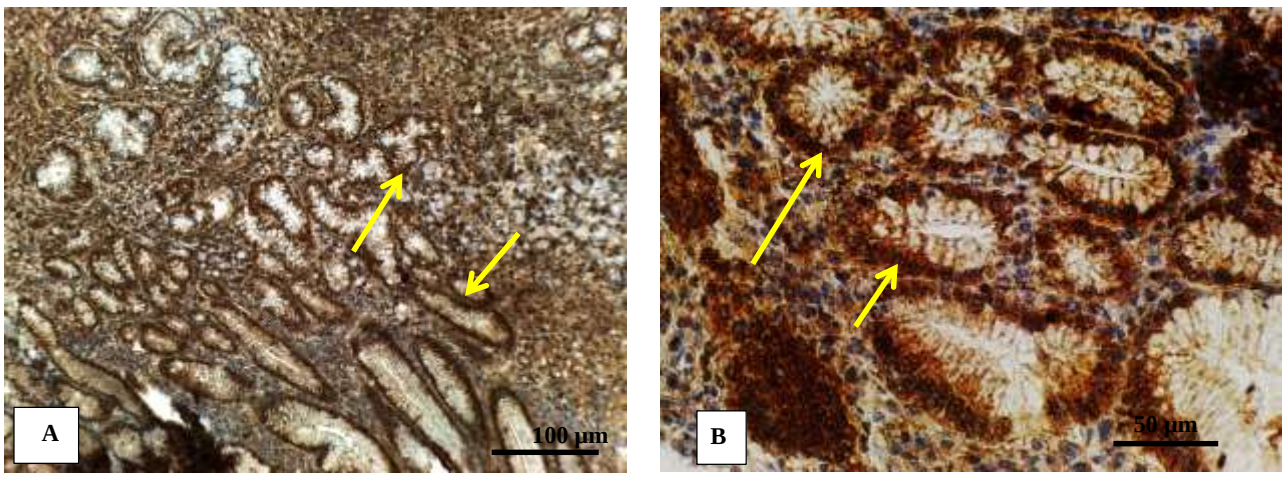

Figure 5. Cross section in gastric cancer tissue, intestinal subtype, moderately differentiated in human stomach wall illustrated gastric cancer tissue positive expression of (CD63) (arrow) at Score +3 (40-100% of cell membrane stained in brown color), strong stain, IHC, with (A) scale bar 100µm, 10X, (B) scale bar 50µm, 40 X.

Tab.3 shows the expression of CD63 correlated with clinic-pathological parameters. This study donates that 60% of total numbers in gastric cancer patients were positively expressed CD63 in males, which was recorded in 24 cases, and (40%) 16 cases for female, statistically, no significant association between gender and the expression of CD63, P- value were larger than 0.05 (P > 0.05). Patients' age ranged from 19 to 83 years, patients from less or equal 50 years were recorded (40%) in expression of CD63, and 60% in patients more than 50 years. P-value = 0.579 larger than 0.05 (P > 0.05) so there was not a significant association between age and the expression of this marker. The current study showed that the total number of patients with intestinal type that positively expressed CD63 were 57.5%, diffuse were 37.5%, and 5% in mixed type. These differences made a significant association statistically at (P ≤ 0.05) P= 0.041. There was an expression of CD63 in patients with gastric cancer at moderately differentiated grade 62.5%, more than in poorly differentiated grade, which recorded 37.5% but there was not a significant correlation (p=0.164). The positive expression of CD63of invasion depth recorded 15% in pT2, and 85% in pT (3 &4), a significant association statistically at P ≤ 0.05, p=0.025. Also, the study indicated that there was 80% of CD63 positive expression in patients involving lymph nodes (N1, N2&N3), whereas in patients without involvement of lymph nodes (N0) patients were 20%. Statistically, these differences made a significant association between involvement of lymph nodes and the expression of this marker, p=0.0001. The highest records of marker positive expression were in advanced stage (III&IV) of gastric cancer in 67.5%, while it was 32.5% in early stage. Statistically, these differences made a significant association between stage of gastric cancer, and the expression of marker at p ≤ 0.05, p= 0.0005.

Table 3. Show the expression of CD63 correlated with clinicopathological parameters

Clinicopathological parameters		Expression of CD63		P-value
		Negative Number (ratio)	**Positive Number (ratio)**	
Gender	Male	0 (0%)	24 (60%)	
	Female	0 (0%)	16 (40%)	P= 0.310*
Age	≤ 50	0 (0%)	16 (40%)	P= 0.579*
	> 50	0 (0%)	24 (60%)	
Histopathological subtype	Intestinal	0 (0%)	23 (57.5%)	P= 0.041*
	Diffuse	0 (0%)	15 (37.5%)	
	Mix	0 (0%)	2 (5%)	
Grade of tumor	Moderately	0 (0%)	25 (62.5%)	P= 0.164*
	Poorly	0 (0%)	15 (37.5%)	
Invasion depth of GC	pT 2	0 (0%)	6 (15%)	P= 0.025*
	pT3 & pT4	0 (0%)	34 (85%)	
Involvement of lymph node	N0	0 (0%)	8 (20%)	P= 0.0001*
	N1,2,&3	0 (0%)	32 (80%)	
Stage of GC (TNM)	II	0 (0%)	13 (32.5%)	P= 0.0005*
	III&IV	0 (0%)	27 (67.5%)	

*Chi square P-value is significant (P ≤ 0.05)

Discussion:

In Iraq malignant neoplasm's represented the second leading cause of death. GC trend demonstrated an instant rise after 2007; it is a fifth of eighteenth cancers in Iraq [2]. GC comprises a universal health issue. It is a malignancy disease with high aggressive nature. It is one of the most prevalent reasons of cancer related death, and takes advantage of an important encumbrance on international health sponsor [1]. Gastric cancer treatment is restricted because of its heterogeneity and genetic complicated [16] so finding of special biomarkers is important for management of the development of gastric cancer, and identified the effective treatments for patients [17]. The current study indicated that the total number of male more than female in about (1.5:1) in (M: F) ratio (the majority cases from males) and the number of older patients (more than 50 years) more than younger (equal or less than 50 years), The result of the current study is agrees with several previous Iraqi studies which indicated that the males are the most gastric cancer incidence than females in about (2.3:1) as in Hermize *et al.*, [18], while the study of Razak *et al.*, [19] declared that the males' higher percentage than females in about 58%, 42% for males, and females respectively. Also, the study of Saeed, [20] which found that males' ratio is more than females, and in another review to the same researcher found that males recorded double in ration (2:1) [21]. Furthermore, the result of this study is corresponded with the different Arab countries

studies: as in Bahrain study, which found that gastric cancer in males recorded a high incidence compared with female about doubles (2:1) [22]. In study from Yamen there was a difference in the incidence in gastric cancer between genders in about (2.5:1) (M: F) [23]. Globally, the results of this study matched with the study of Lou *et al.* [24], which is showed that men are higher incidence than women, and the study of Li *et al.*, [25] that founded that males recorded a significant higher incidence than female in (number of patients, stages, and grade) of gastric cancer. Moreover, as in the Globocan 2012 report, standardized rates of age of gastric cancer was twice more in men than in women [26]. The study of Radkiewicz *et al.* [27] demonstrated that rise of risk for gastric cancer is associated with male sex, the portion of cancer explicated by factors correlated to male sex is huge, and males suffer poorer survival in most cancer sites. A probable exposition is either the preventative impact of estrogen in women, or other effects as diversity in diet and occupational exposure may participate to raise gastric cancer incidence in males [28, 29]. This study indicated that the higher ratio of gastric cancer incidence at ages more than 50 years in ratio 60% with mean 65.625, which matched with several Iraqi studies as the study of Hermiz *et al*., [18], which mentioned that the ratio of gastric cancer incidence increases with age, and the most diagnosis cases were up to 50 years in age ranged from 30-80 years. In study of Lafta and Al-faisal, [30] the two researchers mentioned that the elderly people are the most incidence exposure to gastric cancer than younger. In Saeed's *et al*., [21], they mentioned that the gastric cancer incidence raised with age in age 50 years and more, compared with patients less than 50 years, and in mean age about 59 years. Furthermore, this study is corresponded to many Arab countries, which found that gastric cancer incidence increases with age. From Egypt the study of Zeeneldin *et al*., [31] mentioned that the mean age in gastric cancer patients was (54.1±12.3) year in age ranged from (21-82) year. In study of Alahmadi *et al*., [32] in Saudi Arabia indicated that the mean age of diagnosis patients was (57.55±19.24) year, while in Yamen the study of Kassim *et al*., [23] found that the mean age of patients was (64.98±15.15) in age ranged from (25-100) year. Moreover, the current study is matches the global studies as the study of Kim *et al*., [33], that recommended that endoscopy series in Korea is important each two years for everyone at age 40 years and up. Gastric adenocarcinoma displays remarkable age diversity, and tends to be repeatedly diagnosed in elderly with mean age 68 years in the USA; and above 95% of all new cases are diagnosed in patients up of 40 years [34]. The gastric cancer incidence rate increases gradually with age; 70 years is the median age to the diagnosis of this disease. However, about 10% of gastric cancer is exposed at the age of 45, or younger. Carcinogenesis is a multistage disease operation particular by the advanced development of mutations, and epigenetic changing in the expression of different genes, which are accountable for the appearance of this disease [5]. The Lauren classification is the most common classification of gastric cancer in Iraqi, Arabic, and global studies. Depending on this division, there are two subtypes of this disease that can display: intestinal, and diffuse. The current study indicated that the common subtype was intestinal subtype in about 57.5%, as shown in Tab.1. This result corresponded with several Iraqi studies. In Lafta and Al-faisal, [30] study, they mention that the intestinal subtype is the general of the most cases with gastric cancer in about 71%, while diffuse was recorded less than 28%. Also, with the Al-obiadie *et al*., [35] study, which indicated that 60% of cases were intestinal subtype. Furthermore, in study by Mohammed and Raziq, [36], a study of 65 cases in Duhok city found that 64.6% of cases were intestinal subtype. Many studies mention that the intestinal type is the general type with rising the risk of adenocarcinoma, in age range (55-80) years, commonly appear in male more than female in ratio (2:1) [37, 38]. Also, in [39] author reminded that (54%) of cases were intestinal subtype, which are located in distal stomach (non- cardiac), and it is the highest ratio compared with diffuse, and mix type. This study corresponded with the most Arabic studies, as in Saudi Arabia study by Alahmadi *et al*., [32], which indicated that 91% of the cases were intestinal subtype, and with Egyptian study by Badary *et al*., [40], which mentioned that cases distributed as follows: intestinal subtype was the common subtype in ration 59.5%, diffuse 21.4%, and mixed 19%. Also, with the study from Yamen by Kassim *et al*., [23], the total number of cases was 130 cases; about 82.5% of these cases were intestinal subtype. The results of this study disagree with some Iraqi studies as Al-Kaptan, [41], which indicated that the most diagnosis cases were diffuse subtype in about 63% among other subtypes. Also, the current study does not match with some Arabic studies, such as the study of Awad *et al.* [42] in Jordin, which refers to that about 52% of cases were diffuse subtype. Globally, this study does not corresponded to a study from Vietnam by Phan *et al.* [43] study, which mentioned diffuse subtype occurs in 52% of study cases, and with a study in Brazil by Braga-Neto *et al*., [44], which declared that diffuse is appearing in

younger patients in about 70%, comparing with elderly intestinal subtype that recorded about 33.7%. The construction of these differences in results is that these two subtypes of gastric cancer develop out of various mechanisms, the intestinal subtype is more correlated to environmental factors as dietary factors, lifestyle, and H. *pylori* infection; the diffuse subtype is more related to genetic factors [19]. Also, these differences in results may be due to environment variation, occupational, and geographical differences, ethics, differences in the size, and type of the samples: some of researchers depends on biopsies, and other on surgical excision samples. The current study referred that the moderately differentiated was the common differentiated grade among diagnostic cases in about 62.5%. This result matches with several previous studies in Iraq. In study of Abdulla *et al*., [45], which indicated that the moderately differentiated is the general differentiated grade among other grades. Also, with the study of Abdul Jabar and Al-Faisal, [46], they mentioned that about 44.4% of diagnosis cases were moderately differentiated. In study of Ashour *et al*., [47], this indicated that about 63.3% of cases were moderately differentiated. Moreover, this study matches with Mohammed and Raziq, [36], which declared that about 52.38% of diagnosis cases were moderately differentiated grade. Furthermore, the current study also agrees with some Arabic, and global studies, as in some Egyptian studies, which refers that about 61.8% was the moderately differentiated grade ratio of diagnosis cases, while poorly differentiated grade ratio about 27.6% (Abdel-Salam *et al.* [48], and Harras and Mowafy [49] study, which indicated that moderately differentiated grade was the common grade among other differentiated grades in about 52%, and 36% for poorly differentiated. In a study from Mexican found that moderately differentiated grades are the common grade comparing with other differentiated grades in ration 55.5%, and 27.7% for poorly differentiated grades [50]. In Indian study by Raj *et al*., [51] demonstrated that in ratio 46% the moderately differentiated were the common differentiated grades among all patients cases.

This study does not correspond with some previous Iraqi studies such as: Lafta and Al-Faisal, [30]study mentioned that about 80% of cases were poorly differentiated. Also, the study does not match with some Arabic, and global studies, in Saudi study by Alahmadi *et al*., [32], which refers to that about 50% of cases were poorly differentiated grade, and in study from Tunis by Gharsall *et al*., [52], which mentioned that about 47.83% of cases were poorly differentiated. Also, the study of Zhang *et al*. [53], which found that poorly differentiated was the domain differentiated. The result of this study refers that advanced gastric cancer (third and fourth) were higher than early stage (second) in about 67.5%, which match with several previous Iraqi studies. In Al-Kaptan, [41] study found that about 75% of diagnosis cases were in advanced stage. In Hermize *et al*., [18] study, which indicated that third advanced stage was the higher than early stage in about 93%. Also, in Ashour *et al*., [47], which declared that the advanced stage (third and fourth) were the highest comparing with early stage (second) in ratio 53.3%. Furthermore, the study of Mohammed and Raziq, [37], which found that 66.15% cases were in advanced stage. The current study is in agreement with some Arabic studies. A study by Alahmadi *et al*., [32] from Saudi Arabia, which refers to the fact that advanced stage, was higher than early stage in about 59.1%, 40.9% respectively. Globally, the result of this study is corresponded with several previous studies. The study of Braga-Neto *et al*., [44], which mentioned that advanced gastric cancer was in high ratio in about 60.9%, comparing with early stage in about 39.1%. The study of Feng *et al*., [54], declared that advanced stage was raised in ratio 70.1%. Also, the Li *et al*., [55] study that indicated about 50.3% of diagnosis cases were in advanced stage of gastric cancer.

The current study is not in agreement with some previous Arabic studies, which demonstrated that early stage of gastric cancer was the general stage comparing with advanced. In Abdel-Salam *et al*., [48] study from Egypt refers to that the ratio of early stage was about 56.6%, and advanced in 43.4%.

Furthermore, when comparing some global studies, the result of this study is not corresponded with it, as a study by Katai *et al*., [56], which mentioned that early stage was the common stage comparing with advanced in ratio 58.7%, while about 41.3% for advanced. Also, a study by Silva *et al*., [57] which refers to that early stage recorded in about 54.2%, while advanced 45.8%. Gastric cancer is a disease that is often times revealed delayed, at a stage when medical treatment is difficult to achieve. The identification and demarcation of early pre-cancer harms more defy than in the esophagus, and colon because of the numerous number of folds, known as rugal folds, that increase surface area of the stomach [58]. GC starts without any apparent signs of symptoms until advanced stages; therefore, it is not easy to diagnose at early stage without routine screening by endoscopy. It is a heterogeneous disease from histological, and molecular estimation, also the diversity, and complexity of this disease is not enough evident understand of molecular mechanisms including in tumorigenesis, tumor

progression, and metastasis of its [59]. This can explain the high ratio of advanced stage comparing with early stage of gastric cancer. Furthermore, the causes of differences in results may be due to partly result by screening, mechanisms, plans that depending on diagnosis patients. Also, using advanced techniques in development countries such as: computed tomography (CT), which can detect early small lesions. Moreover, the differences in the type of samples, and the number of studying samples. Tab.3 showed the expression of CD63 correlated with clinicopathological parameters, which indicated that there was not significant association in positive expression of the marker, and age, gender, and grade, but significant in invasion depth of tumor, and lymph node involvement; these foundlings resemble the results study of Miki *et al*. [14]. CD63 have a major role in niche formation metastasis [60]; this study mentioned that this marker is a tissue inhibitor of metalloproteinase-1(TIMP1) receptor, which generates a tumor niche in a small environment of the liver, causing in metastasis of pancreas cancer cells in liver. Knockout of TIMP1 or CD63 in mice was not consistent liver metastasis by cancer cells of pancreas injected. This leads to infer that TIMP1 (pancreatic cancer cells) use CD63 receptor to activate stellate cells (liver), which intermediate the formation of niche to create metastasis. Furthermore, CD63, and TIMP1 coexpression have been recorded in glioblastoma, and astrocytoma patients [61]. Moreover, CD63 has also been involved in coordination with the roles of the tumor development-associated protein, membrane-associated type-1 MMP in extracellular matrix transformation, which leads to rise cell invasion, and metastasis [62]; this can explain the significant correlation between positive expression of CD63, and invasion depth of GC, involvement of lymph node, and stage of GC (TNM) of the current study. The interaction between CD63, and numerous other proteins as CD9, CD81, and β1 integrins participating to the downstream of the signaling pathway of the cell [63]. Seubert *et al* [64] remind that this marker rise the intrinsic metastatic predisposition of tumor cell by initiating β-catenin-dependent in the epithelial- mesenchymal transition, that influence on the plasticity of the cell in ovary cancer, gastric cancer in human, and mouse melanoma cells. Furthermore, as in study of Mohammed *et al*., [65], which mention that Fumonisin B1 (FB1) (a mycotoxin produced in some grains specially in corn) by *Fusarium* species has a significant effect on TGF-β1 and p16 protein expression, so its role in cancer development is proposed. Moreover, a decrease in E-cadherin together with raised Vimentin, MMP-2 and MMP-9 are significant markers that association with poor prognosis of transitional cell carcinoma TCC, also increase in men comparing to women in age up 50 years [66].

Conclusions:

The results of the currents study indicated that CD63 could be a good prospective marker for Iraqi patients with gastric cancer. Cancer cells exosomes include many contents as: (proteins, DNA, mRNA, miRNA, lncRNA, and circRNA). Some of them act as biomarkers; we can take these features for cancer: early detection, early diagnosis, prognosis prediction, and therapeutic efficacy evaluation, and develop new strategies relying on engineered exosomes carrying with tumor-suppressing proteins, nucleic acid components, or targeted drugs function as precision medicine.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Authors sign on ethical consideration's approval.
- Ethical Clearance: the project was approved by the local ethical committee in Baghdad University, with No 3035

Authors' contributions statement:

Kifah Hamdan Abdul Ghafour conceived of the presented idea, and diagnosis the expression of CD63 in patients' gastric cancer cases. Leith Abdul Hussein Abdullah contributed to sample collection. Amal Khudair Abbas supervised the project. Raghad Khalid Mwafaq carried out the experiment, wrote the manuscript, and performed the statistical analysis. All authors discussed the results and contributed to the final manuscript.

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التقييم المناعي الكيميائي النسيجي للـ CD63 في مرضى سرطان المعدة في العراق

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الخلاصة:

يتميز CD63بأنه أحد افراد عائلة التتراسبانين البروتينية، إذ يعد سمة مميزة للحويصلات الخارجية كونه ينعدم وجوده في الانواع الاخرى من الحويصلات. يتم التعبير عنه في الاغشية الخلوية للخلايا السرطانية، فضلا عن سايتوبلازم الخلايا الحشوية (بين الخلوية). تهدف الدراسة الحالية إلى تقييم التعبير الإيجابي لـ CD63 في مرضى سرطان المعدة. وما إذا كان بالامكان استخدامه كدلالة تنبؤية للمرض. علاوة على ذلك، ايجاد العلاقة بين تعبير CD63, والمؤشرات السريرية المرضية مثل: الجنس، والعمر للمرضى، وعمق الغزو الورمي, والأنواع النسجية المختلفة للورم، واصابة العقد الليمفاوية، ودرجة تمايز الورم، ومراحل الورم (TNM). تم اجراء هذه الدراسة بأثر رجعي من الفترة الزمنية (2018-2020), على 50 مريضاً تم اختيارهم عشوائياً 10 عينات كمجموعة سيطرة, و 40 عينة لمرضى سرطان المعدة) مع التقاريرالطبية والتشخيصية من مختبر علم الأمراض في مستشفى أمراض الكبد والجهاز الهضمي التعليمي, في مدينة الطب، ومن بعض المستشفيات الخاصة.تم تصبيغ المقاطع النسجية لشريحتين من كل قالب بارافيني احدها H&E والثانية بـ CD63. أما التحليل الإحصائي فقد تم انجازه بواسطة نظام SPSS عند ($P \leq 0.05$). وجدت فروق معنوية ذات دلالة إحصائية بين مجموعة السيطرة، ومجموعة المرضى في التعبير الإيجابي لـ CD63، كما كان هناك ارتباط معنوي بين تعبير CD63، والأنواع النسجية للورم، وعمق الغزو الورمي، واصابة العقد الليمفاوية، والمراحل المختلفة للورم في المرضى، بينما لم يكن هناك اي فروق ذات دلالة احصائية بين عمر، وجنس المرضى، ودرجة التمايز، والتعبير الإيجابي لـCD63. تشير هذه النتائج إلى أن CD63 يمكن أن يستخدم كدلالة تنبؤية جيدة لمرضى العراق المصابين يسرطان المعدة.

الكلمات المفتاحية: CD63، المؤشرات السريرية المرضية، FFPE، سرطان المعدة، مناعة كيميائية نسجيه.