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Assessment of CD276 and HER2 Immunohistochemical Expression in Bladder Carcinoma: A Comparison Between Pathologist Interpretation and Aperio Digital Software

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ABSTRACT

Background:
Urinary bladder cancer (UBC) is the most common urinary tract malignancy, ranking tenth worldwide. HER2 is a member of a family of epidermal growth factor receptors, which are transmembrane receptor tyrosine kinases involved in cell proliferation, survival. The B7-H3 (also known as CD276), a member of the B7 family of immune checkpoint proteins, is highly expressed in cancer cells and activated tumor-infiltrating immune cells, it helps cancer cells to evade the surveillance of cytotoxic T-cells and natural killer cells.
Objectives:
To comparison the immunohistochemical expression of (CD276) and (HER2) in various bladder tissue sections from Iraqi patients with UBC by pathological and aperio digital.
Materials and Methods:
A total of 140 paraffin-embedded tissue blocks of bladder cancer were obtained from the archive of the Histopathology Unit of the Martyr Ghazi Al-Hariri, Hospital of Medical City, Al-Yarmouk Teaching Hospital, and several private laboratories in Baghdad, Iraq. In addition, 20 control bladder tissues with no significant pathology, collected from the Forensic Medicine Department for comparison purposes after taking the official approvals.
Results:
The results revealed the Digital Aperio Program was inaccurate for interpreting the immunohistochemistry scoring for both markers since it read and measured every stained spot in the sections regardless the type of target cells.
Conclusion:
The Digital Aperio Program gave false positive results for both markers compare about pathological scoring making it an accurate scoring for IHC.

KEYWORDS: Urinary Bladder Cancer, Cluster of Differentiation 276, Human Epidermal Growth Factor Receptors, Immunohistochemistry, Clinicopathological characteristics.

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INTRODUCTION

Urinary Bladder cancer (UBC) is the tenth most common malignancy in the world, bladder cancer is mainly a male tumor as the incidence is up to four times that in women (1). Urinary Bladder cancer had gone through various natural historical events differentiated from other diseases. It is a tumor developed in the bladder lining as tissues have grown abnormally, and in some cases, it spread into the bladder muscle directly (2). According to histopathological features, UBC has three main subtypes; transitional cell carcinoma (TCC) is the most common subtype (>90%), squamous cell carcinoma (SCC) and adenocarcinoma which are less frequent of all UBC cases (less than 5%, 2%) (3). Several risk factors have been documented for UBC, including smoking, age, occupation, sex, polycyclic aromatic hydrocarbons exposure, infection of the urinary tract, and exposure to heterocyclic amines and arsenic (4). Human Epidermal growth factor Receptor 2 (HER2/ERBB2) is a member of a family of epidermal growth factor receptors, along with HER1/EGFR, HER3, and HER4, which are transmembrane receptor tyrosine kinases involved in cell proliferation, survival, and mobility via the downstream activation of different intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K/Akt) pathways. The HER2 proto-oncogene, located at the long arm of chromosome 17 (17q12), codes for the HER2 protein, and its mutation and amplification mostly result in HER2 overexpression (5). When HER2 is overexpressed, the tyrosine residues in the heterodimer's cytoplasmic domain get autophosphorylated, which sets off a number of signaling pathways that promote tumor growth and proliferation (6). The immunological checkpoint molecule B7-H3, also referred to as CD276, is the most evolutionarily conserved member of the B7 family to date and is expressed worldwide in a wide variety of organisms. Subsequent research revealed that CD276 is an immune inhibitory molecule that affects T cell activation and proliferation, despite its initial classification as an immune costimulatory molecule (7).

MATERIALS AND MATHODS

A total of one hindered forty paraffin-embedded tissue blocks of malignant bladder tumors with their information were collected from the archive of the Histopathology Unit of the Department of Martyr Ghazi al-Hariri Hospital, Hospital of Medical City, Al-Yarmouk Teaching Hospital, and some private laboratories in Baghdad, Iraq. The type of biopsy was transurethral resection of bladder tumor (TURBT). Addition twenty samples from cadavers in the Forensic Medicine

Department for comparison purposes after taking the official approvals.
They were preserved in 10% formalin before being subjected to the standard tissue processing sequence and being turned into paraffin blocks. All the preparations for slide sectioning, Hematoxylin/Eosin staining, and IHC staining were performed at a university Al-Nahrain of collage medicine department pathology and forensic medicine.

Immunohistochemistry staining:
The Immunohistochemichal staining process followed exactly as specified by the kit manufacturers' protocols for CD276 (Abcam, anti-CD276 antibody, [SP206] ab227670 and PolyExcel, HRP/DAB Detection System) but for HER2 the kit manufacturers protocols (Dako, primary antibody,colne number A0485, Endogenous peroxidase blocking ,Secondary antibody) . The general protocol was adopted in accordance with Magaki et al., 2019(8). First, the tissue sections were de-waxed by heating the slides in an oven at 60-65°C for 30 minutes, followed by rapid rinsing in xylene and re-heating in the oven for 10 minutes. The sections were rehydrated via immersing them in decreasing concentrations of alcohol. Then, slides were treated with hydrogen peroxide block solution for 10 minutes and the slides were treated with protein blocker for 10 mins. in room temperature. The primary antibodies of CD276 were diluted to (1:200) also the primary antibody of HER2 were diluted to 1:600 and applied to the tissue sections, incubated at room temperature in a humidity chamber for 30 minutes. Next, the complement was added to the slides which were then incubated for 15 minutes at room temperature in the humidity chamber. The slides were then coated with horse reddish peroxidase (HRP) that have protein biotin/ avidin associated with complement and incubated in the humidity chamber for 20 minutes at room temperature. Afterwards, DAB substrate was applied to the slides and incubated in a humidity chamber at room temperature for 10 minutes until the brown stains appeared. The tissue sections were then washed two times with Phosphate-Buffer-Saline (PBS) for 5 minutes each, then counterstained with drops of Mayer' Hematoxylin for 1-2 minutes before being carefully cleaned in distilled water. The slides were then dehydrated, cleared, mounted and cover slipped, being ready for microscopic examination.

Scoring system:
The scoring system for CD276 expression was done according to (9) and the results were assessed and read by a specialist pathologist. The cut-off proportion of positive expression was 5% in each specimen. The semiquantitative H-score of the membrane staining intensity was calculated as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). While the scoring system for HER2 expression was done according to (10). The cut-off proportion of positive expression was > 10% of tumor cells was calculated as 0,1 (negative), 2(equivocal) and 3 (positive)
The scoring was achieved by applying the Aperio Image Scope software v12.3.3.5048V12 positive pixel count algorithms program (Aperio Technologies Inc, USA downloaded from www. Leicabio system). It is used to quantify the amount of a specific color for detecting Anti-CD276 and Anti-HER2 in a tissue section. It involved semi quantitative scoring (Ordinal scale of scores) of combined stain intensity and percent of cells positive of HER2 and CD276 with quantitate score (Aperio system):
1. Intensity from 1(Weak), 2 (Moderate) to 3 (Sever).
2. Immunoreactive cells score 0 for less than 10%, score1 (+) for 10-30%, score 2 (++) for 31-50%, score3 (+++) for 51-70 % and score 4 (++++) for more than 71% of reactive cells.

Statistical Analysis
The Statistical Analysis System SPSS-22 (Statistical Packages for Social Sciences—Version 22) was used of the study. The Chi-square test was used to make a significant comparison between percentages in the different groups (11). Probabilities in this study are: significant (P≤0.05)*, highly significant (P≤0.01) ** and NS (non-significant).

RESULTS

Immunohistochemical expression of CD276 in bladder tumors:
The positive expression of CD276 was seen as a brown membrane staining (with or without cytoplasmic staining) in tumor cells. Results by pathologist scoring showed significant differences (P=0.038) in the expression of this marker between the groups of study table (1-1) while the scoring by aperio digital results showed significant differences in the expression of CD276 (P=0.002) between malignant and control groups, seen of table (1-2) and figure (1.1).

Table (1-1) Scoring of CD276 expression for bladder cancer and control samples by pathologist

Score	Malignant		Control	
	N	%	N	%
0	41	58.57	10	100.00
+1	6	8.57	0	0
+2	12	17.14	0	0
+3	11	15.72	0	0
P value	0.038S*			

Table (1-2) Aperio Scoring of CD276 expression for bladder tissue samples.

Score	Malignant		Control	
	N	%	N	%
0	3	4.29	10	100.00
+1	5	7.14	0	0
+2	20	28.57	0	0
+3	22	31.43	0	0

+4	20	28.57	0	0
P value	0.002 S**			

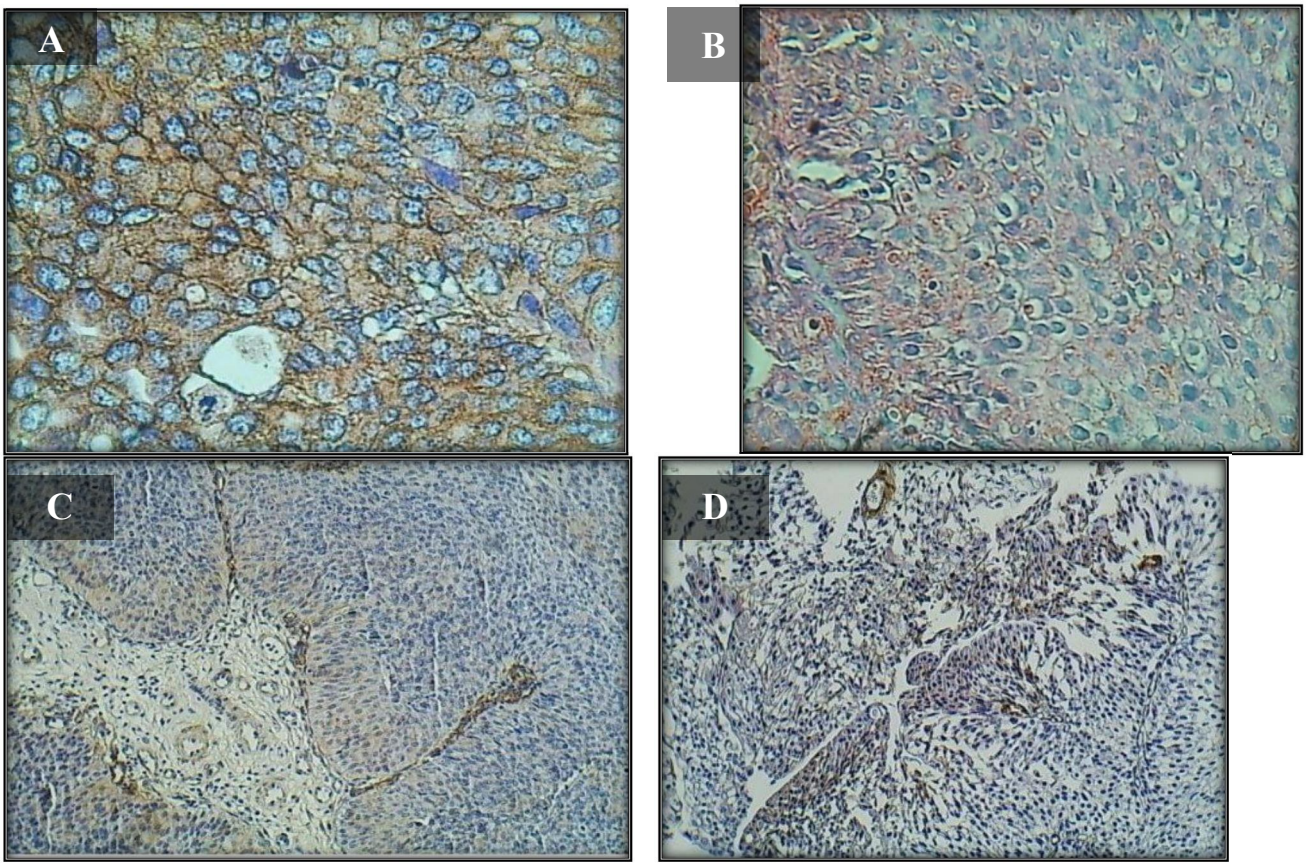

Figure (1.1) Immunohistochemical assessment of CD276 in different bladder tissues. A: positive expression membranous score 3 in urothelial with squamous differentiation (400X). B: Negative expression membranous score 0 in papillary urothelial cancer(100X).C: positive membranous expression score 1 in papillary urothelial cancer(100X). D: positive membranous expression score 2 in papillary urothelial cancer(100X).

Immunohistochemical expression of HER2 in bladder tumors:

The positive expression of HER2 was seen as a brown membranous staining (with or without cytoplasmic staining) in malignant cells. Regarding the score of HER2 expression, the current study declared significant differences(P=0.025) between groups of the study; 28.57%(20 cases) of studied cases had strong HER2 expression within score 3, while 48.57% had negative expression (table (1-3), figures (1.2) while in aperio scoring the expression of HER2 revealed significant differences(P=0.001) between the groups table (1-4).

Table (1-3) Scoring of HER2 expression in groups of study.

Score	Malignant		Control		P value
	N	%	N	%	
0	34	48.57	10	100.00	0.025S
+1	5	7.14	0	0	
+2	11	15.71	0	0	
+3	20	28.57	0	0	

Table (1-4) Aperio Scoring of HER2 expression for bladder tissue samples

Score	Malignant		Control		P value
	N	%	N	%	
0	6	8.57	10	100.00	0.001 S
+1	4	5.72	0	0	
+2	10	14.29	0	0	
+3	25	35.71	0	0	
+4	25	35.71	0	0	

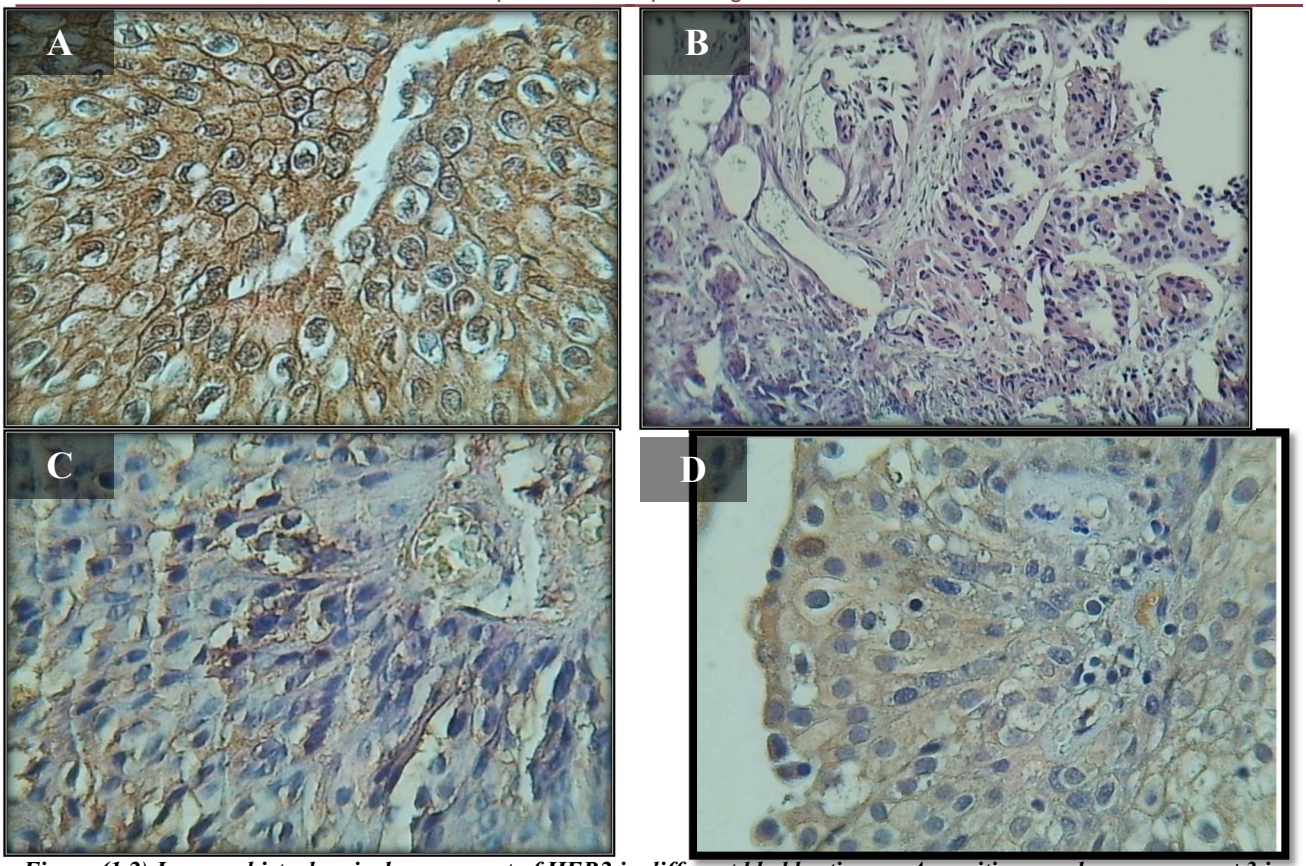

Figure (1.2) Immunohistochemical assessment of HER2 in different bladder tissues. A: positive membranous score +3 in papillary urothelial cancer (100X), B: Negative membranous score 0 in urothelial with squamous differentiation cancer (100X). C: negative membranous score 1 in papillary urothelial cancer(400X). D: equivocal membranous score +2 in urothelial with squamous differentiation cancer (100X).

DISSCUSSION

Although immune checkpoint inhibitors have revolutionized the treatment of certain solid malignancies, only a small percentage of patients with specific tumor types will benefit clinically from immune checkpoint therapy. Immunotherapy, which focuses on modifying or enhancing the immune system to stop tumor growth, is the most successful cancer treatment and represents a novel strategy. Strong immunosuppressive checkpoint CD276 is extensively expressed in many solid tumors and facilitates tumor cells' immunological escape from the lethal effects of TNF-α and interferon-gamma (IFN-γ)(12).To our knowledge, the study to investigate the expression of CD276 in bladder tumors by IHC. Although B7-H3 levels in healthy tissues are relatively low, the molecule is abundantly expressed in numerous cancer types, such as lung cancer, esophageal squamous cell carcinoma (ESCC), gastric cancer, pancreatic cancer, colorectal cancer, liver cancer, breast cancer, brain tumors, and prostate cancer (13). The study that initially identified CD276 demonstrated that it exhibits a co-stimulatory effect on T-cells(14)and a further in vitro study demonstrated that it increased the proliferation of CD4 and CD8 T-cell populations and selectively stimulated IFN-γ production(15).

By contrast, other studies demonstrated that CD276 acts as a T-cell co-inhibitor. In the majority of studies conducted thus far, it was found that human and mouse CD276 inhibit CD4 T-cell activation, and reduce the production of effector cytokines, such as IFN-γ and interleukin-4(16). Moreover, it may function as a protective factor in natural killer cell-mediated cytolysis (17).

Currently, the mechanisms underlying B7-H3's role in tumorigenesis and development remain unclear. However, several potential mechanisms have been proposed. Firstly, B7-H3 affects tumor cell metabolism, as evidenced in triple-negative breast cancer where decreased B7-H3 expression reduces tumor cell glycolytic capacity and increases sensitivity to AKT/mTOR inhibitors(18). Secondly, B7-H3 suppresses T-cell-mediated antitumor immunity by inducing M2-type polarization of tumor-associated macrophages in hepatocellular carcinoma, thereby promoting tumor development through the STAT3 signaling pathway(19). Thirdly, B7-H3 plays a role in tumor promotion by influencing cell proliferation, metastasis, invasion, and epithelial-to-mesenchymal transition (EMT).

Assessment the expression of HER2 in bladder cancer in Iraq was performed previously by (20) who observed that this marker was positively expressed in 41.66% of cases, with significant correlation (P=0.05). Also a research by Mahdi, 2020(21) noted that HER2 was positively expressed in 62.9% of total. The current outcome recorded significant differences in HER2 expression (P = 0.027) between malignant and control groups. Activation of HER2-mediated signaling pathways occurs by heterodimerization with ligand-activated epidermal growth factor receptor family (EGFR) or by homodimerization when it is present in high concentrations such as in cancer. This dimerization would lead to the phosphorylation of tyrosine residues, which initiates downstream signaling cascades such as: phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling cascade, rat sarcoma/mitogen-activated protein kinase/extracellular signal-regulated kinases (Ras/MEK/ERK), and Janus kinase/signal transducer and activator of transcription (JAK/STAT), these pathways regulate cell survival, proliferation,

differentiation, motility, apoptosis, survival, invasion, migration, adhesion, and angiogenesis(22).ErbB/HER family receptor hetero-dimerization causes tyrosine residues in the heterodimer's cytoplasmic domain to become auto phosphorylated, which sets off a complicated chain of events that promotes cell growth and carcinogenesis (23). Because of the important prognostic and therapeutic significance of HER-2/neu in breast cancer, it had been studied in several other human cancers, including in bladder Transitional cell carcinoma (TCC). In foremost studies, there was an increase in HER2 amplification and overexpression in bladder cancer according to Coogan et al., 2004(24) who recorded that the expression of HER2 in bladder cancer was 26% in studied cases.

These major variations in comparison to the low rate of HER2 overexpression in our result could be explained by a number of factors; of them, is the variability in immunohistochemistry assays, related to the heterogeneity between kits, antibodies, protocols, interpretations of staining or cut-off values.

Also, in current study, the lowest percentage of HER2 expression was in score +2 which is named as equivocal; in which HER2 status of the tumor is not clear and needs to be further tested to clarify the result. Fluorescent and/or chromogen in situ hybridization methods are recommended in order to validate HER2 immunohistochemistry results for score +2 positive cases in bladder cancer according to Cimpean et al.,2017(25). Scoring systems are variable and there is no consensus on the definition of HER2 overexpression in bladder carcinoma. Most authors used criteria of Wolff et al. which reported for breast carcinoma (ASCO scoring system) (26).

The Aperio Scan Scope Software System is used to scan and quantify the IHC staining (27). It is a scanning platform that is recently used in a wide range to read the scoring of IHC assays worldwide. In this program, the scores +1, +2, +3, +4 are considered as positive, while score 0 is considered as negative (Aperio Technologies Inc, USA downloaded from www. Leicabio system) (28). Some studies use Aperio program for analysis of markers staining, of them. They compared the outcome of these methods with manual counts and they found that Aperio digital analysis didn't agree with manual count. However, the digital reading was free downline and it required acquisition of digital images of the slides with whole-slide scanners (29). Using digital slide images obviates the need for transporting and handling physical slides afterward. Viewer software includes support tools, such as distance measurement or digital annotations, and checks whether the entire slide has been viewed (30). Though, the problem that shows up when analyzing the tumor regions of interest using Aperio is that the algorithm struggled to accurately distinguish between tumor cells and other cell types within the ROI, such as immune and stromal cells. This resulted in high variability between raters and overestimation of manual counts (31). In a study by (32), they compared between the expression of HER2 by conventional manual microscopy reading and Aperio digital reading in breast cancer, and noticed that the percent agreement of HER2 expression by manual microscopy from 76.3% to 91.3%, while the percentage using aperio digital from 70.0% to 86.0%. While in current study, it had been found that this program did not discriminate tumor cells from other cells during the scanning process and only provided the score for the quantity of stain in the slide sections regardless to the type of cells. Therefore, and after the comparison of Aperio readings with those obtained by professional pathologist using microscope and the huge variations in the gotten results, it should be declared that this program is unreliable to give accurate score that can be obtained using a microscope.

CONCLUSION

The present investigation reported CD276 and HER2 variable expression in bladder cancer and may play important roles in tumor progression and immune evasion. Although the Aperio digital system provides rapid and convenient assessment of IHC staining, its inability to accurately differentiate tumor cells from surrounding non-neoplastic cells resulted in marked discrepancies compared with microscopic evaluation by professional histopathologists. Therefore, conventional histopathological examination remains the more reliable method for accurate interpretation and scoring of CD276 and HER2 expression in bladder cancer.

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