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Immunohistochemical Detection of TNF-α and IFN-γ Expressions in the Lungs of Sheep with Pulmonary Adenocarcinomas

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Abstract

This study aimed to examine the tumor necrosis factor alpha and interferon gamma expressions in the tumor microenvironment of ovine pulmonary adenocarcinomas of different growth patterns and stages by immunohistochemistry and to investigate the effects of these cytokines on the tumor progression. The material of the present study consisted of lung tissue samples of 26 sheep. Lung tissues were fixed in 10% neutral buffered formalin, and later, routine procedures tissues were embedded in paraffin wax. To detect histopathological changes, 5-µm tissue sections were cut and stained with hematoxylin and eosin. Avidin-biotin peroxidase method was used for immunohistochemistry . Tumoral cells showed acinar, papillary, or mixed-type growths in ovine pulmonary adenocarcinomas. No expressions of tumor necrosis factor alpha and interferon gamma was observed in the control group, whereas all ovine pulmonary adenocarcinomas were immunohistochemically positive for tumor necrosis factor alpha and interferon gamma. In advanced-stage cases, the reactions were much more severe than in early-stage cases. The reactions were particularly concentrated in the cytoplasm of alveolar macrophages around the tumoral areas. Tumor necrosis factor alpha and interferon gamma can be remarkable markers to evaluate the severity of disease.

Keywords: Cytokines, IFN-γ, inflammation, ovine pulmonary adenocarcinoma, TNF-α

Introduction

Ovine pulmonary adenocarcinoma (OPA), also known as Jaagsiekte, is a contagious neoplastic lung disease of sheep caused by jaagsiekte sheep retrovirus (JSRV), an exogenous betaretrovirus (Karagianni et al., 2019; Shi et al., 2021). Jaagsiekte sheep retrovirus induces neoplastic transformation of lung epithelial cells such as type 2 pneumocytes and club cells and shares many histological features with human lung cancers in terms of growth patterns (De Las Heras et al., 2021; Lee et al., 2017; Toma et al., 2020).

Increasing evidence supports a strong link between inflammation and the development of cancer (Hsieh et al., 2021; Xie et al., 2014). Inflammatory cells in the tumor microenvironment and cytokines that exhibit a broad spectrum of biological activity can enhance tumor growth and progression and suppress anti-tumor responses (Boldrini et al., 2006, Hsieh et al., 2021; Larruskain et al., 2015).

Tumor necrosis factor alpha (TNF-α), a multifactorial proinflammatory cytokine produced primarily by macrophages, is associated with a wide range of cellular responses, ranging from regulating cell survival, cell proliferation, cell differentiation, cell death, apoptosis, inflammation, and immune activity (Deniz et al., 2018; Zhu et al., 2018). Tumor necrosis factor alpha, which is released from malignant cells and cells in the tumor microenvironment, has been reported to have both pro-tumorigenic and anti-tumorigenic activity in animal model studies (Gong et al., 2021; Ohri et al., 2010; Shang et al., 2017).

Interferon gamma (IFN-γ), an important pleiotropic cytokine, plays a dominant role in immunity against intracellular pathogens and has antiviral activity against numerous viruses (Karachaliou et al., 2018; Larruskain et al., 2012). Interferon gamma is released by cytotoxic T cells and type 1 T helper (Th1) cells that can exert broad pro- or anti-tumor effects on the immune response (Kamimaki et al., 2021; Zhao et al., 2020). The anti-tumoral activity of IFN-γ can be briefly summarized as enhancement of cancer-specific immune effects on dendritic cells, natural killer cells, and T cells; induction of anti-proliferation and anti-angiogenesis; recruitment of tumor-infiltrating macrophages; and pro-apoptotic effect against cancer cells (Higgs et al., 2018; Teranishi et al., 2020).

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The mechanism underlying the poor immune response of sheep to JSRV infection is not fully evaluated (Archer et al., 2012; Karagianni et al., 2019; Summers et al., 2005). In this study, we aimed to examine the TNF-α and IFN-γ expressions in the tumor microenvironment of OPAs of different growth patterns and stages by immunohistochemical methods and to investigate the effects of these cytokines on tumor progression.

Methods

Ethical Approval

This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (Date: June 24, 2021. KAU-HADYEK-202 1/109).

Tissue Samples

The material of the present study consisted of 26 lung tissue samples (control group: 6 sheep and OPA group: 20 sheep) from the paraffin block archive of the Pathology Department. Because tissues were selected from paraffin blocks, no gross pathology findings were available. The cases had been taken between years 2009 and 2017 in Kars, Turkey. Control group consisted of six cases which died of non-pulmonary problems, and no lung lesions were present. Histopathological examination and immunohistochemical staining were used as golden standard when selecting OPA group tissues. Both histopathological examination and immunohistochemical methods were performed and only positive cases with both methods were included in the study. Histopathological and immunohistochemistry results of the cases were compliant, and all cases were positive for each method.

Histopathological Examinations

Lung tissues were fixed in 10% neutral buffered formalin (Merck, Darmstadt, Germany) and, following routine procedures, tissues were embedded in paraffin wax (Merck). Five-micrometer tissue sections were cut and stained with hematoxylin and eosin (H&E) (Merck) to detect histopathological changes. Sections were examined under a light microscope and photographed. Histopathological classification and staging of the tumors were based on Yener et al. (2016).

Immunohistochemical Examinations

The routine streptavidin–biotin-peroxidase complex method was used according to the manual instructions of the kit (Thermo Fisher Scientific, San Diego, California, USA, Histostain-Plus IHC Kit, HRP, broad spectrum, REF: TP-125-HL). Anti-JSRV-CA antibody (supplied by Massimo Palmarini, polyclonal, dilution ratio: 1/1500), anti-TNF-α antibody (MyBioSource, San Diego, California, USA, MBS2025729, monoclonal, dilution ratio: 1/100), and anti-IFN-γ (MyBioSource, MBS2091397, polyclonal, dilution ratio: 1/100) were used after antigen retrieval and nonspecific protein blocking. The reactions were detected with aminoethyl carbazole (AEC) chromogen (Thermo Fisher Scientific, TA-125-HA). Counterstainings were conducted using hematoxylin. Glass slides were then mounted with AEC special adhesive and coverslip. For control sections, Phosphate Buffered Saline (PBS) was applied instead of the primary antibodies.

Slides were examined under a light microscope (Olympus, Tokyo, Japan, Bx53) and photographed via the Cell^P program (Olympus, Soft Imaging Solutions GmbH). Analyses of the images were done with Image J Program (National Institutes of Health, Maryland, USA, 1.51j8). Inflammation, JSRV-CA, TNF-α, and IFN-γ expressions were analyzed by examining ten representative fields of labeled neoplastic cells under ×200 magnification. Fields were chosen among areas where immunostaining was most intensive. Rating system was designated as negative (–): 0%, low (+): 1–10%, moderate (++): 11–59%, or severe (+++): >60% cells stained positive (Beytut, 2017).

Results

Histopathological Results

No histopathological lesions were found in the lungs of the sheep in the control group, and the tissues preserved their normal histological structure. Histopathological types, tumor stages, and inflammation scores of the OPA group are given in Table 1. No correlation was observed between tumor types and stages. Of the 20 cases, 10 were acinar (50%), 6 were papillary (30%), and 4 were mixed (acinar and papillary, 20%). Of the 20 cases, 16 were diagnosed as advanced-stage (80%) and 4 as early-stage (20%) OPA. Tumor foci of varying sizes were observed in the lung tissues of sheep belonging to the OPA group. Neoplastic epithelial cells were proliferated in the bronchiolar and alveolar areas. Tumor cells were mostly cuboidal or columnar in type, with growth patterns as acinar in some areas and papillary extensions in others (Figure 1a-d). It was remarkable that these cells did not disrupt the alveolar wall structure and proliferated in a lepidic pattern. Mitotic figures were very few. The thin tumor stroma around the alveolar and bronchiolar structures was thickened in some areas due to the proliferation of mononuclear cells and connective tissue. Numerous very large alveolar macrophages were concentrated in the peritumoral areas. In some cases, there was extensive neutrophil infiltration.

Immunohistochemical Staining Results

JSRV-CA, TNF-α, and IFN-γ positivity scores of OPA groups are shown in Table 1. The control group was negative for JSRV-CA, TNF-α, and IFN-γ expressions. JSRV-CA positivity was detected in the cytoplasm of cuboidal or columnar cells that formed acinar and papillary tumoral foci, especially in the alveolar and bronchiolar lumens (Figure 2a and b). Intracytoplasmic TNF-α-positive reactions were much more predominant in alveolar macrophages. In addition, the connective tissue cells and mononuclear cells forming the tumor stroma and endothelial cells were positive for TNF-α expression. Acinar type tumors were the most severe cases in terms of TNF-α expression. In advanced-stage cases, the reaction was more severe than in early-stage cases. In advanced cases, tumor cells and peritumoral alveolar macrophages were immunohistochemically positive (Figure 3a-f). Similar to TNF-α results, IFN-γ immunoreactivity was more intense in alveolar macrophages in the peritumoral area. In addition to alveolar macrophages, positive immunohistochemical reactions were also detected in cells in the tumor stroma and in the cytoplasm of neoplastic cells forming acinar/papillary structures. In some cases, IFN-γ immunoreactivity was detected only in tumoral cells, while in some cases, there was IFN-γ expression only in alveolar macrophages. IFN-γ expression was quite severe in cases of acinar type OPA and weaker in the papillary type cases. In advanced-stage cases, the reaction was quite strong in macrophages closer to the tumoral areas compared to the early-stage cases. In addition, the severity of the reaction was weak in inflammatory cells far from the tumoral areas (Figure 4a-f).

Discussion

Various immune factors such as cytokines and inflammatory cells are important factors influencing tumor progression through the

Table 1.
JSRV-CA, TNF-α, and IFN-γ Positivity Scores of OPA Group

Case No	Histopathological Type	Stage	Inflammation	JSRV-CA	TNF-α	IFN-γ
Case 1 (OPA)	Acinar	Advanced	+++	+++	+++	++
Case 2 (OPA)	Acinar	Advanced	+++	+++	++	+++
Case 3 (OPA)	Papillary	Early	+	++	+	++
Case 4 (OPA)	Acinar	Advanced	++	+	++	+++
Case 5 (OPA)	Acinar + papillary	Advanced	+++	+++	++	++
Case 6 (OPA)	Acinar	Advanced	++	+++	+++	++
Case 7 (OPA)	Papillary	Early	++	+++	+	+
Case 8 (OPA)	Acinar + papillary	Advanced	++	++	++	+++
Case 9 (OPA)	Papillary	Advanced	+++	+++	++	++
Case 10 (OPA)	Acinar	Advanced	+++	+++	+++	+++
Case 11 (OPA)	Acinar	Advanced	+++	+++	++	+++
Case 12 (OPA)	Acinar	Advanced	++	+	+++	++
Case 13 (OPA)	Acinar + papillary	Advanced	++	+	+	++
Case 14 (OPA)	Papillary	Advanced	+++	+	+++	++
Case 15 (OPA)	Papillary	Early	+	+++	+	+
Case 16 (OPA)	Acinar + papillary	Advanced	++	+++	++	+++
Case 17 (OPA)	Acinar	Advanced	++	++	+++	+++
Case 18 (OPA)	Acinar	Advanced	++	++	+++	+++
Case 19 (OPA)	Papillary	Early	+	+	+	+
Case 20 (OPA)	Acinar	Advanced	+++	+++	+++	++

Note: JSRV = jaagsiekte sheep retrovirus; TNF-α = tumor necrosis factor alpha; IFN-γ = interferon gamma; OPA = ovine pulmonary adenocarcinoma.

differentiation of immune cells and their chemotactic effects (Wang et al., 2020). Tumor necrosis factor alpha is a major inflammattory cytokine produced by tumor cells and/or tumor-associated immune cells. It also plays a critical role in inflammation, immune response, and cancer development (Gong et al., 2021; Hsieh et al., 2021). Depending on the context, its role against different types of cancer varies. Tumor necrosis factor alpha can also trigger other forms of programmed cell death, such as apoptosis or necroptosis, and has been used as anti-tumor agent for advanced soft tissue sarcoma, melanoma, colon carcinoma, and renal carcinoma (Deniz et al., 2018; Kumar et al., 2010; Zhu et al., 2018). Conversely, constitutive TNF-α expression in the inflammatory tumor microenvironment has been suggested to induce cancer cell survival and proliferation pathways and plays a protumorigenic role by promoting angiogenesis, tumor

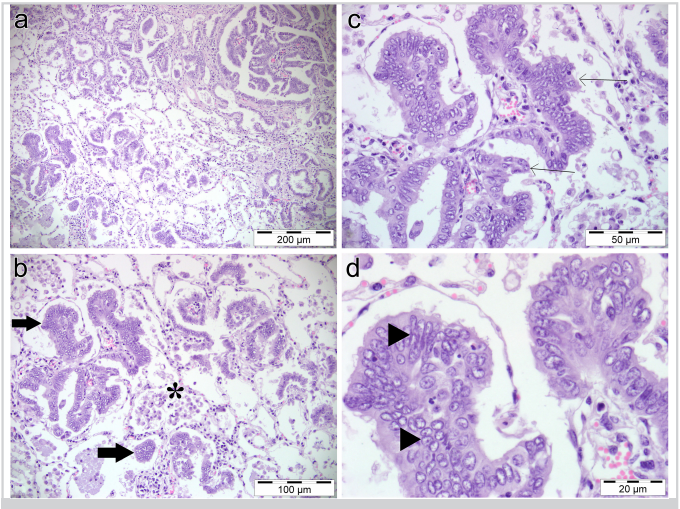

Figure 1.
(a) Lung Tissue, Large and Small Tumoral Areas, H&E, Bar = 200 µm. (b) Acinar Proliferations in Alveolar Lumens (Arrows) and Peritumoral Alveolar Macrophages (Star), H&E, Bar = 100 µm. (c) Papillary Proliferations of Neoplastic Epithelial Cells (Thin Arrows), Bar = 50 µm. (d) Tumor cells of Cuboidal and Columnar Types (arrowheads), H&E, Bar = 20 µm. Note: H&E = Hematoxylin and Eosin.

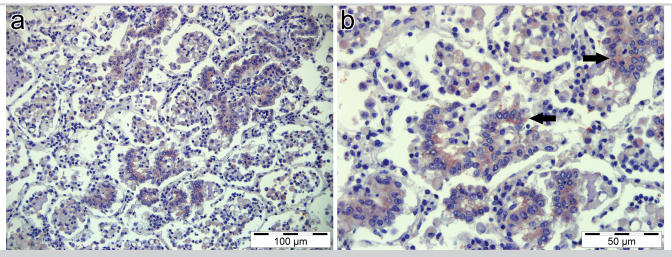

Figure 2.
JSRV-CA, IHC, AEC. (a-b) Intracytoplasmic Positive Reactions in Tumoral Foci (Arrows) Localized Within the Alveolar Lumens. Note: JSRV = Jaagsiekte Sheep Retrovirus; IHC = Immunohistochemistry; AEC = Aminoethyl Carbazole.

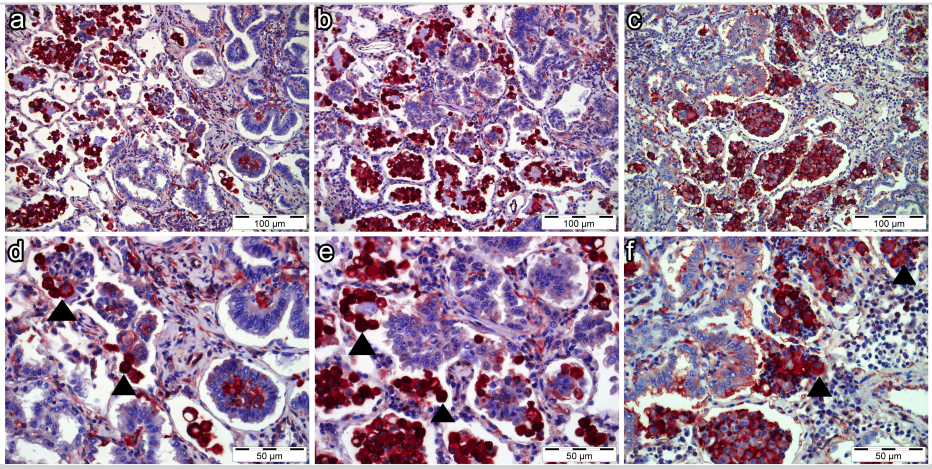

Figure 3.
TNF-α, IHC, AEC. (a, d) Early-Stage OPA. Intracytoplasmic Positive Reactions in Alveolar Macrophages (Arrowheads). (b, e) Advanced-Stage OPA. Severe Expression in Alveolar Macrophages (Arrowheads). (c, f) Advanced-Stage OPA. Intense Intracytoplasmic Immunoreactivity in Tumoral Cells, Tumor Stroma, and Alveolar Macrophages (Arrowheads). Note: TNF-α = Tumor Necrosis Factor Alpha; IHC = Immunohistochemistry; AEC = Aminoethyl Carbazole; OPA = Ovine Pulmonary Adenocarcinoma.

cell migration, and invasion (Hsieh et al., 2021; Xie et al., 2014). In addition, TNF-α-mediated inflammation increases tumor growth and inhibition of TNF-α reduces tumor progression (Zhu et al., 2018).

In the literature review, no study was found in which TNF-α expression in OPAs was evaluated by immunohistochemical methods. In veterinary medicine, only two studies evaluated TNF-α gene analyses in OPA (Karagianni et al., 2019; Larruskain et al., 2015). Karagianni et al. (2019) reported that TNF is downregulated in host–gene expression. Additionally, according to their RNA-Seq analysis results, these authors thought that JSRV infection induces significant immunological changes in lung tissue, including an increase in macrophages associated with tumor foci, and altered expression of numerous cytokines, chemokines, and complement factors. They commented that local immune-modulatory mechanisms might also suppress the immune response against JSRV. In human medicine, there are many studies examining TNF levels in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) (Boldrini et al., 2006; Kumar et al., 2010; Ohri et al., 2010; Shang et al., 2017; Wang et al., 2020; Xie et al., 2014). Wang et al. (2020) reported that inflammatory factors such as TNF-α, interleukin 2 (IL-2), and interleukin 6 (IL-6) play an important role in NSCLC progression. Shang et al. (2017) found that TNF-α expressions were significantly increased in NSCLC groups compared to control groups, both in serum and in tissues. In addition, they revealed that TNF-α levels were positively correlated with proximal and distant tissue metastases. In another study, Xie et al. (2014) suggested an essential link between the TNF-α gene 308G>A polymorphism and the risk of lung cancer in Asians. In a different

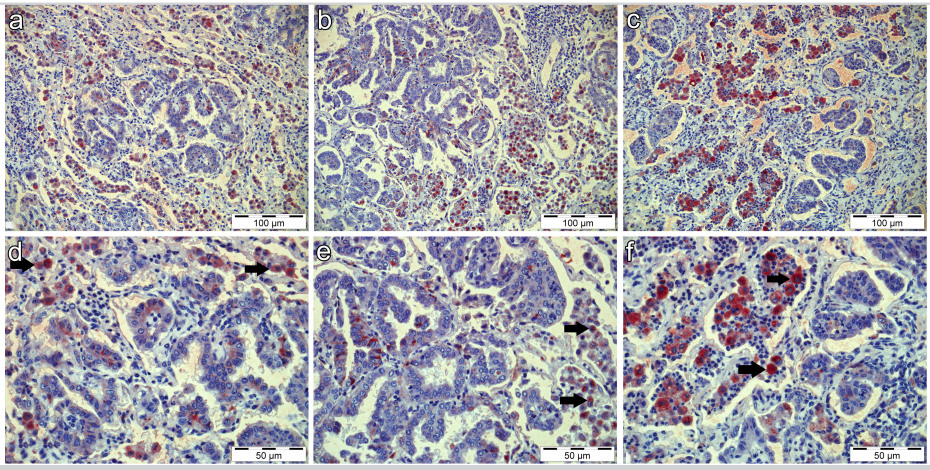

Figure 4.
IFN-γ, IHC, AEC. (a, d) Early-Stage OPA. Intracytoplasmic Immune Positive Expressions in Alveolar Macrophages (arrows). (b, e) Advanced-Stage OPA. Dark Red Positive Reactions in the Cytoplasm of Alveolar Macrophages (Arrows) in the Peritumoral Area. (c, f) Advanced-Stage OPA. Severe Positive Immunoreactivity in the Cytoplasm of Macrophages (arrows) Near Tumor Foci. Note: IFN-γ = Interferon Gamma; IHC = Immunohistochemistry; AEC = Aminoethyl Carbazole; OPA = Ovine Pulmonary Adenocarcinoma.

study, Ohri et al. (2010) noted that expressions of TNF-α in tumor islets of patients with NSCLC were associated with improved survival, suggesting a role in the host anti-tumor immunological response. Kumar et al. (2010) reported that TNF-α expression increased in the NSCLC group compared to the control group. Still, TNF-α levels were not correlated with survival and chemotherapy response, and TNF was not a reliable marker in this context. Boldrini et al. (2006) found a positive correlation between high TNF expression and favorable prognosis in patients with NSCLC. Consistent with the literature data (Kumar et al., 2010; Shang et al., 2017), TNF-α expressions, which were negative in the control group, also increased significantly in OPA cases in this study. The current study determined that TNF-α expression was quite severe in alveolar macrophages in the tumor microenvironment, especially in advanced-stage and acinar type cases. A positive increase in TNF-α expression correlated with tumor stage was interpreted as the contribution of this cytokine to the progression of OPA.

Interferon gamma, a key pleiotropic cytokine produced by NK, CD4+, and CD8+ T cells, has a variety of physiological functions and orchestrates the immune response (Chung et al., 2014; Higgs et al., 2018; Huang et al., 2018). Interferon gamma performs dramatic antiviral, anti-tumor, and immunomodulatory tasks by increasing the activity of immune cells, upregulating antigen presentation and pathogen recognition, inhibiting cellular proliferation, and increasing the sensitivity of tumor cells to apoptotic signals (Deniz et al., 2018; Zhao et al., 2020). It has been reported that IFN-γ, which has a direct cytotoxic and anti-proliferative effect against tumor cells, is effective in treating various neoplastic diseases such as prostate, renal cell carcinoma, melanoma, and ovarian cancer (Chen et al., 2012; Karachaliou et al., 2018). Contrary to these, different researchers have reported that IFN-γ can downregulate the anti-tumor response by promoting regulatory T-cells (Treg) function and suppressing effector T-cell function via enhancement of indoleamine 2,3-dioxygenase secretion from cancer cells and antigen-presenting cells (Kamimaki et al., 2021).

There are very few studies evaluating the IFN-γ expressions in OPAs in veterinary medicine (Karagianni et al., 2019; Larruskain et al., 2012; Larruskain et al., 2015; Summers et al., 2005; Summers et al., 2012). Karagianni et al. (2019) reported that IFN-γ expressions did not increase significantly in experimental OPA cases. However, RT-qPCR results showed that IFN-γ expressions increased in advanced natural cases. In a similar study, Larruskain et al. (2015) reported that three candidate genes IFN-γ, CCR5, and MX1 were significantly associated with OPA progression. In addition, they suggested that in the progression of the disease, the increase in IFN-γ expression, as well as the polymorphism in the gene, affects the process. In another study, Larruskain et al. (2012) genotyped four microsatellites located in the immune-related regions, the major histocompatibility complex (MHC) region, IFN-γ, and interleukin-12p35 to determine their association with maedi-visna and OPA viral diseases. These researchers found that the microsatellites in the MHC were the most diverse, while those in the cytokines were less polymorphic. In the case of IFN-γ, the study results revealed the presence of null alleles. In previous studies, Summers et al. (2005, 2012) reported that the expression of IFN-γ in macrophages closer to tumor areas was more intense than in macrophages far from tumor areas. These researchers detected that tumor cells were also weak in terms of IFN-γ immunoreactivity. In this study, very severe IFN-γ expression was detected in macrophages in the peritumoral areas, especially in advanced cases, in line with the literature data (Summers et al., 2005, 2012). In addition to macrophages, tumor cells and cells in the tumor stroma were positive for IFN-γ immunoreactivity. The increase in IFN-γ expression in the tumor microenvironment, especially in advanced cases compared to early-stage cases, suggested that this cytokine may be effective in OPA progression and tumor-related inflammatory response.

Conclusion and Recommendations

In conclusion, it is thought that the data obtained from the current study can contribute to the literature in terms of detailing the immune response and pathogenesis of OPA. The fact that the increase in TNF-α and IFN-γ expressions is more severe, especially in advanced cases, has the potential that these cytokines can be remarkable markers to evaluate the severity of disease. In future studies, it would be useful to evaluate the relationship between these cytokines and inflammation in the tumor microenvironment together with various pro- and anti-inflammatory interleukins to examine in a more complex way how they contribute to the development of the disease.

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Declaration of Interests: The authors declare that they have no competing interest.

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