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Evaluation of the relationship between inflammatory reaction and interleukins in ovine pulmonary adenocarcinomas

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Abstract

In this study, it was aimed to investigate the association between inflammatory reaction of tumoral microenvironments with interleukin responses in ovine pulmonary adenocarcinomas (OPAs). Material of the study consisted of 26 sheep lung tissue samples being brought to the Pathology Department for routine diagnosis. Cases were collected between years 2009 - 2021; pre-diagnosis was based on clinical symptoms, anamnesis and gross lesion of the lungs. These tissues were designated in two groups as control (n = 6) and OPA (n = 20) groups. Choice of immunohistochemical staining was avidin-biotin peroxidase method. Reverse transcription polymerase chain reaction (RT-PCR) was used to confirm Jaagsiekte sheep retrovirus from paraffin-embedded tissues. On gross examination of OPAs, lesions seen were mostly in the caudal lobes of the lung, 1.00 - 2.00 cm in diameter as gray-white consolidated foci and in microscopic observation, tumor cells showed acinar, papillary or mixed growths. No expressions of interleukin (2 and 8) were observed in the control group. All OPAs cases were positive for interleukins (2 and 8) expressions. A total of eight tissue samples were detected as positives through RT-PCR. In conclusion, in this study, it was determined that interleukin-2 and interleukin-8 were produced from tumor microenvironment elements, especially tumor-associated macrophages, and these interleukins showed pro-inflammatory effects. Interleukins and the inflammatory reaction may promote the development of OPA.

Introduction

Ovine pulmonary adenocarcinoma (OPA) is a transmissible cancer affecting sheep lung tissues, and is caused by an exogenous betaretrovirus called Jaagsiekte sheep retrovirus (JSRV).[1-3] The OPA affects many sheep breeds as well as goats and moufflons all over the world. Onset of the infection follows a long sub-clinical course; disease symptoms are especially seen in sheep between the ages of 2 and 4 years.[4-6] The disease is mostly transmitted through the respiratory route and probably via milk and colostrum.[3,7] Loss of condition, respiratory distress and weight loss despite the presence of normal appetite are the main clinical symptoms being seen in OPA.[2,8,9] In advanced cases, drainage of the fluid collected in the lungs through the nostrils when the head of the animals is lowered is present. The JSRV mostly has a tropism to alveolar type 2 pneumocytes and rarely to Clara cells, leading to neoplastic transformation of epithelial cells.[3,4,10] The OPA is particularly similar to human bronchioalveolar cell carcinoma in its morphological, pathological and epidemiological features.[1,9] Anamnesis, clinical findings, gross pathology and histopathology (gold standard), electron microscopy, polymerase chain reaction (PCR), ultrasonography and computed tomography can be very useful in the diagnosis of OPA.[5,8,10]

Inflammation, one of the hallmarks of cancer, is critically associated with tumor development and progression.[11-13] Inflammation is a very complex process involving different cells and mediators.[14] Interleukins, one of the cytokines, play a central role in tumor development by regulating the expression of various molecules being involved in inflammation.[15-17]

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Interleukin-2 (IL-2) causing T-lymphocyte activation, proliferation, differentiation and growth is produced predominantly by T cells.[18-20] The IL-2 boosts $CD8^+$ T cell and natural killer (NK) cell cytotoxicity as well as differentiation of naive $CD4^+$ T cell into T helper-1 and T helper-2 cells.[21] Detection of IL-2 expression in tumoral areas is very useful for predicting tumor progression and prognosis in various cancer types such as renal cell carcinoma, metastatic melanoma and non-small cell lung cancer (NSCLC).[13,22] Interleukin-8 (IL-8), another pro-inflammatory cytokine, has a chemotactic effect on neutrophils and macrophages.[17,23] The IL-8 is over-expressed in various human cancers such as colon carcinoma, melanoma and ovarian and prostate cancers.[24] The IL-8 is also a potential angiogenic and autocrine growth factor and plays a serious role especially in the progression and metastasis of lung cancers.[15,25,26]

In this study, it was aimed to investigate the association of inflammatory reactions of tumoral microenvironments with interleukin responses in OPAs.

Materials and Methods

Animals. Twenty-six lung tissue samples were chosen as the study material. These tissues were taken from sheep being brought to Pathology Department for routine diagnosis between years 2009 - 2021. Pre-diagnosis of the animals was based on clinical symptoms, anamnesis and gross lesion of the lungs. These tissues were designated in two groups as control (n = 6) and OPA (n = 20) groups. This study was approved by the Kafkas University Animal Experiments Local Ethics Committee, Kars, Türkiye (KAU-HADYEK-2021/109).

Nucleic acid extraction from paraffin-embedded tissues and reverse transcription PCR (RT-PCR) of the samples. Nucleic acid was acquired from paraffin-embedded tissues as described by Pikor *et al.*[27] This method is intended for DNA extraction; but total RNA is also propagated. Differing from the original method, we did add RNase A to use the viral RNA being present in the aqueous phase. The RT-PCR is selected to confirm the histopathological findings. Following the extraction, PCR of the samples was conducted with primer pair under conditions specified by Mansour *et al.*[28] However, there were minor changes in protocols being described formerly. Reverse transcription of samples was performed separately with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA). The PCR was performed with DreamTaq DNA Polymerase (Thermo Scientific, Waltham, USA). Both products were used according to manufacturer's datasheets. Electrophoresis of the PCR products was done using 1.00% agarose gel stained with ethidium bromide (Sigma-Aldrich, St. Louis, USA) via 100V current. Products were visualized under ultraviolet-based imager.

Histopathological examination. Following gross examinations, lung tissues were fixed in 10.00% neutral buffered formalin (Sigma-Aldrich) and routinely processed. Following routine procedures, tissues were embedded in paraffin wax (Sigma-Aldrich). For histopathological analyses, paraffin sections of 5.00 µm thickness were stained with Hematoxylin and Eosin (H&E; Merck, Darmstadt, Germany). Light microscope (Bx53; Olympus, Hamburg, Germany) is also used for examination and taking photographs. Inflammation was analyzed by examining five representative fields of labelled inflammatory cell infiltrates with the 20 X magnification. Rating system was designated as negative (–; 0.00%), low (+; 1.00 - 10.00%), moderate (++; 11.00 - 59.00%) or severe (+++; > 60.00%).

Immunohistochemical examination. The routine streptavidin–biotin peroxidase complex method was used according to the manual instructions of kit (Histostain-Plus, Thermo Scientific). Anti-IL-2 (Biorbyt, Cambridge, UK) and polyclonal anti-IL-8 (MyBioSource, San Diego, USA) antibodies were used after antigen retrieval and non-specific protein blocking. The reactions were detected with aminoethyl carbazole chromogen (Thermo Scientific). Hematoxylin is also used for counterstaining. After this procedure, glass slides were mounted with Entellan (Merck) and a coverslip. For control sections, phosphate-buffered saline was applied as drops on the sections instead of the primary antibodies.

Prepared slides were examined under the light microscope and photographed via the CellΛP Software (Olympus). Analyses of the images were done with ImageJ Program (1.51j8, LOCI, University of Wisconsin). The IL-2 and IL-8 expressions were analyzed through examining five representative fields of labelled immune positive cells with the 20X magnification. Rating system was designated as negative (–; 0.00%), low (+; 1.00 - 10.00%), moderate (++; 11.00 - 59.00 %) or severe (+++; > 60.00%).

Statistical analysis. Statistical analysis of the results was performed using the SPSS Software (version 26.00; IBM Corp., Armonk, USA) program. According to the cell infiltration scoring, Kruskal-Wallis H test was used for multiple comparisons of IL-2 and IL-8 and Mann-Whitney U test was used for pairwise comparisons. Obtained results were given as mean ± standard error. The $p < 0.05$ expression was considered statistically significant in the evaluation of the results.

Results

Gross findings. The lesions were mostly in the caudal lobes of the lung, 1.00 - 2.00 cm in diameter as gray-white consolidated foci. The lesions had a glossy or ground-glass appearance and did not form a distinct bulge in the pleura. The presence of diffuse bright gray-white colored nodular structures of varying sizes on the

cross-sectional surface of the lung was remarkable. In some cases, purulent or verminous pneumonia lesions were also present (Figs. 1A and 1B).

Polymerase chain reaction results. A total of eight samples had appropriately sized amplicons and deemed as positives (Fig. 2).

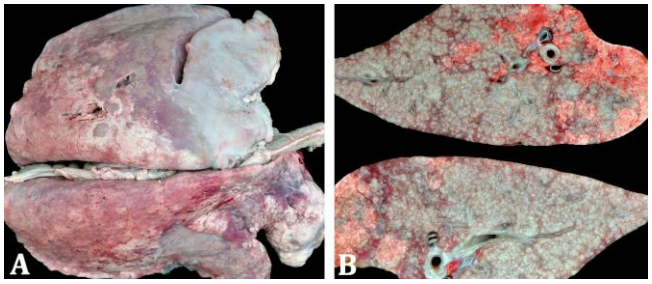

Fig. 1. A) Consolidated areas of grayish (ground glass appearance) in the cranial and dorso-caudal regions of the lung, not forming a prominent bulge in the pleura; **B)** Whitish nodular lesions with prominent protrusion on the cross-sectional face of the lung.

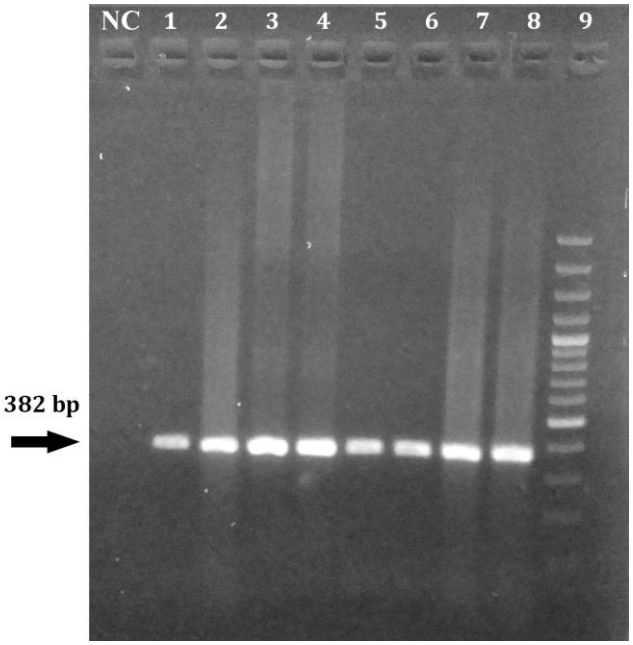

Fig. 2. Image of the amplicons after polymerase chain reaction for Jaagsiekte sheep retrovirus. Expected amplicon size is situated at 382 bp and negative control (NC) is placed in further left lane; eight samples are placed in lanes 1 to 8; and DNA ladder (100 bp plus, Thermo Scientific) is placed in lane 9.

Histopathological findings. No histopathological lesions and inflammatory cell infiltration were detected in the lungs of sheep from control group. Two cases (of 20) had metastases to lymph nodes. In addition, 16 of 20 cases were diagnosed as advanced stages; only four cases were classified as early stage OPAs. Disseminated tumor foci of varying sizes were observed in the lung tissues of sheep belonging to the OPA group. Tumor cells, mostly cuboidal or columnar, were found to form acinar and papillary neoplastic structures within the bronchioles or alveoli. In many tumoral foci, acinar and papillary structures were together. In addition to these reproduction patterns, there were fibromyxoid growths around the interstitium or bronchioles. While very severe inflammatory infiltration was observed around the tumoral structures in 12 of 20 cases, the severity of the inflammation was quite low in the remaining eight cases. Most of the inflammatory infiltration was alveolar macrophages. Other cells were lymphocytes, plasma cells and histiocytes. In some cases, intense neutrophil infiltration was also detected. It was observed that the stroma surrounding the tumoral areas thickened due to the presence of connective tissue and mononuclear cells (Figs. 3A and 3B).

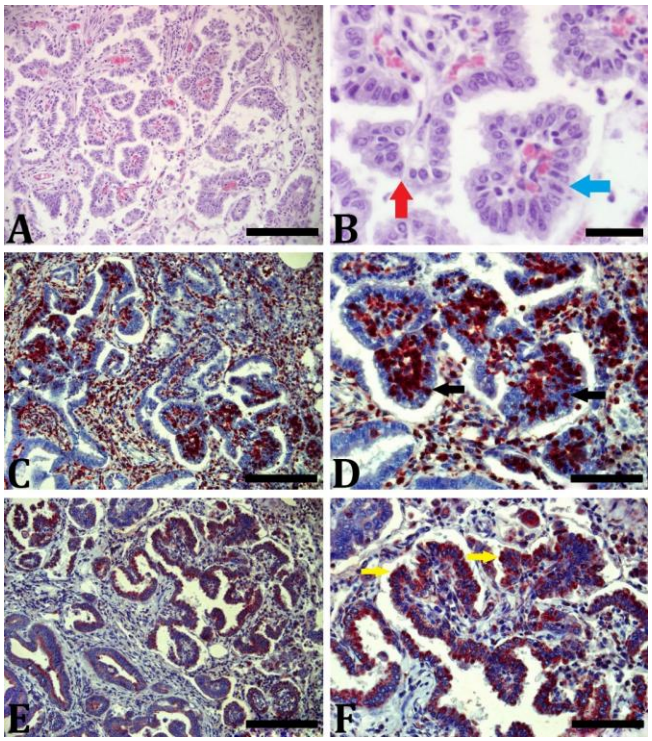

Fig. 3. A) Large tumor focus (H&E; bar = 100 μm); **B)** Higher magnification showing acinar (blue arrow) and papillary (red arrow) structures elongated into the lumen (H&E; bar = 20 μm); **C** and **D)** Interleukin-2 expressions in the cytoplasm of neoplastic cells forming papillary proliferation (black arrows), (H&E; bars = 100 and 50 μm, respectively); **E** and **F)** Intra-cytoplasmic interleukin-8 reactions in neoplastic cells (yellow arrows) within alveolar lumens (H&E; bars = 100 and 50 μm, respectively).

Immunohistochemical findings. The immuno-positivity scores of all groups are given in Table 1. The control group was negative for the expressions of IL-2 and IL-8. The IL-2 and IL-8 immunoreactivities in the OPAs with severe inflammation and the OPAs with low inflammation were statistically increased compared to the control group. Expressions were much more severe in advanced cases compared to early-stage cases. Positive reactions for all markers were present in almost the same

type of cells and same localizations. Immune positive reactions were detected in the cytoplasm of tumoral cells, inflammatory infiltration around the tumoral foci, especially in alveolar macrophages with large-sized cytoplasm located in these areas, and in fibrocyte, fibroblasts, endothelial cells and mononuclear cells forming the tumor stroma (Figs. 3C and 3F).

Table 1. Immunopositivity scores of all groups.

Groups (n)	IL-2	IL-8
Control (6)	0.00 ± 0.00[a]	0.00 ± 0.00[a]
OPA with low inflammation (8)	1.56 ± 0.18[b]	1.44 ± 0.18[b]
OPA with severe inflammation (12)	2.73 ± 0.14[c]	2.09 ± 0.09[c]
***p*-value**	< 0.001	< 0.001

IL: Interleukin; OPA: Ovine pulmonary adenocarcinoma.
[abc] Different letters represent statistically significant differences among the groups ($p < 0.001$).

Discussion

Gross pathology and histopathological findings are accepted as the gold standard in the diagnosis of OPA. As well, anamnesis, clinical findings, immunohistochemistry, electron microscopy, PCR analysis of blood or bronchoalveolar lavage fluid, ultrasonography and also computed tomography can be used to detect the disease.[5,8,10] The gross pathology and histopathological findings of the current study were consistent with the literature data. [1,4,6-8,10] Eight samples (of 20) were deemed as positives after PCR. It is likely that embedding and extraction processes caused degradation and decrease of the number of nucleic acids; therefore, some of the samples can be considered as false negatives.

About 25.00% of human cancers are related to chronic inflammation.[14] Inflammatory cells, immune cells and macrophages are mostly found in the tumor micro-environment.[22] Inflammatory mediators, another important component of the tumor micro-environment, play a serious role in tumor progression. In the tumor micro-environment, cytokines are released by immune, non-immune, or tumoral cells.[29]

The IL-2 is a cytokine mostly produced by $CD4^+$ T cells as well as $CD8^+$, NK and activated dendritic cells, and has beneficial effects on survival rates in many advanced cancer cases in anti-cancer therapies administered alone or in combination.[19] There is only one literature study in veterinary medicine investigating IL-2 in detail in sheep with pulmonary adenocarcinoma, and in this study Larruskain *et al.* detected a polymorphism in the IL-2 gene and thought that this gene might contribute to the progression of OPA.[3] In contrast, in humans, Bersanelli *et al.* reported that the use of IL-2 improves survival in patients with advanced NSCLC.[18] In a similar study, Tian *et al.* determined that IL-2 is an independent prognostic parameter of overall survival for NSCLC patients.[13] In another study, Xu *et al.* found that IL-2 is beneficial in tumor size reduction in a nude mouse lung cancer model.[21]

Mi *et al.* noted that the combination therapy of IL-2 or induced killer cells is highly effective in the treatment of NSCLC and positively affects the overall survival rate.[20] A study evaluating the behavior of IL-2 in patients with advanced NSCLC revealed that all relevant subjects showed higher serum IL-2 levels compared to control.[22] Li *et al.* reported that IL-2 is effective in the migration and progression of NSCLC.[22] In this study, IL-2 expressions were significantly increased in OPA groups compared to the control group, and it was observed that cells in the tumor micro-environment were immune-positive. In addition, IL-2 immunoreactivity increased in a direct proportion to the severity of the inflammation. According to Larruskain *et al.*, it is thought that IL-2 may play a role in the progression of OPA and this marker can be used in the diagnosis of the disease.[3]

The IL-8, an essential pro-inflammatory mediator involving in tumor development, acts as an angiogenesis growth factor, thereby promoting cell proliferation and angiogenesis.[17,23] In addition, it is involved in epithelial-mesenchymal transition and cancer cell stemness during tumor progression.[26] The IL-8 is critical for neo-vascularization and its over-expression is associated with angiogenesis, tumor progression, metastasis and poor survival in many human cancers such as NSCLC.[15,24,25] No literature study evaluating IL-8 expressions in OPA was found. Rafrafi *et al.* stated that the IL-8 promoter polymorphism is related to the risk of NSCLC.[23] Shiau *et al.* found that human papillomavirus type (HPV) 16 E6 up-regulates pro-angiogenic matrix metalloproteinase-2 (MMP-2) and MMP-9 by inducing IL-8 expression in lung cancer cells.[25] They reported that cytokines such as IL-8 induced by HPV infection promote growth and angiogenic and metastatic characteristics of infected cells. Umekawa *et al.* found that NSCLC patients have significantly lower plasma IL-8 levels after treatment than at diagnosis.[17] Sunaga *et al.* suggested that IL-8 is a potential prognostic marker for lung adeno-carcinomas.[26] Liu *et al.* also found that a wide variety of chemokines such as IL-8 is involved in the tumorigenesis of lung carcinomas, and over-expression of these chemokines indicates poor tumor staging.[15] Lee *et al.* suggested that IL-8 is a significant target in the treatment of advanced cancers.[24] According to the results of the current study, IL-8 immunoreactivity was significantly higher in OPA cases than control group, suggesting that IL-8 is a useful marker in the diagnosis of OPA, similar to human lung cancers.[26]

In conclusion, in this study, it was determined that various interleukins were produced from tumor micro-environment elements, especially tumor-associated macrophages, and these interleukins showed pro-inflammatory effects. Interleukins and the inflammatory reaction may promote the development of OPA. However, this study had a small sample size and only two inter-

leukins were investigated. More detailed studies and analyses are needed to confirm whether these parameters directly contribute to the tumoral progression of OPA (like IL-1, IL-6, IL-10 and IL-12). In addition, it was concluded that the data obtained from this study will contribute to the literature in terms of detailing the immune response against OPA. These interleukins are considered to be quite useful in the diagnosis of OPA.

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Conflict of interest

The authors declare that they have no conflict of interest.

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