EVALUATION OF PD-L1 EXPRESSION IN TUMOR TISSUE

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Abstract: Introduction: The interaction between the immune system and cancer cells is complex. Cancer cells can avoid immune destruction by suppressing the immune system. Tumors can create an immunosuppressive microenvoirment and therefore cytotoxic T lymphocytes (CTL) can't lysate them. Avoidance of the immune response is through the inhibitory ligand PD-L1, which is found on the surface of cells of the immune system, mesenchymal, epithelial, and endothelial cells as well as tumor cells. PD-1 is a surface cell receptor, part of the immunoglobulin family. Its ligand PD-L1 is expressed by tumor cells and stromal tumor infiltrating lymphocytes (TIL). This information has been used to increase the therapeutic possibilities for many malignant diseases.
Objectives: 1.Optimization of immunohistochemical method (IHH) with antibody staining, anti PD-L1.
2. Optimization of the evaluation method while using different clones of antibodies.
Material and methods: In this study are included 44 samples from 3 different types of cancer. 24 triple-negative breast cancers (TNBC), 10 non-small cell lung cancer (NSCLC) and 10 malignant melanoma. Monoclonal mouse primary antibodies have been used by manufacturers Quartett (QR1) and Ventana (SP 142, SP263). To visualize the reaction, an ultraView Universal DAB Detection Kit from Ventana was used on an automated platform for immunohistochemical staining Ventana BenchMark GX. The statistical analysis was done with the statistical package SPSS for Windows Version 22.0.
Results: With comparing the sensitivity of two different clones on the same tissue samples from triple-negative breast cancer (TNBC), it can be conclude that the Clone QR1 gives more positive results than the Clone SP142, but there is no statistically significant difference.
With comparing the sensitivity of two different clones in the same tissue samples from malignant melanoma, it can be conclude that Clone SP263 gives more positive results than Clone QR1, but there is no statistically significant difference.
With comparing the sensitivity of two different clones in the same tissue samples from non- small cell lung cancer, we concluded that Clone QR1 gives more positive results than Clone SP142, but there was no statistically significant difference.
Conclusion: Use of any of these clones of the manufacturers Quartett and Ventana give satisfactory results and can be used in the determination and evaluation of PD-L1 status in patients, and thus to determine further therapy and outcome of patients.
Keywords: breast cancer, malignant melanoma, non-small cell lung cancer, immunohistochemistry, antibodies

1. INTRODUCTION

The immune system in the human body recognizes and eliminates cancer cells. The interaction between the immune system and cancer cells is complex process. Cancer cells can avoid immune destruction by suppressing the immune system. Cellular immunity, particularly activation of T cytotoxic lymphocytes, determines the quality of the anti-tumor immune response. T cell receptor (TCR) molecules recognize the antigen along with the major histocompatibility complex (MHC), which is located on the cell surface. T cells attacks tumor cells that have specific tumor antigens on their surface and the immune system can recognizes them as foreign.
Tumors can produce an immunosuppressive microenvironment. Avoiding the immune response is through the PD-L1 inhibitory ligand (1).
PD-1 is a surface cell receptor, that belongs to family of immunoglobulins (Ig). PD-1 (CD279) belongs to the CD28 (Cluster of Differentiation) family, encoded by the PCCD1 gene located on the long arm of chromosome 2 (2q37.3).It is present on the surface of B and T cells. PD-1 is a check-point for the immune system and protects against neoplastic processes and autoimmune diseases through two mechanisms. The first mechanism is promotion of apoptosis (programmed cell death) through antigen-specific T cells in the lymph nodes. The second mechanism is reducing the apoptosis of regulatory T cells that have anti-inflammatory action. Inhibition of the PD-1 / PD-L1 axis is an important factor in disease outcome. Targeting this axis enhances the response of T cells that kill tumor cells (2).
Its ligand, PD-L1 is expressed by tumor cells and stromal tumor infiltrating lymphocytes (TILs). The physiological

role of this protein is to bind to the PD-1 receptor that is expressed in cytotoxic T lymphocytes also known as a CD8+ T-cell or killer T cell.

This information has been used to increase the therapeutic possibilities in a number of malignant diseases: non-small cell lung cancer (NSCLC), triple-negative breast cancer (TNBC), malignant melanoma, kidney cancer, bladder cancer, cervical cancer, gastric cancer. The main feature of tumors is the avoidance of the immune response. Target therapy is useful because it targets tumor cells and reduces the side effects of the drug and reduces collateral damage to physiologically healthy tissues and organs (3).

Despite all the research and clinical studies that are being done, few patients respond to this target therapy with monoclonal antibodies. To optimize and improve the effectiveness of therapy PD-1 / PD-L1 is usually combined with another therapeutic agent. Determination of PD-L1 expression in tumor and immune cells is a useful biomarker for determining the effectiveness of cancer therapy (4). Antibodies used for therapy are directed at blocking PD-1 pembrolizumab, levodlumab and cemipipinab or at blocking PD-L1 atezolizumab, avelumab and durvalumab (5). Several clinical trials have tested pembrolizumab in preterm and terminally ill patients to prolong and improve quality of life.

PD-L1 expression varies in different types of tumors. It is regulated by various intrinsic and extrinsic signals, such as chromosomal alterations, epigenetic modifications, under the influence of certain oncogenes and tumor-suppressor genes, inflammatory cytokines, or genetic modifications in transcription, post-transcription, or transcription. Patients who have multiple copies of the PD-L1 gene have increased PD-L1 expression and are more likely to develop one of these cancers (6).

In our research we included 3 tumor types:

1.**Triple-negative breast cancer (TNBC)** is aggressive tumor, with frequent relapses and very often metastasizes to the brain and lungs, rarely to the bones. 15-20% of all breast cancers are TNBC (9). This subtype is diagnosed with immunohistochemical method (IHC), with finding of negative estrogen receptor (ER), negative progesterone receptor (PR), and negative human epidermal growth factor receptor 2 (HER2). These patents can carry a mutation in the BRCA1 gene (10). Patients with TNBC have a worse short-term prognosis than other subtypes of breast cancer, because there is no targeted therapy for these tumors. Patients diagnosed with metastatic or locally advanced triple-negative breast cancer may be tested for PD-L1 status to determine if these patients benefit from an anti-PD-L1 inhibitor. In these group inhibitors is atezolizumab which is PD-1 antagonist that selectively binds to PD-L1, prevents interaction with the PD-1 receptor and thus altering the suppression of immune cells. PD-L1 expression, its staining, and clinical pathological features are not clearly defined with survival rate. Some studies have shown that cytoplasmic positivity of PD-L1 was associated with a lower risk of mortality (11). In all studies, the variability of PD-L1 expression was strongly associated with the different antibody clones used. It has been suggested that tumor cell staining is associated with 'disease-free survival' (12). This term is defined as the time from randomization to tumor relapse or death. TNBC is resistant to the conventional treatment regimen, so there are a lot on going clinical trials to identify new molecular prognostic markers.

The presence of tumor infiltrating lymphocytes (TIL), CD8 + cells, high CD8 + / FOXP3 + ratio is a predictor of better prognosis and disease outcome (13). Immune cells should occupy> 1% of the tumor. Immune cells can be individually distributed or in a group.

Lung cancer is one of the leading causes of death in both genders. More than half of patients diagnosed with lung cancer die in less than a year. 5-year survival is 17.8% (14). The two main subtypes of lung cancer are small cell lung cancer and non-small cell lung cancer. Small cell carcinoma consists of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma.

The most common of these is adenocarcinoma, which accounts for about 40%. Risk factors are smoking, exposure to radon, asbestos, uranium, pesticides oncogenic viruses, and air pollution. Small cell lung cancer predominates in people working in uranium mines.

Occupational exposures related to lung cancer include the use of arsenic and arsenic products such as insecticides, herbicides. Asbestos and cigarettes are the best example of occupational exposure interaction (15).

Radiologists are also part of the risk group for occupational exposure to beryllium and beryllium oxide. A family history of lung cancer is also a risk factor. Carriers of mutations in the TP53 gene and mutations on chromosome 15 also carry the risk of lung adenocarcinoma.

Melanoma is a malignant tumor that originates from melanocytes. It most commonly occurs on the skin, but can occur on the mucous membrane of the eye, oral and nasal mucosa, anorectal and vulvar mucosa, gastrointestinal mucosa, and central nervous system (CNS). One of the main factors for high incidence is increased exposure to ultraviolet rays, especially in places where there is an ozone depletion. Ultraviolet radiation is the cause of melanoma in 86% of cases. UV radiation has a genotoxic effect and is in the range of 100 to 400nm. 2% of all skin cancers are melanoma with high mortality rate. Australia has the highest incidence of 55 / 100,000 inhabitants.

Melanoma may originate from a pre-existing nevus (25% of cases), but may also occur de novo (6). Melanoma can occur without prior sun exposure. The CDKN2 gene, located on the short arm of chromosome 9, is most commonly mutated in people with a family history of melanoma. This gene encodes a tumor suppressor protein that regulates RB. Other important genes in the carcinogenesis of melanoma are the BRAF gene (in 60% of patients) and the NRAS gene (in 20% of patients). These two gene mutations are not found in the same tumor (17).

2. OBJECTIVES

In our research we set two objectives.

1. Optimization of immunohistochemical method (IHH) with antibody staining, anti PD-L1.
2. Optimization of the evaluation method while using different clones of antibodies.

3. MATERIALS AND METHODS

In this study are included 44 samples of tumor tissue. We collected the samples from the surgical department in the Clinical Hospital Acibadem Sistina, Skopje, North Macedonia for a period of 3 months. These samples are from the following types of tumors:

-24 specimens of triple negative breast cancer (TNBC)

The specimens are treated with two different clones of PD-L1:

1.Ventana SP142, Ventana Medical Systems, Roche, Tucson, Arizona, United States 2.QR1, Quartett, Germany.

-10 specimens of malignant melanoma

The specimens are treated with two different clones of PD-L1:

1.Ventana SP263 Ventana Medical Systems, Roche, Tucson, Arizona, United States 2.QR1, Quartett, Germany.

-10 specimens of non-small cell lung cancer (NSCLC),

The specimens are treated with two different clones of PD-L1:

1. Ventana SP142 Ventana Medical Systems, Roche, Tucson,Arizona, United States 2.QR1, Quartett, Germany.

Comparison of the percentage of stained tumor and immune cells was made. The comparison of the percentage of tissue cells from the same patient with different antibody clones was made.

4μ thin sections were cut from paraffin blocks of selected specimens, than applied to the subject glass, previously treated with adhesive (poly-l-lysine). Monoclonal mouse antibodies from manufacturers Quartett and Ventana were used for this phase of the study. An ultraView Universal DAB Detection Kit from Ventana, on an automated immunohistochemical platform Ventana BenchMark GX , was used to visualize the reaction. In table 3 we can see the type of antibodies, clones and dilutions.

Table 1. Presenting the name, clone and dilution of the antibodies that have been used

Primary used Antibody	Clone	Dilution
Monoclonal Mouse Primary Antibody anti-PD-L1 (Ventana)	SP263	RTU*
Monoclonal Mouse Primary Antibody anti-PD-L1 (Ventana)	SP142	RTU*
Monoclonal Mouse Primary Antibody anti-PD-L1 (Quartett)	QR1	1:50

*- ready to use

The procedure of immuno-histochemical detection and visualization of proteins is in three stages: pretreatment, detection and visualization.

1. Pre-treatment

The preparation of slides takes place on the platform VentanaBenchMarkGX, according to the protocol recommended by the manufacturer, for a duration of 30 minutes.

2. Detection

The detection phase begins with the application of primary mouse antibodies, diluted according to the manufacturer's recommendation (Quartett) or RTU (Ventana), at a temperature of 37 °C on the BenchMarkGX platform for 32 minutes. Then reagents are applied from the appropriate detection kit according to the protocol recommended by the manufacturers. This cycle lasts 8-20 minutes.

3. Visualization

The reaction is visualized by applying DAB + Chromogen at room temperature for 8 minutes. Hematoxylin was applied as a dye opposite to DAB + Chromogen. After that the stained slides were dehydrated in increasing concentrations of alcohol to xylene. At the end we have covered with a permanent coating medium (Enthellan by

Merck).
Positive reaction was noted when a brown discoloration in the membrane of the tumor cell and in the cytoplasm of immune cells appeared.
In the quantitative analysis, the cases are divided into the following groups:

- negative, no expression (≤1%)
- positive, low expression (≥1% - 49%)
- positive, high expression (≥50% - 100%)
- The intensity of repainting is quantified as:
- 0 - no repainting;
- + (light colors);
- ++ (moderate colors);
- +++ (intense colors).

4. RESULTS

Table 2. Comparison of the percentage of treated tumor cells with different clones of antibodies, 24 tissue samples from triple negative breast cancer (TNBC).

	Clone QR1	Clone SP142
≤1%	24	24
≥1 - 49%	0	0
50 - 100%	0	0

In table 2 we compared the two clones, and there is no difference in using either of these two clones.

Table 3. Comparison of the percentage of treated immune cells with different clones of antibodies, 24 tissue samples from triple negative breast cancer (TNBC).

	Clone QR1	Clone SP142
≤1%	16	18
≥1 - 49%	6	6
50 - 100%	2	0
$\chi 2$ = 2,11	df=2	p=,347

In Table 3 we compared two clones and determined $\chi 2$.
The value of $\chi 2$ = 2.11, two degree of freedom df=2, p = ,347 is not statistically significant. This indicates that there are no differences in the percentage of stained immune cells with different antibody clones, 24 specimens from triple-negative breast cancer (TNBC).

Table 4. Comparison of the percentage of treated tumor cells with different clones of antibodies, 10 tissue samples from malignant melanoma.

	Clone QR1	Clone SP263
≤1%	7	5
≥1 - 49%	3	5
50 - 100%	0	0
$\chi 2$ = ,833	df=1	p=,325

In Table 4 we compared two clones and determined $\chi 2$.
The value of $\chi 2$ = ,833, degree of freedom df=1, p = ,325 is not statistically significant. This indicates that there are no differences in the percentage of stained immune cells with different antibody clones, 10 specimens from malignant melanoma

Table 5. Comparison of the percentage of treated immune cells with different clones of antibodies, 10 tissue samples from malignant melanoma.

	Clone QR1	Clone SP263
≤1%	4	2
≥1 - 49%	6	8
50 - 100%	0	0
$\chi 2$ = ,952	df=1	p=,314

In Table 5 we compared two clones and determined $\chi 2$.
The value of $\chi 2$ = ,952, degree of freedom df=1, p = ,314 is not statistically significant. This indicates that there are no differences in the percentage of stained immune cells with different antibody clones, 10 specimens from malignant melanoma.

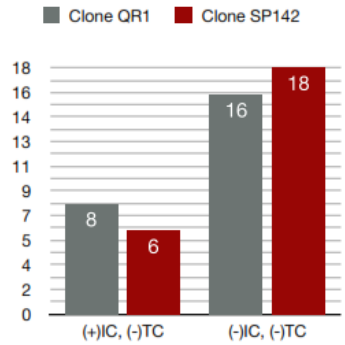

Chart 1. TNBC, PD-L1 expression of immune cells (IC) and tumor cells (TC) treated with Clone QR1 and Clone SP142

Comparing the sensitivity of two different clones in the same specimens(tissue samples) from non-small cell lung cancer (NSCLC). It can be seen that Clone SP142 gives more positive results, compared to Clone QR1, but there is no statistically significant difference.

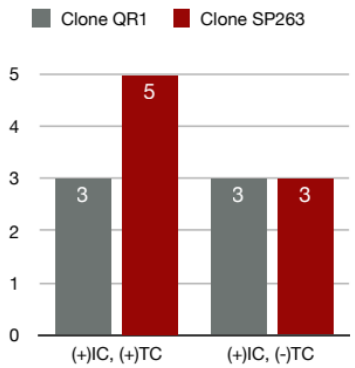

Chart 2. Malignant melanoma, PD-L1 expression of immune cells (IC) and tumor cells (TC) treated with Clone QR1 and Clone SP263

Comparing the sensitivity of two different clones in the same specimens (tissue samples) from malignant melanoma. It can be seen that Clone SP263 gives more positive results, compared to Clone QR1, but there is no statistically significant difference.

Table 6. Comparison of the percentage of treated tumor cells with different clones of antibodies, 10 tissue samples from non-small cell lung cancer (NSCLC).

	Clone QR1	Clone SP142
≤1%	8	9
≥1 - 49%	2	1
50 - 100%	0	0
$\chi 2$ = ,392	df=1	p=,500

In Table 6 we compared two clones and determined $\chi 2$.
The value of $\chi 2$ = ,392, degree of freedom df=1, p = ,500 is not statistically significant. This indicates that there are no differences in the percentage of stained immune cells with different antibody clones, 10 specimens from non-small cell lung cancer (NSCLC).

5. DISCUSSION

From our results we concluded that the use of Ventana or Quartett antibodies can be used in selection of patients to determine their PD-L1 status. This information can be also used in further treatment. The results are matched with the results obtained by Karnik T. et al.
Determining PD-L1 status
Essay approved to determine the PD-L1 status of TNBC patents is Ventana SP142 by the US Food and Drug Administration (FDA). The tested cells should be from the non-necrotizing zone of the cancer and stromal part from the tumor that has tumor infiltrating lymphocytes (TIL).
Patients with high levels of TIL after treatment have a reduction to recurrence of metastases. TILs are important before and after treatment (18). PD-L1 immune cell positivity is a good prognostic factor. Immune cells and cancer cells do not always give the same results. There for the immune cells may be positive for PD-L1 and the tumor cells may not negative like in some specimens in our research (19).

6. CONCLUDING REMARKS

Two different clones of Ventana SP142 and QR1 were compared in triple-negative breast cancer (TNBC) specimens. There are differences in the results obtained after the treatment with different clones. We got better results in the samples treated with the QR1 clone, but they are statistically insignificant. The use of any of the clones gives satisfactory results and can be used to determine and evaluate PD-L1 status in patients, and thus to determine further therapy and patient outcome.
A comparison of two different clones of Ventana SP263 and QR1 was made in malignant melanoma specimens. There are differences in the results obtained after the treatment with different clones. We got better results in the samples treated with clone SP263, but they are statistically insignificant. The use of any of the clones gives satisfactory results and can be used to determine and evaluate PD-L1 status in patients, and thus to determine further therapy and patient outcome.
Two different clones of Ventana SP142 and QR1 were compared in samples from non-small cell lung cancer(NSCLC). There are differences in the results obtained after the treatment with different clones, ie there are better results in the samples treated with the QR1 clone, but they are statistically insignificant.
The use of any of the clones gives satisfactory results and can be used in determining and evaluating PD-L1 status in patients, thereby determining further therapy and patient outcome. To determine the golden standard, which antibody is most appropriate for PD-L1-positive cancers it is necessary a clinical double-blind randomized trial of a larger group of patients treated with PD-L1.

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