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Overexpression of IL-6 and STAT3 may provide new insights into ovine pulmonary adenocarcinoma development

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Abstract

Background Ovine pulmonary adenocarcinoma (OPA) is caused by Jaagsiekte sheep retrovirus (JSRV) and is considered an important potential animal model for human lung cancer. The precise mechanisms of OPA oncogenesis are still uncertain. The transcription factor signal transducer and activator of transcription 3 (STAT3) is activated by interleukin-6 (IL-6) in many cancers, but this aspect is unknown in OPA. We therefore aimed to evaluate the expression of IL-6 and STAT3 in OPA for its potential role in pulmonary carcinogenesis.

Results Lung tissues from 9 grossly normal and JRSV-negative sheep and 20 cases of JSRV-positive OPA sheep were included in the study. Tissue samples were stained with antibodies against IL-6, STAT3, and JSRV-MA. IL-6 and STAT3 were further quantified in both groups using Western Blot (WB). Immunohistochemically, IL-6 was expressed in stromal, inflammatory, and epithelial cells in all cases of OPA, while STAT3 immunoexpression was restricted to epithelial cells. In the OPA group, the percentage of immunolabelled cells for STAT3 accounted for a mean value of 96%. Using the H-SCORE method, 95% of cases were considered positive for STAT3 expression. Control tissues showed multifocal and weak immunoexpression for both markers. Using WB analyses, a highly significant amount of both IL-6 ($p = 0.0078$) and STAT3 ($p < 0.0001$) proteins were present in lung neoplasms, by comparison to the control lungs.

Conclusions Our data showed overexpression of IL-6 and STAT3 in lung tissues from OPA compared to lungs from JSRV-negative sheep. These results suggest a potential role of IL6-STAT3 in OPA carcinogenesis.

Keywords Immunohistochemistry, Interleukin-6, Jaagsiekte sheep retrovirus, Oncogenesis, Signal transducer and activator of transcription 3, Western blot

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Background

Ovine pulmonary adenocarcinoma (OPA), also known as ovine pulmonary adenomatosis, or Jaagsiekte, represents a chronic, progressive and contagious neoplastic pulmonary disease in small ruminants, particularly in sheep [1]. The role of exogenous Jaagsiekte sheep retrovirus (exJSRV) in the oncogenesis of OPA is well-known [2–4], but the mechanisms underlying this process are not fully elucidated. OPA consists of neoplastic transformation of both type II pneumocytes and club cells, forming acinar and papillary structures, and occasionally containing myxoid growths [5].

OPA shares many similarities with human non-small cell lung cancer (NSCLC), including histological features and activation of common cell signaling pathways, representing an important animal model for understanding the mechanisms of pulmonary oncogenesis [6]. Additionally, the size and organization of human lungs are much closer to those of sheep, than other animal models, facilitating experimental approaches in this species [7].

In humans, NSCLC lung cancer is considered to be the most common lung cancer in the world, with an incidence of 1,350,000 new diagnosis each year and mortality of 1,180,000 deaths each year [8, 9]. In the last decade, research has been focused on evaluating new prognostic markers in lung cancer patients and for identification of potential therapeutic targets. Recently, the role of signal transducer and activator of transcription 3 (STAT3) in the initiation and growth of human lung cancer has been described [10]. STAT3 is an intracytoplasmic latent transcription factor, activated by tyrosine phosphorylation, which results in genetic activation [11]. STAT3 also induces the expression of factors that promote angiogenesis, such as vascular endothelial growth factor (VEGF), determining the infiltrative character and/or metastasis of lung cancer. In addition, STAT3 can also promote immunosuppression by activation of IL-10 and transforming growth factor beta (TGFβ) [12–14]. The oncogenic mechanism by which STAT3 is most commonly activated in cancers is due to mutations in the EGFR gene that regulates cytokine IL-6 expression, which in turn activates the gp130/JAK/STAT3 pathway [15]. In humans, IL-6 is found in increased amounts in approximately 40% of lung adenocarcinomas [16]. Furthermore, phosphorylated-STAT3 (p-STAT3) overexpression is significantly correlated with advanced TNM stages, lymph node metastasis, and poor overall survival of lung cancer patients. Thus, p-STAT3 is an important biomarker of poor prognosis in lung cancer [17]. In animals, STAT3 expression and activity are upregulated in various cancers, including canine lymphomas [18], prostate cancer [19], osteosarcoma [20], feline oral squamous cell carcinoma [21], and mammary tumors [22].

Although the IL-6-STAT3 pathway plays a critical role in human lung cancer, no studies have evaluated this pathway in naturally occurring pulmonary cancer in sheep. Due to the close similarities between OPA and human lung cancer, in this study, we aimed to evaluate the expression of IL-6-STAT3 in JSRV-related pulmonary adenocarcinoma and to explore the potential role of the STAT3 signaling pathway in OPA oncogenesis.

Results

Histological examination

Data regarding histological features of each pulmonary lesion included in this study are summarized in Additional file 1. In the control group, consisting of pulmonary tissue from 9 JSRV-negative sheep, no significant histological findings were identified. The alveoli of the normal lung were lined by poorly discernible type I pneumocytes, on a discrete basal membrane. Type II pneumocytes were easier observable, as rare cuboidal to polygonal cells, with a central round nucleus, intermingled with type I pneumocytes (Fig. 1a). The bronchioles were lined by simple columnar to cuboidal epithelium, underlined by a thin smooth muscle layer.

In the OPA group, the neoplastic lesions were characterized by highly cellular masses composed of cuboidal to columnar epithelial cells organized in variably sized acini, and occasionally showing papillary projections, associated with a variable amount of fibrovascular stroma. Based on WHO classification, the pulmonary tumors were classified as acinar ($n=14$) and papillary ($n=6$) adenocarcinomas [23]. The neoplastic cells showed a moderate amount of pale acidophilic, finely granular cytoplasm, and a moderate N/C ratio. Anisokaryosis and anisocytosis were mild to moderate. The nuclei were round to oval, centrally or paracentral located, with lacy chromatin and 1–2 distinct, basophilic, regular nucleoli. Mitoses were rarely observed. In some samples, the stroma was severely infiltrated with macrophages, small lymphocytes, few plasma cells and eosinophils (Fig. 1b).

Immunohistochemistry

The normal lung tissues showed no JSRV-MA immunoexpression. JSRV-MA immunolabeling was observed in neoplastic epithelial cells, in all OPA cases (Additional file 2).

For IL-6 all normal lung tissues ($n=9$), showed a diffuse and mild to moderate expression in the cytoplasm of both type I and type II pneumocytes and bronchial epithelial cells (Fig. 1c). The intensity of IL-6 immunolabelling was recorded as moderate (grade 2) in 3 cases (33.33%), and weak (grade 1) in 6 cases (66.66%).

In all cases of pulmonary adenocarcinoma ($n=20$), IL-6 expression was observed in the neoplastic epithelial cells, inflammatory cells associated with tumors, alveolar

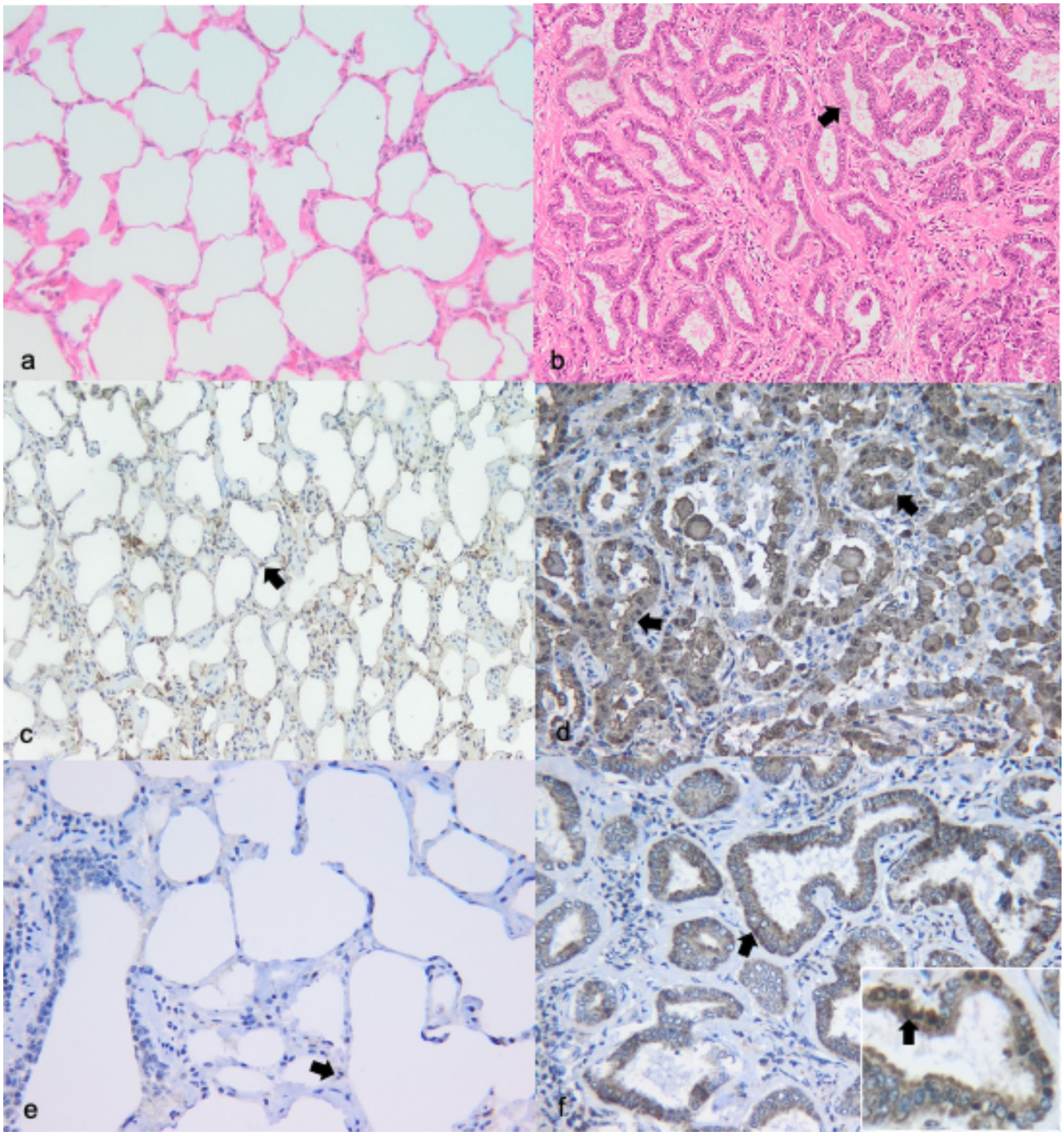

Fig. 1 (a-f). Hematoxylin and eosin and immunohistochemistry for IL-6 and STAT3. **a**. Normal lung tissue, control group, case 2. **b**. Pulmonary adenocarcinoma, acinar type, OPA group, case 3. The tumor is composed of cubical to polygonal cells arranged in variably sized and irregular acini (arrow), associated with moderate fibrovascular stroma. **c**. Normal lung tissue, control group, case 2. IL-6 immunoexpression was observed in rare type I and type II (arrow) pneumocytes with low intensity. **d.** Pulmonary adenocarcinoma, acinar type, OPA group, case 3. There is strong immunolabeling for IL-6 in the cytoplasm of almost all neoplastic epithelial cells (arrows). **e**. Normal lung tissue, control group, case 2. Mild immunolabeling for STAT3 in the cytoplasm of scattered type II pneumocytes cells is present (arrow). **f**. Pulmonary adenocarcinoma, acinar type, OPA group, case 1. There is strong immunolabeling for STAT3 in the cytoplasm of almost all neoplastic epithelial cells. Nuclear immunoreactivity (arrow) is also present in some areas of tumors (inset)

macrophages, and stromal cells (Fig. 1d). The percentage of IL-6 immunopositive cells was evaluated in the neoplastic tissue, and the intensity of expression was also recorded. The intensity was high (grade 3) in 45% of cases, moderate (grade 2) in 45% of cases, and weak (grade 1) in 5% of the examined samples. One case (5%) showed no reactivity for IL-6. In 6 cases, more than 50% of the neoplastic epithelial cells expressed IL-6, and in

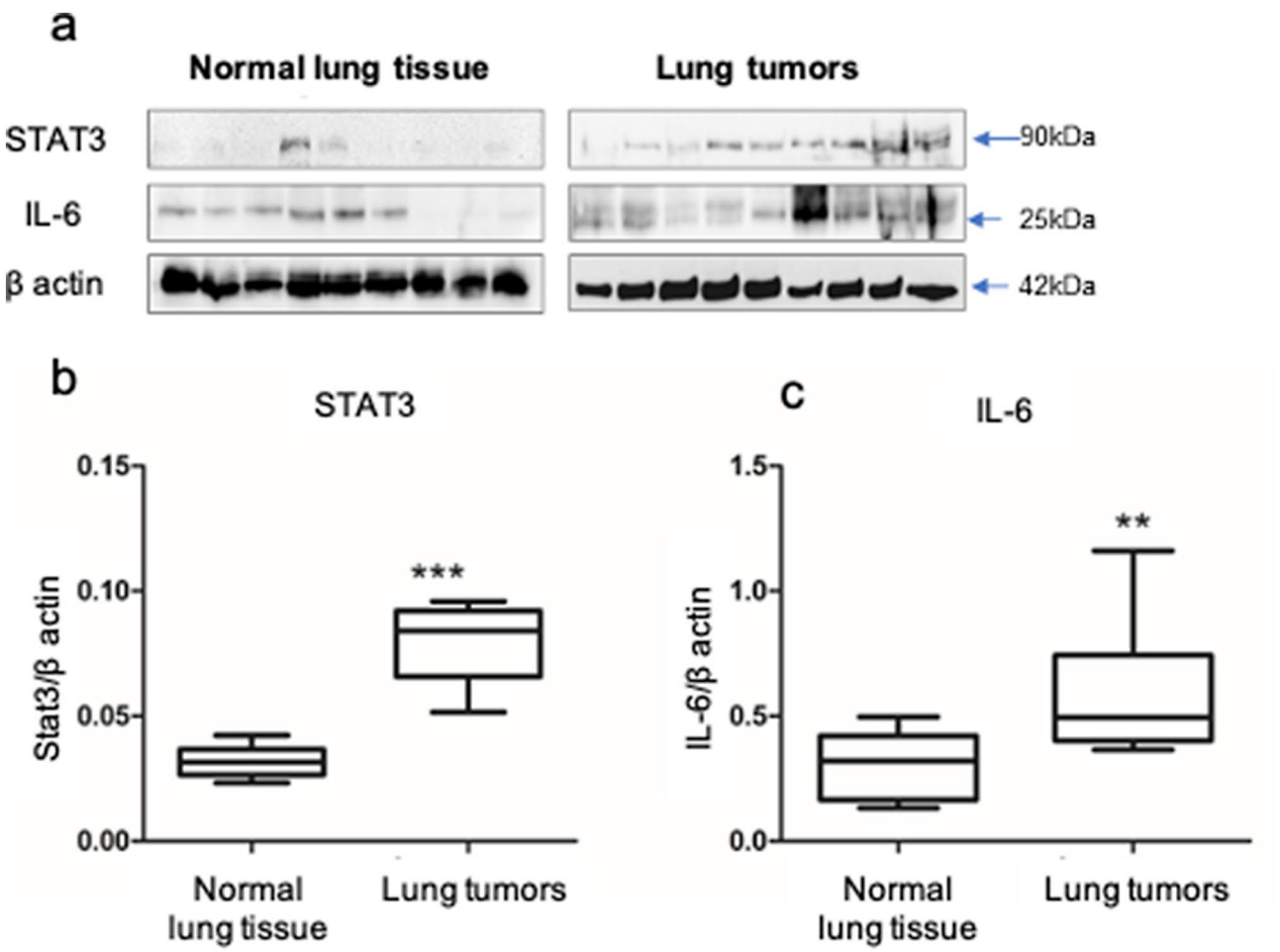

Fig. 2 **(a-c)**. Western Blot analysis of the protein expression of IL-6 and STAT3. Gels of the two groups were run separately, in parallel and in identical conditions. **a**. Image analysis of WB bands was done by densitometry, and results were normalized to β actin. Upper panel - WB images of normal lung tissue (left), and lung neoplasms (right). **b**. Graphical representation of the quantitative WB results shows a higher expression of IL-6 in lung tumors than in normal lung tissue. **c**. Graphical representation of the quantitative WB results shows a higher expression of STAT3 in lung tumors than in normal lung tissue. Each bar represents mean ± standard deviation ($n = 9$). ***= $p < 1e^{-3}$, **= $p < 0.01$, compared to control. Statistical significance of the protein expressions (lung neoplasm compared to control) was analyzed by Mann-Whitney nonparametric test

13 cases the percentage of labeled cells varied between 25% and 50%, thus being scored as grade 3 and grade 2 respectively. In 1 case less than 25% of the neoplastic epithelial cells expressed IL-6.

STAT3 was weakly expressed in all normal lung tissues. The intensity of the reaction was recorded as weak (grade 1) in all evaluated samples ($n = 9$) (Fig. 1e). In these cases, STAT3 immunolabelling was characterized by pale cytoplasmic expression in few type II pneumocytes and some bronchial epithelial cells. No nuclear labelling was observed.

In the OPA group, 5 cases (25%) showed high (grade 3) STAT3 cytoplasmic expression (Fig. 1f); 14 cases (70%) showed moderate (grade 2) immunolabelling, and 1 case (5%) showed weak (grade 1) immunolabelling. Nuclear labeling was present in all cases with an average of 1.71%, with the results ranging between 0.01 and 4.47%. The percentage of neoplastic epithelial cells that were positive for STAT3 was variable, accounting for a mean value of 96%, varying between 91.34% and 97.99%. Using the H-SCORE method, 19/20 (95%) cases were considered positive for STAT3 expression, and only 1 case (5%) was considered negative.

Western blotting

As seen in Fig. 2a, the protein levels of IL-6 and STAT3 in the normal lung tissue, were low. Comparatively, in the OPA group, STAT3 and IL-6 were significantly increased. Mann-Whitney test showed a significant difference between the two groups in what concerns IL-6 protein expression ($p = 0.0078$) (Fig. 2b) and STAT3 ($p < 0.0001$) (Fig. 2c). The original blots are available as Supplementary Information.

Discussion

Over the last few decades, numerous tumor markers have been investigated in human and veterinary medicine to improve the diagnosis, prognosis, and the monitoring of various types of cancers. Identifying new molecules and signaling pathways involved in lung carcinogenesis is still required for treatment outcomes and prognosis.

In human medicine, pulmonary cancer is classified into small-cell lung cancer (SCLC), originating from bronchial neuroendocrine cells, and NSCLC, originating from bronchoalveolar epithelial cells [24]. The most common type of NSCLC is represented by adenocarcinoma, accounting for 40% of cases [25, 26]. In sheep, OPA lesions are those of a well-differentiated bronchoalveolar adenocarcinoma with a papillary or acinar predominant growth pattern [27, 28]. In our cases, similar results were observed and the predominant histological type was represented by acinar.

The main signaling pathways involved in human lung cancer include the following: growth-promoting pathways (EGFR/Ras/PI-3-Kinase), growth inhibitory pathways (p53/Rb/P14ARF, STK11), apoptotic pathways (Bcl-2/Bax/Fas/FasL), DNA repair and immortalization genes [29–33]. In OPA, the Ras-MEK-ERK, PI3K-AKT-mTOR, EGFR, RON-HYAL2, and heat shock proteins are described as commonly activated pathways [34, 35]. Recently, increased gene expression of anterior gradient 2 (AGR2), amphiregulin (AREG), yes-associated protein 1 (YAP1) has also been reported, suggesting a role for Hippo pathway. However, significant differences have been observed between natural and experimentally infected cases [36]. In comparison to human lung cancer, an important and unique aspect of JSRV biology is the *env* protein that directly activates cellular signaling mechanisms leading to neoplastic transformation and proliferation [4, 6, 37]. Thus, JSRV is unique due to the *env* protein being directly oncogenic, as opposed to other viruses that cause neoplastic transformation by insertional mutagenesis or oncogene capture [4, 38]. Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway plays an important role in several physiological processes, such as immunity, cell growth, and differentiation [39]. The important role of the IL-6-STAT3 pathway has been intensely studied in human cancers, particularly in lung tumors [40, 41]. It has been demonstrated that STAT3 has multiple effects in pulmonary cancer including preventing apoptosis, promoting cell proliferation and angiogenesis, and helping tumor cells to evade anti-tumor immunity. JAK/STAT3 signaling is commonly activated by IL-6 and dysregulation of the interleukin IL-6-mediated JAK/STAT3 signaling pathway is closely related to the development of various human tumors and is often associated with poor patient outcomes [42]. According to the authors' knowledge, the IL-6 and STAT3 expression has not previously been evaluated in spontaneous OPA cases.

This study evaluated the immunoexpression of IL-6 and STAT3 in two groups of sheep pulmonary tissues: (1) healthy tissue (control group); and (2) neoplastic lesions (OPA group). By immunohistochemistry, IL-6 expression was observed in the neoplastic epithelial cells in 19 of 20 (95%) OPA cases, while STAT3 immunolabeling was demonstrated in all pulmonary adenocarcinomas (100%). In the healthy tissue control group, scattered epithelial cells showed weak expression of both IL-6 and STAT3. A statistical correlation was not performed between the control and OPA groups because the counting error for type II pneumocytes was considered high, in the absence of the double immunohistochemistry technique.

Furthermore, Western Blot analyses revealed upregulation of IL-6 ($p = 0.0078$) and STAT3 ($p < 0.0001$) in neoplastic tissue by comparison with normal lung tissue. This technique is based on molecular weight, which aims to identify specific proteins from a complex mixture extracted from cells [43]. Western blot analysis revealed bands at the appropriate molecular weight for both IL-6 and STAT3 (25 kDa and 90 kDa respectively), indicating that the two antibodies used in this study are correctly reacting with this species.

IL-6 participates in numerous signaling pathways one of which is Janus kinase (JAK) [44]. JAK is activated by ligation of IL-6 with IL-6R causing phosphorylation of STAT3. This phosphorylation starts homodimerization and allows STAT3 to enter the nucleus, where it affects the expression of STAT3 target genes, which encode proteins that play a role in cell proliferation (cyclin D1), and/or their survival (BCL2-like protein 1) [45]. STAT3 also induces IL-6 expression, thus causing a cyclic autocrine feedback mechanism [46].

Sources of IL-6 are variable, but in a neoplastic process, IL-6 is produced by several cell types including stromal cells, inflammatory cells, and neoplastic cells. In inflammatory processes, the main sources are macrophages and T cells [23, 24, 39, 43, 47–49]. High IL-6 serum levels were reported in human patients with pulmonary tumors including NSCLC [16, 50]. In the present study, we were able to show immunohistochemically that OPA neoplastic epithelial cells are also capable of producing IL-6. In addition to the neoplastic epithelial cells, IL-6 was also produced by alveolar macrophages within perineoplastic areas, intratumoral inflammatory infiltrates, as well as neoplastic stromal tissue. Alveolar histiocytosis is a commonly observed feature in OPA, and the influx of macrophages is most likely the first event following JSRV infection [30]. Even though distinguishing macrophage subtypes was not an explicit objective of this study, alveolar and intratumoral macrophages frequently immunoexpressed IL-6, suggesting that these macrophages may also

have an important role in IL-6-STAT3 expression in OPA cases. Recently, numerous genes related to macrophage immunoregulatory function were found to be activated in experimental and natural cases of OPA, suggesting an important role in the pathogenesis of the disease [36]. Moreover, IL-6 overexpression and increased levels of IL-2, IL-1β, IL-10, IL-12β, tumor necrosis factor alpha and interferon gamma were recently demonstrated in OPA microenvironment with tumor-associated macrophages and occasionally the neoplastic cells representing the main sources [51–53].

Previous studies of experimental OPA carcinogenesis showed that by applying JAK inhibitors, the neoplastic transformation of fibroblast cell cultures by JSRV env protein was not affected [56]. Western Blot analyses presented in this study showed significantly higher levels of IL-6 and STAT3 in the OPA group compared to the control group, and immunohistochemically the neoplastic cells expressed both markers. One possible explanation might be a difference in the microenvironments of cell culture versus cancer, and as mentioned earlier, IL-6 is produced in large amounts by inflammatory cells within the tumor microenvironment.

An increased STAT3 activity represents a negative prognostic marker in human neoplasms including lung cancer, particularly NSCLC [54, 55]. Therefore, the possibility of targeting the STAT3 pathway as a possible therapy for treating such tumors is the subject of an ongoing investigation.

The main limitation of this study is the lack of assessment of the phosphorylated variant of STAT3. However, the STAT3 antibody labels the total STAT3, and moreover, nuclear staining was observed in all cases from the OPA group, suggesting activation and translocation of STAT3 to the nucleus. Another limitation is the low number of animals in the study group, as only 20 animals with OPA were available. However, in the countries where is present, OPA has a low prevalence rate. The previously reported prevalence of affected animals within the Romanian sheep population was 1.26% [5]. Similar studies regarding the prevalence of OPA in sheep population are reported in other European countries. For instance, the prevalence of OPA in the United Kingdom in 2015 was reported as 0.9%, and 0.5% from 369 animals tested in the Republic of Ireland [56, 57].

Conclusion

Overall, the findings of this study demonstrated increased levels of both IL-6 and STAT3 in ovine pulmonary adenocarcinomas compared to normal pulmonary tissues, suggesting a potential role of these proteins in pulmonary carcinogenesis, as in human beings.

Methods

Samples and inclusion criteria

Twenty samples of sheep pulmonary tumors were collected after pulmonary masses were identified during routine meat inspection in two slaughterhouses from Transylvania (Romania). Additionally, pulmonary tissues from 9 sheep without gross lesions were post-mortem collected from the abattoir. All samples were subdivided and either fixed in 10% buffered formalin for microscopical investigations or the tissues were stored at -20 °C for molecular tests.

The selection criteria for samples included: (1) all pulmonary masses were grossly classified as the classical form of OPA according to Garcia-Goti et al. (2000) and Toma et al., (2019) [5, 58]; (2) all tumors were histologically diagnosed as pulmonary adenocarcinomas without myxoid growths [23, 59]; (3) JSRV infection was confirmed in all tumors by immunohistochemistry and PCR [5]; (4) JSRV-negative pulmonary tissues that had no significant histological changes were used as controls.

Histopathology and immunohistochemistry

For the histological analysis, tissues were routinely processed for paraffin embedding. The pulmonary neoplasms and hyperplastic changes were classified and described from 3-µm-thick hematoxylin-eosin–stained sections according to WHO classification [23, 59].

Immunohistochemistry was performed using the manual protocol and Novolink™ Polymer Detection System kit (Leica Biosystems). Briefly, the paraffin sections were dewaxed, followed by rehydration, epitope retrieval in sodium citrate buffer (pH-6), and protein block. Slides were incubated with anti-IL-6 rabbit polyclonal antibody (Abcam, ab6672), diluted 1:600, anti-STAT3 mouse monoclonal antibody (clone F-2, Santa Cruz Biotechnology, sc-8019), diluted 1:200, and anti-JSRV-MA (kindly provided by Prof. Massimo Palmarini, University of Glasgow), diluted 1:1500, overnight at 4°C, followed by placing the secondary antibodies. The immunolabelling was visualised using 3,3'-diaminobenzidine (DAB) chromogen (Novolink™ DAB, Leica Biosystems). The sections were counterstained with Mayer's haematoxylin.

All samples were independently evaluated by three pathologists (RP, CT and MT) using an Olympus BX-41 light microscope and the Stream Basic imaging software (Olympus Corporation, Tokyo, Japan). When there was a divergence of opinion, an agreed diagnosis was reached through simultaneous evaluation at a multi-head microscope (Zeiss Axio Scope A1).

For OPA group, STAT3 immunoexpression was assessed by the semi-quantitative H-SCORE method, which involves multiplying the percentage of labeled cells (0-100%) evaluated on three consecutive 40× fields, by the expression intensity (0, 1, 2 or 3). Thus the obtained

results were in a range between 0 and 300 [60]. The obtained score was considered positive if it was greater than 100, and negative if it was less than 100 [61]. The immunolabeling intensity was established after the initial evaluation of all the samples and graded as follows: (0) negative - absence of immunolabelling; (1) weak - low intensity, but diffuse cytoplasmic expression, variable observable at low magnification; (2) moderate - medium intensity, visible at low magnification; (3) high intensity, easily observable with 4× objective, representing diffuse and intense staining of cytoplasm of neoplastic cells.

IL-6 expression was also semi-quantitatively scored by assessing 5 consecutive high power fields, summing the percentage of positive cells with the staining intensity level. The percentage of positive cells was recorded as follows: (0) – the absence of marking, or less than 5%; (1) 5–25% of cells were labeled; (2) 25–50% labeled cells; (3) > 50% of the cells were labeled. The staining intensity was graded as: (0) negative; (1) weak; (2) moderate and (3) high. The obtained results were considered negative for scores between 0 and 2, and positive for scores between 3 and 6 [62].

Western Blot (WB) analysis

Fresh-frozen samples were selected for quantitative evaluation of the two proteins and included: 9 individuals from the control group and nine OPA. The tissue samples were prepared as previously described [63], and total protein content was measured by the Bradford method (Biorad, Hercules, CA, USA [64]. Lysates (20 µg protein/lane) from nine different normal lung tissue samples and respectively from nine different lung tumors were separated by electrophoresis on SDS PAGE gels and transferred to polyvinylidenedifluoride membranes, using Biorad Miniprotean system (BioRad). Gels were run in parallel in identical conditions. Blots were blocked and then incubated with antibodies against anti-IL-6-rabbit polyclonal to IL-6 (Abcam, ab6672, Cambridge, UK) and anti-STAT3 (F-2)-mouse monoclonal antibody (Santa Cruz Biotechnology, sc-8019, Heidelberg, Germany), clone F-2, then further washed and incubated with corresponding secondary peroxidase-linked antibodies (Santa Cruz Biotechnologies). Proteins were detected using Supersignal West Femto Chemiluminescent substrate (Thermo Fisher Scientific, Rockford IL, USA), and a Gel Doc Imaging system equipped with an XRS camera and Quantity One analysis software (Biorad). β actin (Santa Cruz Biotechnologies) was used as a protein loading control.

Statistical analysis

For Western Blot analysis, the statistical significance of the differences between the control group (normal lung tissue) and the pulmonary neoplasm groups was assessed with Mann Whitney nonparametric test. Statistical tests were performed using GraphPad Prism Software version 8.3.0 (San Diego, California). The results were considered statistical significant at $p < 0.05$.

Abbreviations

OPA	Ovine pulmonary adenocarcinoma
ExJSRV	Exogenous Jaagsiekte sheep retrovirus
NSCLC	Non-small cell lung cancer
STAT3	Signal transducer and activator of transcription 3
VEGF	Vascular endothelial growth factor
IL-10	Interleukin 10
TGFβ	Transforming growth factor beta
EGFR	Epidermal growth factor receptor
IL-6	Interleukin 6
gp130	Glycoprotein 130
JAK	Janus kinase
p-STAT3	Phosphorylated-STAT3
TNM	Tumour, Node, Metastases
JSRV	Jaagsiekte sheep retrovirus
WHO	World Health Organization
N/C ratio	Nuclear-cytoplasmic ratio
JSRV-MA	Jaagsiekte sheep retrovirus - matrix
SCLC	Small-cell lung cancer
Ras	"Rat sarcoma virus"
PI-3-Kinase	Phosphatidylinositol 3-kinase
Rb	Retinoblastoma protein
P14ARF	STK11
Bcl	2/Bax/Fas/FasL
DNA	Deoxyribonucleic acid
MEK	Mitogen-activated protein kinase
ERK	Extracellular signal-regulated kinases
AKT	Protein kinase B
mTOR	Mammalian target of rapamycin
RON	Recepteur d'origine nantais
HYAL2	Hyaluronidase-2
AGR2	Anterior gradient 2
AREG	Amphiregulin
YAP1	Yes-associated protein 1
VEGFR	Vascular endothelial growth factor receptor

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions

CT, CG RP, and AN were responsible for sample collection and histological diagnosis. CT was responsible for the original draft preparation, which was revised by MT and AW. LC performed the statistical analyses. IB performed the Western Blot analyses and interpretation. CT, DB, and IA participated in immunohistochemical interpretation and immunomarker quantification. MT participated in the sample collection, and pathological studies, and contributed to the design and supervision of the study. All authors read and approved the final manuscript.

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Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
The current study did not involve any live animal experiments. All samples used were collected from national approved slaughterhouses, therefore request from the ethics committee approval was not required.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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