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Validity of novel techniques (modified tissue ELISA and IHC) in correlation and comparison for detection of patients with gastric cancer using cut off values of check point inhibitor (PD-1 & PD-L1)

Hind H. Al-Ammiri
BVM&S, MSC, Immunology, Microbiology department, collage of Vet. Medicine, Baghdad University
Corresponding author email: Dr.hindhamid@gmail.com

Mohammed M. Mohammed
MBChB, MSc, FICMs/Path, Microbiology department, collage of Medicine, Baghdad University

Ameer D. Hameedi
MBChB, FICMS/ Path, Microbiology department, collage of Vet. Medicine, Baghdad University

Nawal M. Alkhalidi
MBChM CABm, FiCMS GE and H, Microbiology department, collage of Medicine, Baghdad University

***Abstract*---**Background: One of the most common malignancies involving the gastrointestinal tract is gastric cancer. It is categorized as an aggressive disease with poor treatment outcome, as most patients remain undetected until later stages, a multifactorial disease involving both genetic and environmental factors in etiology; a wide variance in the occurrence of gastric cancer in various geographical regions (Abdi-Rad et al., 2006, Abuderman, 2019). Checkpoint inhibitor therapy is revolutionizing the oncology disease management process. despite the fact that gastric cancer is a common malignancy with a poor prognosis, relatively little attention has been drawn and attracted to the treatment and diagnosis of this disease by checkpoint inhibitor therapy (Kang YK, 2017). Objectives: The aims of this study To compare between (a modified ELISA method) and immunohistochemistry (IHC) for those immunological markers PD-1,

PD-L1 as a best rapid, sensitive and specific diagnostic methods in patients with Gastric Cancer. Patients and methods: Thirty gastric cancer patients were included, they were among patients who attending the Histopathology department -GIT hospital and Histopathology department- Teaching laboratories/ medical city teaching complex Baghdad / Ministry of Health, during the period from August 2020 to April 2021. Another 30 patients diagnosed with benign tumor, In addition, 30 apparently healthy person were chosen as a healthy control group. For these three groups, PD-1, PD-L1, using tissue ELISA and IHC technique was carried out. Results: Correlation analysis of check point inhibitors (PD-1 & PD-L1) by ELISA and IHC staining; the study investigated the correlation between their mean concentration in ELISA values and IHC status of expression in matched samples based on ELISA /IHC assay, however the means values of check point inhibitors (PD-1 & PD-L1) by ELISA (133.413±53.126) (151.175±47.641) respectively is highly rather than that in IHC means (45.0±18.3) (47.9±29.9) respectively in gastric cancer groups. For the relation between the ELISA and the final scores in IHC the result give strong point to their association with p-values (0.0001) for each high and low expression of final score PD-1and PD-L1 on gastric cancer using IHC in comparison with ELISA. Conclusions: In conclusion, our study explored the clinical significance of PD-1, PD-L1 in gastric cancer and also, the study found that modified tissue ELISA, was more correlated with gastric cancer disease progression than IHC. In addition, this study discovered for the first time PD-1, PD-L1 values in modified tissue ELISA concentration is strong relation p-values (0.0001) with gastric cancer for each high and low expression of PD-1and PD-L1 of final score on gastric cancer using IHC.

***Keywords*---**checkpoint inhibitors (PD-1, PD-L1), gastric cancer, validity.

Introduction

Gastric cancers considered the most fatal malignancies worldwide and have a wide pathological and biological variety (Werner, M., et. al., 2001). The proteins appearance profile of gastric cancers is essential to provide therapeutic targets on individual basis and diagnostic molecular markers for gastric cancer (Kim B., et al., 2003). PD-1 is an immune checkpoint and two pathways guard in cancer Firstly, it promotes apoptosis of T-cells and Secondly, in regulatory T cells, apoptosis decreases (Francisco et al., 2010, Fife BT, 2011). Programmed death-ligand 1 (PD-L1) is a protein that is encoded in humans by the CD274 gene, also known as (CD274) differentiation cluster 274 or B7 homolog 1 (Kalim et al., 2020).To modulate activation or inhibition, PD-L1 binds to its receptor. Said et al. showed that PD-1, up-regulated on activated CD4 T-cells, can bind to monocyte-expressed PD-L1 and eventually induces the production of IL-10 that inhibit T-cells proliferation and activation (Said et al., 2010).

Materials and methods

Subjects
Patients study group

Thirty gastric cancer patients with age range from (24-75) years with Mean±SD (57.3±12.4) were included in this study, another 30 patients with age range from (20-73) years with Mean±SD (53.3±14.6) diagnosed with benign tumor, these patients were diagnosed clinically, histopathologically and immunohistochemistry by specialists, they were among patients who attending the Histopathology department -GIT hospital and Histopathology department- Teaching laboratories/ medical city teaching complex Baghdad / Ministry of Health, during the period from August 2020 to April 2021. Ethical permission to conduct the research was obtained from this center. Thirty apparently healthy individuals, with age range from (8-76) years with Mean±SD (47.7±17.9) who have no clinical evidence of malignant diseases, were chosen. For these three groups, PD-1, PD-L1, using each tissue ELISA and IHC techniques was carried out.

Kits and reagents: Human Programmed Death 1 (PD-1) ELISA Kit and Human Programmed Death Ligand-1 (PD-L1/CD274) ELISA Kit.

Primary antibody for IHC technique

1. Monoclonal antibodies: Mouse monoclonal Anti-PD-L1 antibody [PDL1/2764]
2. Polyclonal antibodies: Rabbit polyclonal Anti-PDCD10/CCM3 antibody

Expose mouse and rabbit specific HRP/DAB detection IHC kit which include

Secondary antibody

Goat anti –rabbit HRP Conjugate: 15 ml of Goat anti-rabbit Conjugate with HRP.

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-27 (Statistical Packages for Social Sciences- version 27).

Results

Assessment of check point inhibitor levels in comparison among these values in each ELISA and IHC Techniques

Correlation analysis of check point inhibitors (PD-1 & PD-L1) by ELISA and IHC staining; the study investigated the correlation between their mean concentration in ELISA values and IHC status of expression in matched samples based on ELISA /IHC assay. the means values of check point inhibitors (PD-1 & PD-L1) by ELISA (133.413±53.126) (151.175±47.641) respectively is highly rather than that in IHC means (45.0±18.3) (47.9±29.9) respectively in gastric cancer groups (table

1 and 2). These results explored the clinical significance of PD-1, PD-L1 in gastric cancer. The study found that ELISA not IHC, was correlated with gastric cancer disease progression. This result agree with Li et al., 2019 who reported that One explanation might be that the expression of PD-1, PD-L1 in tumor sample is heterogeneous (Li et al., 2019). In addition, further large-sampled studies exploring the relationship between them.

Table 1: The ranges and mean values of PD-1 and PDL-1 in tissues of gastric cancer patients, benign tumor group and control healthy group

	Malignant (n=30)	Benign (n=30)	Healthy control (n=30)	P value
PD-1	133.413±53.126 (40.531-227.395)	29.905±12.634 (0.744-43.053)	21.775±12.489 (1.419-39.950)	0.0001^
PDL-1	151.175±47.641 (49.278-232.667)	72.565±9.945 (60.001-94.285)	82.102±12.642 (54.090-100.823)	0.0001^

-Data were presented as Mean±SD (Range)
#Significant difference between two independent means using Students-t-test at 0.05 level.
^Significant difference among more than two independent means using ANOVA-test at 0.05 level.

Table 2 : The ranges and mean values of PD-1, PDL-1 in tissues of gastric cancer patients, benign tumor group and control healthy group

	Malignant	Benign	Healthy control	P value
PD-1 percentage	45.0±18.3 (20-80)	27.7±27.1 (5-80)	26.7±14.4 (10-50)	0.046^
PDL-1 percentage	47.9±29.9 (2-80)	21.2±19.3 (5-60)	31.2±15.5 (20-50)	0.011^

-Data were presented as Mean±SD (Range)
#Significant difference between two independent means using Students-t-test at 0.05 level.
^Significant difference among more than two independent means using ANOVA-test at 0.05 level.

The relation between PD-1,PDL-1 tissue ELISA values in these three groups' gastric cancer patients, benign tumor group and control healthy group according to (PD-1, PDL-1) final score values

Regarding the relation between PD-1 mean concentration in tissue and final PD-1 score in gastric cancer group, a higher level was notice in final score 3 (209.38±) and increasing with advancing scores (126, 131, 134, 144 respectively), but it did not reach the level of significance statistically (P=0.682) . The mean concentration of PD-1 of patients with malignant gastric tumor (209.383±) was higher than those with benign tumor (32.63±.) of Score 3 with (P=0.0001); There was a statistically significant positive (P=0.0001) between mean concentration of PD-1 values in each gastric cancer high expression (2-12) (141.21±) incomparable to benign groups (23.93±) and healthy control group (30.46±) (table 3).

Table 3. The relation between PD-1 tissue ELISA values in these three groups' gastric cancer patients, benign tumor group and control healthy group according to (PD-1, PDL-1) final score values

		PD-1						
		Malignant		Benign		Healthy control		P value
		No	Mean±SD	No	Mean±SD	No	Mean±SD	
Detailed Final PD-1 score	0	16	126.59±49.41	20	31.95±13.33	18	21.36±14.26	0.0001^
	1	-	-	4	28.65±3.63	9	19.71±9.99	0.116
	2	4	131.48±52.35	-	-	-	-	-
	3	1	209.38±	4	32.63±.43	-	-	0.0001#
	4	6	134.62±74.16	2	6.54±0.28	3	30.46±0.26	0.035^
	6	3	144.66±35.98					-
	P value		0.682		0.045^		0.439	
Final H&L PD-1 expression	High expression (2-12)	14	141.21±57.93	6	23.93±13.48	3	30.46±0.26	0.0001^
	Low expression (≤1)	16	126.59±49.41	24	31.40±12.25	27	20.81±12.82	0.0001^
	P value		0.462		0.201		0.211	
#Significant difference between two independent means using Students-t-test at 0.05 level.								
^Significant difference among more than two independent means using ANOVA-test at 0.05 level.								

Regarding the relation between PDL-1 mean concentration in tissue and final PDL-1 score in gastric cancer group, a higher level was notice in final score 4 (205.19±.), but it did not reach the level of significance statistically (P=0.233) (Table 4). There was a statistically significant positive (P=0.0001) between mean concentration of PDL-1 values in each gastric cancer high expression (2-12) (153.05±) incomparable to benign groups (70.50±) and healthy control group (93.69±). There was a statistically significant positive (P=0.0001) between mean concentration of PDL-1 values in each gastric cancer low expression (≤1) (147.42±) incomparable to benign groups (73.32±) and healthy control group (80.81±).

Table 4. The relation between PDL-1 tissue ELISA values in these three groups' gastric cancer patients, benign tumor group and control healthy group according to (PD-1, PDL-1) final score values

		PDL-1						
		Malignant		Benign		Healthy control		P value
		No	Mean±SD	No	Mean±SD	No	Mean±SD	
Detailed Final PDL-1score	0	7	159.88±51.36	20	74.23±11.57	22	79.30±12.87	0.0001^
	1	3	118.34±59.93	2	64.22±0.28	5	87.45±10.33	0.248
	2	6	150.85±56.00	6	68.80±2.39	-	-	0.005#
	3	1	197.92±	-	-	-	-	-
	4	2	205.19±.14	-	-	3	93.69±2.23	0.0001#
	6	5	116.79±24.53	2	75.59±0.28	-	-	0.075
	9	6	160.61±36.04	-	-	-	-	-
	P value		0.233		0.407		0.103	

Final H& L PDL-1expression	High expression (2-12)	20	153.05±45.29	8	70.50±3.74	3	93.69±2.23	0.0001^
	Low expression (≤1)	10	147.42±54.40	22	73.32±11.39	27	80.81±12.67	0.0001^
	P value		0.766		0.502		0.094	
#Significant difference between two independent means using Students-t-test at 0.05 level.								
^Significant difference among more than two independent means using ANOVA-test at 0.05 level.								

Discussion

Earlier studies have reported that PD-L1 is expressed in 65% of gastric cancer. Furthermore, PD-L1 expression in tumor cells has been reported in 14–24% of patients with gastro-oesophageal cancer. There are several ongoing studies in phase 1–3 with various combinations and different settings against PD-L1 positive gastric cancer (Junttila, A., et. al., 2020). Perhaps the most prevailing strategy to evaluate Tumor microenvironment involve IHC detection for markers like PD-L1, Biomarkers, in gastric cancer patients include PD-L1 high expression, who may benefit from therapies and could be used as prognostic biomarkers in patients with gastric cancer (Rong, X., et. al., 2021) (Zhang, C.,et. al., 2020).

High concentrations of check point inhibitor were detected in patients with gastric cancer, seemingly indicative of significant pro-tumorigenic immunosuppression (Sundar, R., et. al., 2020). Interactions between PD-L1 and PD-1 are thought to maintain peripheral tolerance. PD-L1/PD-1 has been shown to mediate T-cell exhaustion, an important mechanism underlying T-cell dysfunction (Kim PS and Ahmed R. 2010) (Zheng, Z.,et. al., 2014). These present results suggest that (PD-1, PD-L1) which can be easily measured in clinical practice, may be an important prognostic factor in gastric cancer and useful for identifying a subgroup of patients with high risk for recurrence or progression. These results suggest a role of (PD-1, PD-L1) in gastric cancer pathogenesis and offer new insights into potential therapeutic strategies.

In the present study compared semi-quantitatively scored IHC determinations with quantitative ELISA measurements. For the relation between the ELISA and the final scores in IHC the result give strong association with p-values (0.0001) for each high and low expression of final score PD-1 on gastric cancer using IHC in comparison with ELISA. The same think appear in PD-L1 with p-values (0.0001) for each high and low expression respectively of PD-1 on gastric cancer using IHC in comparison with ELISA. These results were in agreement with Ferrier, C.M., et. al., 1999 who reported that; although a higher IHC score category was always associated with an increased mean ELISA value, there was an overlap of ELISA values from different scoring classes. Hence, for the individual tumor cases the relation between ELISA and IHC is ambiguous (Ferrier, C. M., et. al., 1999). In conclusion, these data indicates that the novel tissue ELISA model is a promising tool for prognosis and clinical decision-making in multiple types of advanced gastric cancer.

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