

The Role of Digital Pathology in The Assessment of PD-L1 Expression in HER2-neu Positive Breast Cancer

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ABSTRACT

Digital pathology is a technology for representing whole stained tissue sections from glass slides and viewing them by a pathologist on a computer. We aim to find out the role of digital pathology in the assessment of "PD-L1 in HER2-neu-positive breast cancer" and the effect of storage time on PD-L1 expression. This is a case series study that evaluates "PD-L1 protein immunohistochemical expression" using monoclonal mouse Anti-PD-L1 (Dako), clone 22C3 on "50 formalin-fixed paraffin-embedded tissues (core biopsy)" in Iraq over 7 months and scored using a combined positive score. "PANNORAMIC® Flash DESK DX slide scanner (3DHISTECH digital pathology firm)" was used to scan the slides. The PD-L1 stained slides were stored for 7 months, then a reassessment of the 50 slides was done using a light microscope in the same methods and compare the results with digital images. The results of reassessment of the 50 glass slides after 7 months under a light microscope found that there is slight fainting in the staining and slight changes in the combined positive score in 11 cases. Digital pathology contributes to documenting the PD-L1 assessment score, the storage time of PD-L1 immunohistochemical slides will cause fainting of the staining.

Keywords: digital pathology; PD-L1; HER2-neu positive breast cancer.

دور علم الأمراض الرقمي في تقييم تعبير PD-L1 في سرطان الثدي الإيجابي HER2-neu

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الخلاصة

علم الأمراض الرقمي عبارة عن تقنية لتمثيل أقسام الأنسجة الملطخة بالكامل من الشرائح الزجاجية وعرضها بواسطة أخصائي علم الأمراض على جهاز كمبيوتر. نهدف إلى معرفة دور علم الأمراض الرقمي في تقييم PD-L1 في سرطان الثدي الإيجابي "HER2-neu وتأثير وقت التخزين على تعبير PD-L1 هذه دراسة من سلسلة الحالات التي تقيم "التعبير الكيميائي المناعي للبروتين "PD-L1 باستخدام الماوس أحادي النسيلة Anti-PD-L1 (داكو) ، استنساخ 22 C3 على "50 من الأنسجة المضمنة بالبارافين الثابتة بالفورمالين (خزعة أساسية)" في العراق على مدى 7 أشهر وسجل باستخدام مجموع نقاط إيجابية. تم استخدام "ماسح الشرائح PANNORAMIC® Flash DESK DX شركة علم الأمراض الرقمية 3 (3DHISTECH) لمسح الشرائح ضوئياً. تم تخزين الشرائح الملطخة PD-L1 لمدة 7 أشهر ، ثم تم إعادة تقييم 50 شريحة باستخدام مجهر ضوئي بنفس الطرق ومقارنة النتائج بالصور الرقمية. وجدت نتائج إعادة تقييم 50 شريحة زجاجية بعد 7 أشهر تحت المجهر الضوئي أن هناك إغماء طفيفاً في التلوين وتغيرات طفيفة في النتيجة الإيجابية المجمعة في 11 حالة. يساهم علم الأمراض الرقمي في توثيق درجة تقييم PD-L1 ، وسيؤدي وقت تخزين الشرائح الكيميائية النسيجية المناعية PD-L1 إلى إغماء التلطخ.

الكلمات المفتاحية : علم الأمراض الرقمي ، PD-L1 ، سرطان الثدي الإيجابي HER2-neu .

INTRODUCTION

Whole slide imaging (WSI), also known as digital pathology or virtual pathology, is a method for acquiring high-speed, high-resolution digital images that represent whole stained tissue sections from glass slides in a format that enables a pathologist to view them on a computer, where the image can be magnified and navigated spatially like that of standard microscopy¹. It appears around 1999². However, it provides many additional features in comparison to a light microscope, such as an overview image to aid navigation within the examined slide, automated image analysis, simultaneous examination of multiple slides, comparison of two staining of the same material side by side, as well as continuous zooming as opposed to discrete stages available with a standard light microscope, translates to additional functionalities like annotations³. It may be used effectively in breast histopathology for diagnoses, research, and quality assurance⁴.

The advantage of WSI for upfront analysis in pathology, before replacing the well-established, well-known, and adaptable microscope plus WSI, numerous legitimate issues necessary to be addressed⁵. One of the major barriers to using DSI for this purpose is the deficiency of systematic validation inappropriately in large research groups, and the "Food and Drug Administration (FDA)" has not approved the use of DSI for primary diagnostics in the United States. As a result, validation of adopting DSI for the first digital diagnostics is still required, and much research has been conducted to demonstrate concordance between diagnosis using WSIs and glass slides^{6,7}.

Breast cancer is the most frequent type of cancer in women and, after lung cancer, the second biggest cause of death⁸.

Overexpression of "HER-2 (human epidermal growth factor receptor-2)" is found in "15 - 20%" of primary breast cancers, reflected by amplification of "proto-oncogene located on chromosome 17" and /or protein overexpression^{9,10}. HER2 testing is performed using "immunohistochemistry", positive cases have (a score of 3+) staining¹¹ or by "ISH (in situ Hybridization)" techniques applied in those with equivocal immunohistochemistry score (+2)¹². This protein's overexpression has been accompanied by a high histologic grade and increased risk of metastasis to axillary nodes¹³.

Anti-HER2 therapy (trastuzumab), is an effective standard therapy against HER2+ tumors, however, resistance to this treatment has been documented in up to 50% of HER2-positive patients¹⁴.

PD-1 is an immunoglobulin superfamily member related to the "CD28/CTLA-4 (cytotoxic T-lymphocyte antigen-4) family of co-stimulatory receptors"¹⁵. T-cells, monocytes, B-cells, natural

killer cells, and dendritic cells are all immune cells that express it¹⁶. It contains two ligands: "B7-H1 (PD-1 ligand 1)" and "B7-H2 (PD-1 ligand 2) (B7-dendritic cells)" that can be detected on non-lymphoid cells in the periphery as well as APC (antigen-presenting cells) and activated lymphocytes¹⁶.

The interplay of PD-1/PD-L1 serves as a regulatory check against excessive antigen-specific immune response and autoimmunity. According to new research, the PD-1 pathway may function as an active immune checkpoint in a variety of cancers. The PD-1/PD-L1 pathway may be targeted to avoid inhibitory T-cell signaling and boost T cells to enhance tumor cell killing¹⁸.

The goal of PD1/PD-L1 immunotherapy is to boost the human immune system by inhibiting the PD-1/PD-L1 signaling pathway in order to eradicate cancer cells¹⁸. PD-L1 expression has previously been studied in the lung, pancreatic, esophageal, kidney, ovarian, colorectal, head and neck, and squamous cell carcinomas, melanoma, and glioma^{17,20}.

Aims

1. To find out the role of digital pathology in the assessment of "PD-L1 in HER2-neu-positive breast cancer"
2. To find out the effect of storage time on PD-L1 expression.

PATIENTS AND METHODS

This is both a "retrospective and prospective" case series study including 50 tru-cut samples of primary her2/neu positive breast tumors assembled for 7 months period from November 2021 to May 2022 from private laboratories in the North of Iraq.

PD-L1 IHC 22C3 pharmDx (Dako) "Monoclonal Mouse Anti-PD-L1", "Clone 22C3" was used for the detection of PD-L1 protein in Formalin-fixed and paraffin-embedded (FFPE) blocks along with "EnVision FLEX visualization system Autostainer Link 48", in a private laboratory in Duhok City/Iraq.

Formalin-fixed and paraffin-embedded blocks were cut into 4 μ thickness, cleared and rehydrated in a succession of alcohols and xylene, and then stained with PD-L1 immunohistochemical stains. To boost staining intensity by unmasking the antigen with "single primary antibodies (epitope retrieval)", "a water bath-based Dako PT (pre-treatment) Link tank" was used in conjunction with "a Dako Target Retrieval Solution (50x) (code K8005) (made by diluting the concentrate 1:50 in distilled or deionized water)".

Following the peroxidase block, specimens are incubated with “the monoclonal mouse primary antibody to PD-L1”, followed by incubation with “a Mouse LINKER”, then incubation with “visualization reagent”, which consists of secondary antibody molecules and horseradish peroxidase molecules attached to a dextran polymer backbone. Tissue staining was visualized using “a 3,3 Diaminobenzidine DAB substrate chromogen solution (brown precipitate)”. Slides are counterstained with “hematoxylin”, dehydrated, and mounted.

The testing includes an external positive control “tonsil”; its parenchyma contains immune cells (lymphocytes and monocytes) that are moderately to significantly PD-L1 positive in the paracortical area or inside the germinal centers, as well as tonsil crypt epithelial cells that are strongly positive. Negative portions of the positive control tissue are also employed as the “Negative Control Tissue”. To check each staining, positive and negative cell line controls were used in each staining run.

After the performance of the staining process, the slides were checked under the light microscope for assurance of the quality of the procedure.

The PD-L1 kit (22C3 Dako PharmDx) guidelines recommend using a “Combined Positive Score (CPS)”, in which both “tumor cells” and “tumor-infiltrating immune cells (TCIC)” are used according to the equation below (Guo et al., 2020) (Erber & Hartmann, 2020), to be deemed adequate for PD-L1 assessment, a minimum of 100 viable tumor cells should be present in the PD-L1 stained slide (Erber & Hartmann, 2020) (Agilent, 2018).

$$\text{CPS} = \frac{\# \text{PD-L1 staining cells "tumor cells, lymphocytes, and macrophages"}}{\text{Total \# of "viable tumor cells"}} \times 100$$

Slides were inspected under a light microscope to assess IHC accuracy in contrast to the positive and negative controls, viable tumor cell adequacy, and assessment of CPS.

After a careful analysis of slides, the slides were scanned using the “PANNORAMIC® Flash DESK DX slide scanner (3DHISTECH digital pathology company)” which is a low-cost slide scanner for clinical routine diagnosis, has exceptional image quality in brightfield scanning, uses flash scanning technology for fast scanning, high-quality objectives, and a “12 megapixel (MP) camera with xenon flash illumination”, can achieve up to “41x or 82x” optical magnification and 0.242 or 0.121 m/pixel resolution. It also had a capacity of 1 slide, speed 30 sec/slide, single and double width slide scanning, “20x objective/40x” optical equivalent magnification, 7500um focus point distance, “15*15mm” average sample size, and single layer local scanning.

Electronic digital pictures of glass slides were created through multiple steps; dry the slides to prevent sticking in racks, transfer the slides to scanning racks and load racks into scanners, then initial tissue capture to ensure that all tissue on the slide will be scanned, slide image captured at high resolution, remove racks from the scanner and remove the slides from the racks, visual inspection of images to ensure adequate quality. If the slide failed to be scanned, an evaluation was made, and the slide was rescanned.

For quality control, the following recommendations were provided: Glass slides should be stained and dried properly, free of pen stains, and not damaged, air bubbles were to be missing, and the coverslips had to be appropriately positioned so that they did not droop over the edge of the glass slide. The glass slide marker should be level and not extend above the slide frame or cover tissue on the slides⁷; however, the quality of the focus may be evaluated by the focus range of movement. The time for adjusting the focus, minimum distance, the method applied to distance measurement, and movement resolution at the z-axis²¹.

After scanning the slides, the digital images were viewed on a computer with “3DHISTECH slide viewer software 2.6 (64-bit version)” for Windows, which allows for a variety of functions such as changing magnification, measuring, annotating, and quantifying immunohistochemical staining cells, mitotic figure counting, and reevaluating “CPS”. Also, the scanned slide was sent digitally to another pathologist for “CPS” evaluation and compare the results with the light microscope.

Lower magnifications (10x); were used to assess the sufficiency of viable tumor cells on the slide, including PD-L1 staining and non-staining tumor cells, to confirm that the sample contained at least 100 viable tumor cells.

At a greater magnification (20x); examine the PD-L1 expression in tumor and immune cells by splitting the slide into several sections, evaluating each region and calculating CPS individually, totaling the total areas, and then taking the mean.

PD-L1 expression was evaluated whether CPS \geq 10 or $<$ 10.

The PD-L1 stained slides (50 slides) were stored at optimal room conditions then after 7 months, a reassessment of the slides was done using a light microscope in the same methods, recalculating the CPS, and comparing the results with digital images.

RESULTS

During seven months, "fifty core needle biopsy specimens" from breast cancer patients were collected, stained, and studied for PD-L1 IHC. PD-L1 is found to be expressed in 17 (34%) of HER2-neu positive cases with "CPS equal to or more than 10", whereas 33 (66%) of cases have a negative expression with CPS less than 10.

After 7 months, the results of reassessment of the 50 glass slides under a light microscope found that there is a slight fainting in the staining of "tumor and immune cells" and slight changes in the combined positive score in 11 of these slides but the percentage of cases with positive PD-L1 still the same (34%) , as mentioned in table 1 and figures (1,2,3 and 4) .

Table 1. Comparison between first assessment and reassessment after 7 months of "PD-L1 stained glass slides".

Slide no.	1st assessment (CPS)	2 nd reassessment (CPS) after 7 months	Percent of change
1	75	70	5%
2	13	11	18%
3	20	17	15%
4	28	25	9 %
5	48	44	10%
6	30	26	12%
7	15	12	20%
8	55	50	9%
9	18	15	16
10	25	20	20%
11	14	12	18%

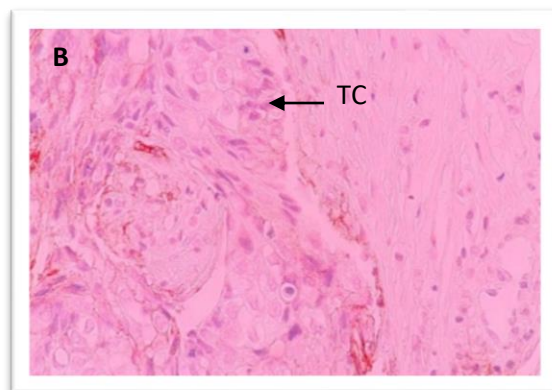
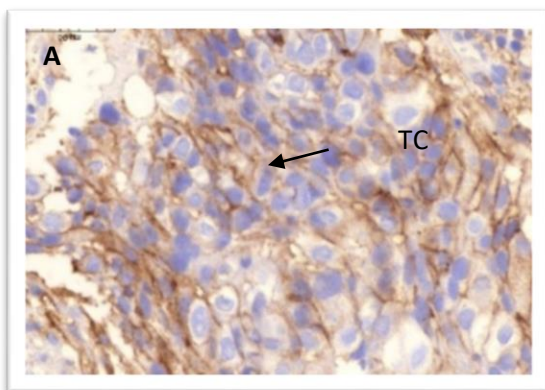


Figure 1. PD-L1 IHC staining slide A.digital slide image showing membranous staining of the tumor cell (arrows) in 1st assessment of case (1) B.light microscopy image in 2nd assessment of the slide after 7 months showing mild fainting of the PD-L1 staining of the same case (400x).

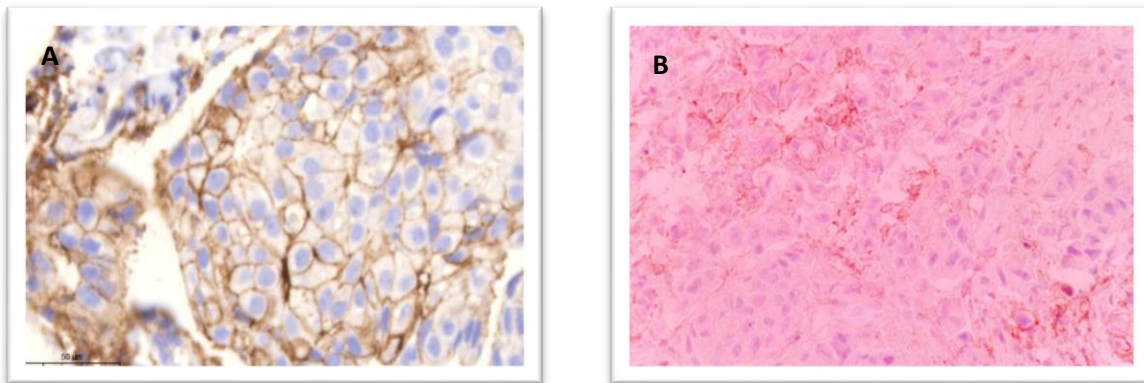


Figure 2. PD-L1 IHC staining slide A.digital slide image showing membranous staining of the tumor cell and cytoplasmic staining of the immune cell in 1st assessment of case (4) B.light microscopy image in 2nd assessment of the slide after 7 months showing mild fainting of the PD-L1 staining of the same case (400x).

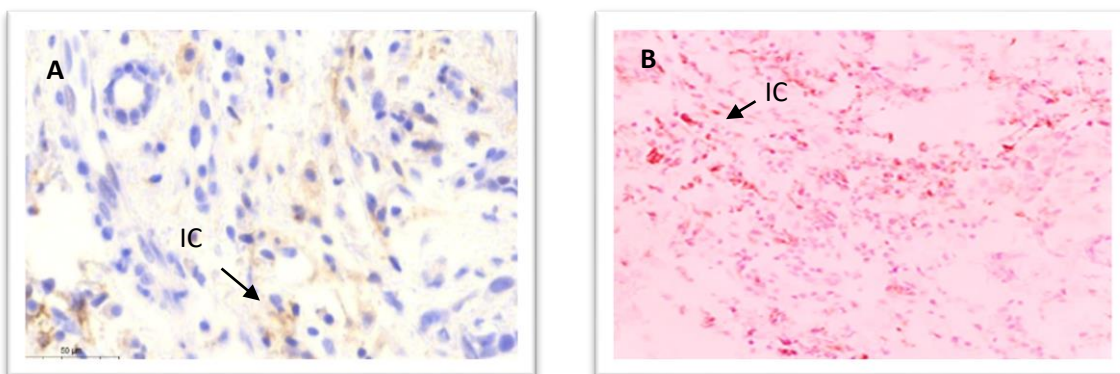


Figure 3. PD-L1 IHC staining slide A.digital slide image showing cytoplasmic staining of the immune cell (arrows) in 1st assessment of case (5) B.light microscopy image in 2nd assessment of the slide after 7 months showing mild fainting of the PD-L1 staining of the same case (400x).

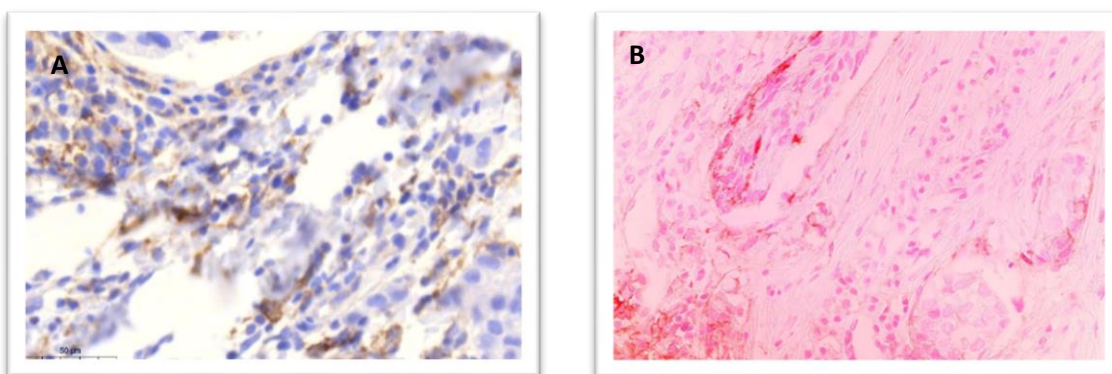


Figure 4. PD-L1 IHC staining slide A.digital slide image showing cytoplasmic staining of the immune cell in 1st assessment of case (8) B.light microscopy image in 2nd assessment of the slide after 7 months showing mild fainting of the PD-L1 staining of the same case (400x).

DISCUSSION

Digital pathology technologies are quickly replacing static images in teaching, diagnostic, and research applications. The digitization of histologic or cytologic slides permits pathologists to cover the complete slide surface, allowing them to analyze the entire section as they would with a "traditional" microscope⁴.

DSI allows their usage for teleconsultation, and web accessibility, speeding up the workflow, and minimizing the time required for sending the glass slide to remote locations⁴. The financial and administrative advantages allow for significant long-term cost reductions in terms of equipment, technical personnel, and laboratory facilities. Individual student microscopes, technical people to maintain or repair microscopes, and concerns about the loss of priceless specimens are no longer required. The presentation will cover several hardware alternatives (light microscope scanning stage systems), commercial e-learning software, copyright issues, and the formation of national repositories for virtual picture archives²².

We found a reduction in the percentage of immunoreactive cells "tumor and immune cells" for PD-L1 IHC in a quarter of cases during this quite short time of the staining process and a slight reduction in the CPS in 11 cases; however the percentage of positive to negative PD-L1 expression cases still the same, this fading may denote that a longer period of storage time may be of significant change that may lead some cases to change from positive to negative status and this will affect the treatment decision, documenting, archiving and slide reviewing. This fading of PD-L1 staining may be ascribed to the processing factors including but not limited to, counterstaining, mounting, material and methods, and slide storage conditions if stored at more than 25° room temperature and exposed to excessive light levels. Another study by Giunchi discovered the key observation is that PD-L1 IHC expression fades in TCs of non-small cell lung cancer in older paraffin tissue blocks, with a substantial decrease in the percentage of immunoreactive cells for PD-L1 in tissues processed in 2014 versus 2015²³.

CONCLUSION

- Digital pathology contributes to documenting the PD-L1 assessment score.
- The storage time of PD-L1 IHC slides will cause fainting of the staining.

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