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Original article

Role of cytotoxic T-lymphocyte antigen 4 (CTLA-4) expression in the pathogenesis of Warthin's tumor growth

Alvionika Nadyah Qotrunnada[1], Tecky Indriana[1], Jane Kosasih[2], Meiske Margaretha[2], Mei Syafriadi[1]
[1]Department of Biomedical Sciences, Faculty of Dentistry, University of Jember, Jember, Indonesia
[2]Department of Patological Anatomy, Dr. Soebandi Hospital, Jember, Indonesia

ABSTRACT

Background: *One of the benign salivary gland tumors is Warthin's tumor, which is a benign tumor consisting of a papillary cystic structure covered by a double epithelial layer cells and lymphoid stroma with germinal center. Several cases have reported the Warthin's tumor transformation into a malignant tumor such as lymphoma that develops from their stromal. Expression of cytotoxic T-lymphocyte antigen 4 (CTLA-4) as part of the immune checkpoint when highly expressed leads to a more rapid development or progression of tumors.* **Purpose:** *To analyze CTLA-4 expression in Warthin's tumors associated with the pathogenesis of its growth through an escape mechanism from immune checkpoints and analyze based on CTLA expression whether this marker has the potential to be used as immunotherapy by administering anti CTLA-4.* **Methods:** *The tissue sections slides of Warthin's tumor (n=8) were stained with Hematoxylin Eosin and immunostained with Recombinant Anti-CTLA4 antibody [CAL49] (ab237712). The slide with positive CTLA-4 is shown as staining on the cell membrane and/or cytoplasm. Observations were carried out using Optilab. The result is presented as figures.* **Results:** *Tumor cells expressed of CTLA-4 show in cytoplasm and/or cell membranes of the epithelial and stromal components of Warthin's lymphoid. CTLA-4 is expressed lymphoid stroma, which is associated with inhibition of T cell activity against tumor cells, while the exact mechanism of CTLA-4 expression in epithelial components is not known but is thought to induce tumorigenesis and inhibit apoptosis.* **Conclusion:** *CTLA-4 is expressed in epithelial and stromal cells of Warthin's tumor and this expression indicates that Warthin's tumor cell growth is through the escape mechanism of the CTLA-4 check point immune. Further research is necessary to investigate whether CTLA-4 expression in lymphoid stroma has relate to their transformation toward a malignant tumor of lymphoma.*

Keywords: *cytotoxic T-lymphocyte antigen-4 (CTLA-4); immune escape; Warthin's tumor*

Correspondence: Mei Syafriadi, Department of Biomedical Sciences, Faculty of Dentistry, University of Jember, Jl. Kalimantan No. 37, Jember, 68121 Indonesia. Email: didiriadihsb@gmail.com

INTRODUCTION

Warthin's tumor is one of the tumors whose incidence rate ranks second among benign salivary gland tumors after pleomorphic adenoma tumors, which is 5%-22% of all neoplasm in the parotid gland.[1,2] Warthin's tumor has a characteristic histopathological, a papillary pattern consisting of a two-layered epithelial structure and a cystic lumen as well as a tumor stroma containing lymphoid tissue with a germinal center.[2,3] Although this tumor is not a malignant tumor, several cases have reported malignant tumors such as lymphoma can develop from the stroma of Warthin's tumor.[3,4]

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) is a surface receptor associated with immune blockade mechanisms in tumor cells.[5] CTLA-4 is one of the immune checkpoints. Tumor cells can evade immune defense by producing excess ligand protein molecules that function as immune checkpoints, so that the excess ligand protein produced by tumor cells can be used by tumor cells to avoid recognition and attack by the immune system.[6,7]

CTLA-4, which is normally produced by cells, functions as an inhibitory receptor to downregulate early-stage T-cell activation. It is well known that T cell activation requires antigen recognition by the T cell receptor to produce CD 28 protein which is stimulated by B7 protein. CD28 and

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CTLA-4 have the same ligand, namely B7 but CTLA-4 has a stronger affinity for B7. The stronger the stimulatory signal resulting from the binding of T cell receptor (TCR) CD28 to B7 on the surface of the antigen presenting cell will induce the production (upregulated) of CTLA-4. If CTLA-4 binds to B7, it will inhibit the immune system from fighting cancer cells (Figure 1).[8]

CTLA-4 which is expressed by tumor cells is triggered by infiltration of conventional T cells or Treg cells and can also be expressed by tumor cells themselves.[9,10] In addition, it was stated that CTLA-4 is considered a "leader" of the inhibition of early activation of T cell, Thus, it plays a very important role in the defense mechanism of the immune system.[11] Expression of CTLA-4 as part of the immune checkpoint when highly expressed leads to a more rapid development or progression of tumors.[12]

Based on the above description, this study aims to understand whether Warthin tumors express CTLA-4 and use the CTLA-4 pathway to inhibit tumor infiltrating cells in order to escape from immune checkpoints for their growth and progression and also to understand the possible use of immunotherapy based on the location of CTLA-4 expression in these tumor-forming cells. Several studies reported that anti-CTLA-4 therapy can be used as immune therapy in certain malignant tumors because CTLA-4 immunotherapy inhibitors of monoclonal antibodies with immune checkpoint blockade mechanisms are being studied and developed.[8–10]

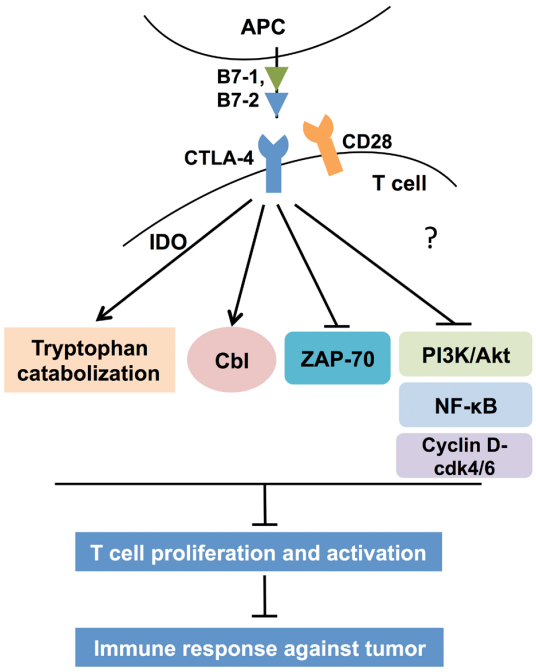

Figure 1. Mechanism of CTLA-4 functions in tumors. CTLA-4 shares the same B7 ligands as CD28, including B7-1 (CD80) and B7-2 (CD86) with a negative effect on T cell activation. After activation of the TCR, CTLA-4 induces idoleamine-2, 3-diocygenase (IDO), promotes expression of the Casitas-B-lineage lymphoma (Cbl)-b protein, inhibits the formation of zeta-associated protein 70kDA (ZAP-70) and also induces inhibitory PI3K/Akt, cyclin D3-cdk4 /6 and NF-κB for negative regulation of T cell proliferation and activation by producing inhibitory signals to attenuate immune responses (Source: Zhao et al).[8]

MATERIALS AND METHODS

This research uses a retrospective method and has received approval from the ethics committee at the Dentistry Faculty, University of Jember No. 1272/UN25.8/KEPK/DL/2021. The samples were paraffin embedded block cases Warthin's tumor patients who diagnosed at the Anatomical Pathology Laboratory, Dr. Soebandi Hospital, Jember and have been treated. The sample selection was carried out using a purposive total sampling technique and obtained eight cases of Warthin's tumor and two cases breast cancer grade III as the positive control of primary antibody. All paraffin embedded-tissue block were cut 4 µm in thick and stained with Hematoxylin Eosin (HE) to confirm the diagnosis of sample and immunohistochemistry (IHC) Anti-CTLA-4.[13]

Immunohistochemical staining was done according to datasheet protocol. All tissue samples after being dried for 3x24 hours on a slide warmer then followed by a series of processes such as removal of paraffin wax, rehydration, and washing with phosphate buffered saline (PBS) pH 7.4. The antigen retrieval site was carried out using a citrate buffer solution (pH 6) which was heated in an autoclave at 121°C for 15 min, following by cooling for 30 min at room temperature. All samples then washed in PBS. Then added drops of H_2O_2 block at room temperature for 10 minutes to inhibit endogenous peroxidase, following by intense washing with PBS. All tissue samples were covered by protein block at room temperature for 10 min and rinsed with PBS. Sections were applied with primary antibody Recombinant Anti-CTLA-4 antibody [CAL49] (ab237712) (Abcam, Cambridge, England) in a ratio of 1:100, then incubated at 4°C overnight and rinsed with PBS. Added Biotinylated Goat Anti-Rabbit IgG (Abcam, Cambridge, England) for the sections, incubation for 10 min at room temperature and rinsed with PBS. Followed by adding streptavidin peroxidase for 10 min at temperature room and adding 3,3'-diaminobenzidine (DAB) chromogen solution (Abcam, Cambridge, England) on the surface of tissue samples and incubating at temperature room for 10 min. Tissue samples were counterstained with Mayer's Hematoxylin as long as 1 min. The tissue samples were dehydrated in a graded series of alcohol (96% ethanol, 100% ethanol), cleared with xylene, covered with mounting medium and deck glass. This observation was carried out by four observers consisting of two anatomical pathology specialists, one consultant Oral and Maxillofacial Pathology specialist and one researcher. The positive expression of PD-L1 was determined as the cytoplasm and/or membrane of immune cells and tumor cells stained by DAB, while the cell nuclei stained purple

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(basophilic) by Hematoxylin. Immunohistochemical and HE staining were evaluated by using Binocular Microscope (Olympus CX43, Tokyo, Japan) and digitally scanned using Optilab (Miconos®, Yogyakarta, Indonesia). The result is presented as figures.

RESULTS

This study assessed the expression of CTLA-4 protein in Warthin's tumors. Based on histopathological analysis with HE staining, it was found that all samples (100%) were Warthin's tumors showing the structure of bilayer oncotic epithelium and lymphoid stroma. All case samples of Warthin's tumor showed immunopositive of CTLA-4 (100%) both in the epithelial cells and in the lymphoid stroma (Figure 2), When viewed in the lymphoid stroma, even though they were immunopositive in the germinal center and dense lymphoid components of CTLA-4, they were expressed with weak to strong expression (Figure 3). In addition, Figure 2 also shows that the expression of dense lymphoid components is less than that of the germinal center. Expression of CTLA-4 was seen in the cytoplasm and/or cell membranes of both epithelial and stromal lymphoid cells. Of the eight case samples of Warthin's tumor, all of the epithelial cells (100%) expressed CTLA-4 in the cytoplasm while six samples (75%) of the epithelial cells expressed CTLA-4 in the cell membrane and there was anomalous expression of CTLA-4 in the epithelial cell nuclei (Figure 4).

DISCUSSION

Based on the results of CTLA-4 Immunohistochemical staining, all samples showed a immunopositive expression of CTLA-4 in tumor epithelial cells (oncocytes like cells) and lymphoid stroma. In T-cells, CTLA-4 was a downregulate of T-cell immune function that could inhibit the early stages of T-cell activation. High CTLA-4 expression leads to more rapid development or progression of tumors.[12] In the early phase of tumorigenesis, CTLA-4 could decrease T cell activity by producing inhibitory signals to weaken the immune system against tumors through binding of CTLA-4 to CD80/CD86.[8] The presence of high expression of CTLA-4 in lymphoid stroma might be related to the pathogenesis of Warthin's tumor, which could transform to malignancy, such as Malignant Lymphoma. There have been several hypotheses regarding Warthin's stromal tumor to date. Thus, further research was needed on the function of the lymphoid stroma of Warthin's tumor and the relationship between CTLA-4 and malignancy.[8]

Tumor cells have the ability to escape immune cells associated with PD-L1/PD-1 and CTLA-4 overexpression.[5] The presence of CTLA-4 expression in the stroma of Warthin tumors (lymphoid and/or germinal centre area) is thought to have a role in the immune escape mechanism. That is, it is likely that the germinal center containing lymphocyte cells in Warthin's stromal tumor is part of the tumor whose function is already abnormal and is not able to block the growth of tumor cells.[8]

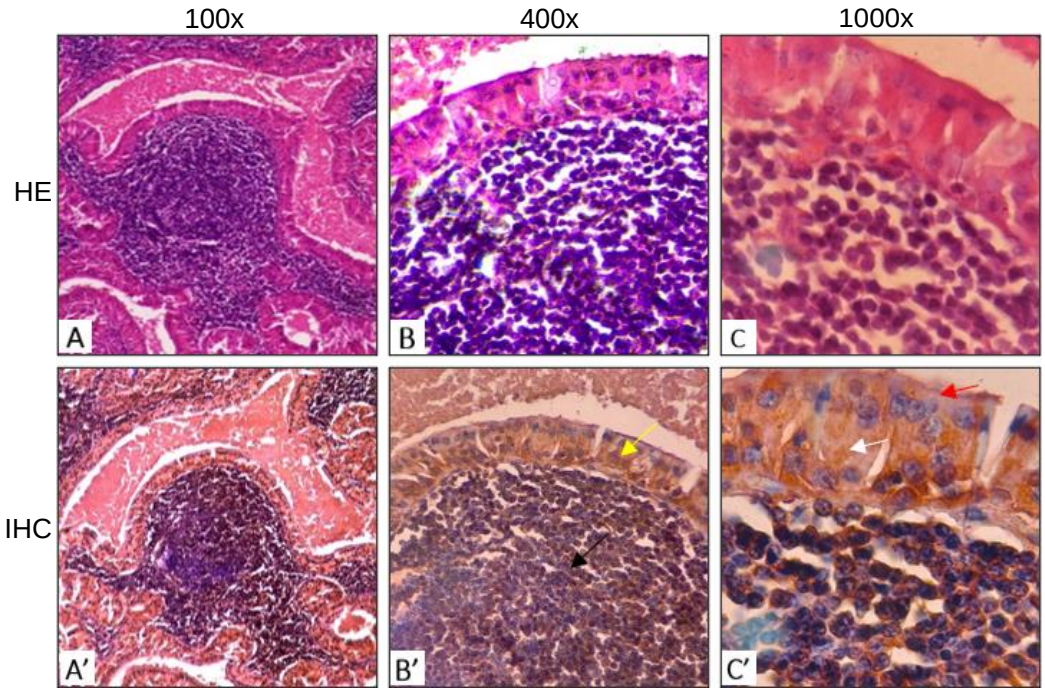

Figure 2. CTLA-4 overexpression in epithelial and stromal cells of Warthin's tumor. Figure (A-C) shows HE staining and image (A'-C') shows IHC staining. On IHC staining, the expression of CTLA-4 protein was stained as brown (A'). CTLA-4 expression is seen in epithelial (yellow arrow) and stromal cells (black arrow) (B'). Warthin's tumor. CTLA-4 expression appears both in the program (white arrow) and cell membrane (red arrow) (C').

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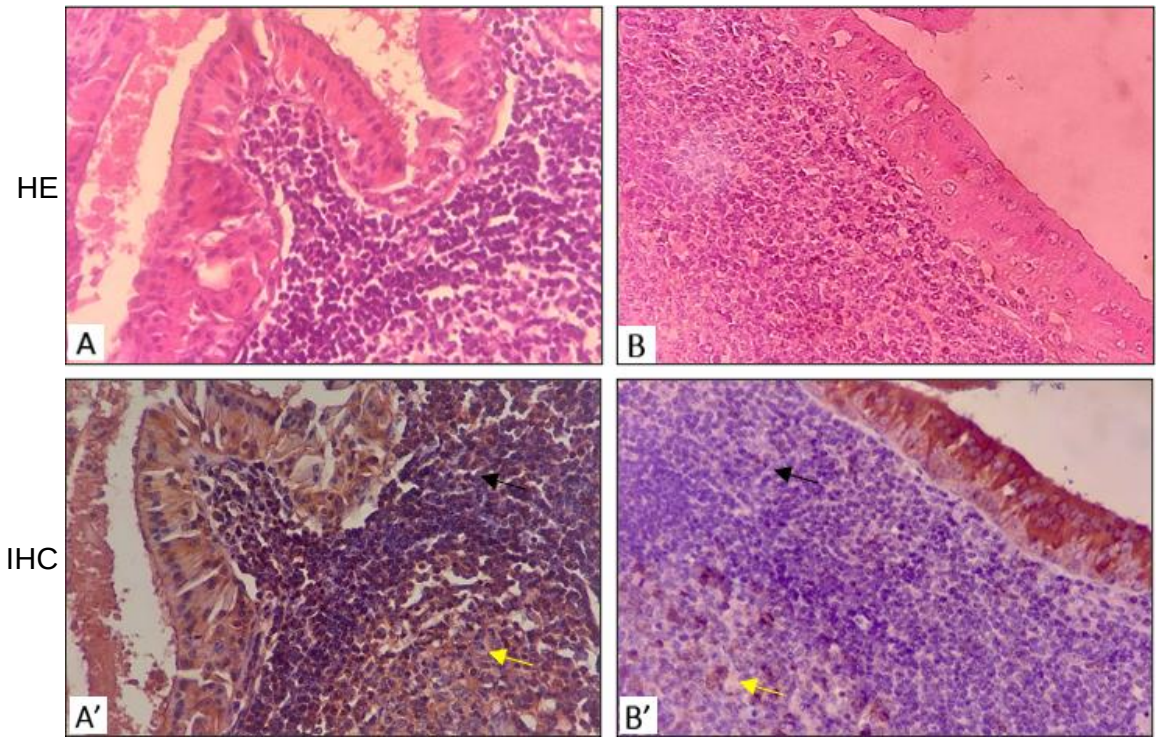

Figure 3. CTLA-4 expression in the lymphoid stroma of Warthin's tumor. Figure (A-B) shows HE staining and image (A'-B') shows IHC staining at 400x magnification. Figure (A') shows CTLA-4 overexpression in the dense lymphoid component (black arrow) and germinal center (yellow arrow). Figure (B') shows less CTLA-4 expression in the dense lymphoid component (black arrow) than in the germinal center (yellow arrow).

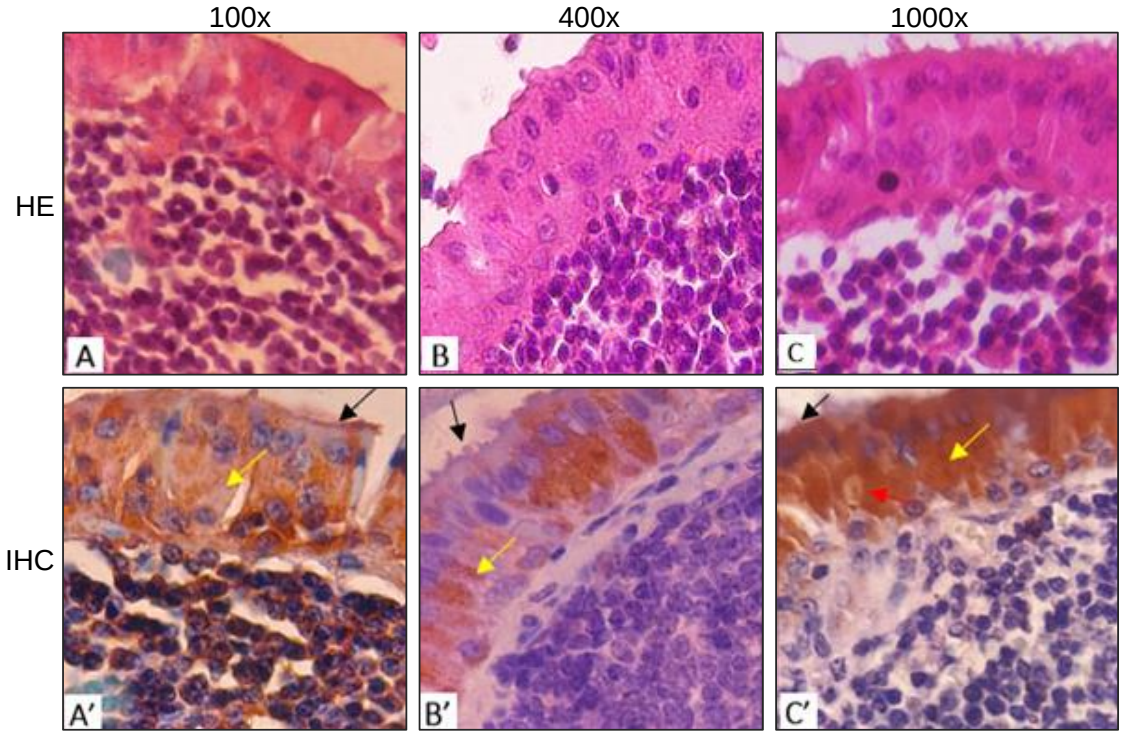

Figure 4. Expression of CTLA-4 on Warthin's tumor epithelial cells. Figure (A-C) shows HE staining and image (A'-C') shows staining at 1000x magnification. The figure (A') shows the expression of CTLA-in the epithelium in the cytoplasm (yellow arrow) and cell membrane (black arrow). The figure (B') shows the expression of CTLA-4 on the epithelium in the cytoplasm (yellow arrow) and not on the cell membrane (black arrow). The figure (C') shows the expression of CTLA-4 on the epithelium in the cytoplasm (yellow arrow), membrane (black arrow), and cell nucleus (red arrow).

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In the lymphoid stroma of Warthin's tumor, expression of CTLA-4 could be found in dense lymphoid components and germinal centers. CTLA-4 is usually distributed in the germinal center and mantle zone of the lymphatic follicles.[14] There were also samples that showed CTLA-4 expression in the dense lymphoid component which was less than the germinal center. Although many literatures suggest that CTLA-4 is expressed on T cells, it is possible that the cells that express CTLA-4 in the germinal center are B cells. CTLA-4 expression on B cells is thought to be involved in the process of inhibiting the production of IgM, IgG, and IgE. However, the specific mechanisms driving the expression and function of CTLA-4 B cells have not been fully accepted beyond their T cell boundaries.[15] Moreover, until now there have not been many references that discuss the relationship between CTLA-4 and the germinal center in a tumor, so further research is needed regarding it and its relation to the lymphoid stroma in Warthin's tumor.

In some samples, CTLA-4 was not expressed homogeneously in the lymphoid stroma. There were several reasons why CTLA-4 is not expressed in T cells. First, the expression level of CTLA-4 could be so low that it is not expressed in T-rest cells. CTLA-4 predominantly appears to be due to T cell activation.[16] Second, the non-expression of CTLA-4 might also be related to mutations in the gene encoding the CTLA-4 protein in the q33 band of chromosome 2.

In addition to the lymphoid stroma, there was a positive expression of CTLA-4 in oncocytic epithelial cells. Until now, the mechanism regarding this is still unknown.[17] Zhang et al.[18] reported that CTLA-4 expression in melanoma cells induces tumorigenesis and inhibits apoptosis.

CTLA-4 expression on epithelial cells also showed strong expression. Cases of malignant transformation in Warthin's tumor are more common from the lymphoid component than from the epithelial component.[19] Thus, further research is needed to determine whether the high expression of CTLA-4 in epithelial oncocytic cells is indeed related to the transformation of Warthin's tumor to malignancy.

T cells or other tumor cells that express intrinsic CTLA-4 tumor cells are considered cells that have had different functions than T cells or other cells; therefore, studies with immuno-4 immuno-4 can bind to the intrinsic CTLA-4 tumor cells and activate the Epidermal Growth Factor Receptor (EFGR) pathway to induce programmed death-ligand 1 (PD-L1) expression so as to trigger apoptosis of tumor cells.[20] The expression of CTLA-4, Programmed Death-1 (PD-1), PD-L1, and EGFR in tumor cells can predict the response of therapeutic immunosupnomic treatments by administering anti-PD-1 or anti-PD-L1 as predictive biomarkers of immunotherapy success. Thus, the expression of CTLA-4 in Warthin's tumor cells, both in tumor epithelial cells and in lymphoid stroma, may have the potential to be a better treatment response.

In this research, it showed CTLA-4 was expressed predominantly in the cytoplasm in both epithelial and stromal lymphoid cells. In T cells themselves, CTLA-4 expressed in the cytoplasm or cell membrane can be seen by assuming that the brown color that approaches or touches the T cell nucleus; this matter indicates the presence of positive CTLA-4.[21] CTLA-4 staining was predominantly cytoplasmic although rarely expressed on the membrane.[16] Structurally, CTLA-4 was the same as CD28, but CTLA-4 was an intracellular protein that can rotate between the cell surface and cytoplasm, in contrast to CD28 which is expressed on the cell surface.[11]

There was also CTLA-4 in the study showing strong expression in the nucleus of Warthin's tumor epithelial cells. This possibility is related to the formation of CTLA-4 proteins due to gene mutations which mean proteins cannot be transported out of the nucleus and accumulate in the nucleus. In the protein synthesis process, the transcription step was carried out in the cell nucleus and produced mRNA, which will be released through the pores of the cell nuclear membrane to the ribosomes by tRNA.[22] This unbalanced or dysfunctional tRNA problem will be related to the pathogenesis of tumor growth where tumor cells will be uncontrolled proliferation, and this is what is characteristic of a neoplasm. It is also known that tRNA abundance can affect mRNA abundance, so that RNA overexpression in cancer can increase protein synthesis.[23] When the amount of tRNA is reduced, there is nothing to carry the mRNA out to the ribosomes, and accumulation occurs in the cell nucleus. Thus, it is possible that there is a relationship between the expression of CTLA-4 in the epithelial cell nucleus of Warthin's tumor with the presence of gene mutations and further research is needed on this matter.

From the results of this study, it can be concluded that CTLA-4 is expressed in epithelial and stromal cells of Warthin's tumor and this expression indicates that Warthin's tumor cell growth is through the escape mechanism of the CTLA-4 check point immune, and it is possible that CTLA-4 expression in these tumor cells can be used as a marker for immunotherapy by administering anti CTLA-4. However, the potential for CTLA-4 to be used as an immunotherapy needs further research.

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