Ovine pulmonary adenocarcinoma- epidemiology, diagnosis methods and mechanisms of oncogenesis

SUMMARY OF THE PhD THESIS

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INTRODUCTION

Ovine pulmonary adenocarcinoma has a unique feature in the animal series, being the only type of lung tumor produced by a viral agent (Jaagsiekte sheep retrovirus) (PALMARINI and FAN, 2003). Jaagsiekte sheep retrovirus exhibits tropism for type II pneumocytes and Clara bronchiolar cells (PLATT et al., 2002; Martineau et al., 2011). This pathology has been reported over time globally with the exception of some countries such as Australia and New Zealand (PALMARINI and FAN, 2003).

Ovine pulmonary adenocarcinoma has been reported in Europe in most countries, such as: Bulgaria (ENCHEV et al., 1958), Ireland (LEE et al., 2017), Italy (KWANG et al., 1995), Spain (MINGUIJON et al., 2013), Scotland (LEWIS et al., 2011), United Kingdom (COUSENS et al., 2015a), Cyprus (TOUMAZOS, 1989), Greece (SEIMENIS et al., 1983), Germany (VOIGT et al., 2007), France (SCHELCHER et al., 1991). Regarding Romania and the neighboring countries, there are no recent studies that confirm the presence of this pathology in the last decades. Moreover, previous reports in Romania did not identify the presence of JSRV in the examined samples (ADAMEȘTEANU et al., 1970; BABA et al., 1980), being also reported primary lung tumors in sheep, not induced by JSRV infection (LEE et al., 2017). Grossly, two forms have been reported: classical and atypical forms (GARCIA-GOTI et al., 2000), the latter being less commonly encountered (GRIFFITHS et al., 2010).

Currently, major concerns related to this pathology are related to the mechanisms of oncogenesis underlying the development retrovirus-induced lung tumors, which are incompletely elucidated (LIU and MILLER, 2007; YOUSSEF et al., 2015). Also, fragments of the JSRV genome have been identified in human lung tumors, assuming a potential role of JSRV in the oncogenesis of human lung tumors, but with inconsistent results (YOUSEM et al., 2001; LINNERTH-PETRIK et al., 2014).

Similarities related to the anatomy and physiology of the respiratory tract between humans and sheep, as well as the characteristics of sheep lung adenocarcinoma, may propose sheep as a perfect species to serve as an experimental model (YOUSSEF et al., 2015). Furthermore, human lung cancer causes an increased mortality rate, currently being ranked as the leading cause of death as a result of a neoplastic process (BASUMALLIK and AGARWAL, 2019; GEJMAN et al., 2019). In Romania, according to the GLOBOCAN database, in 2018 there are 11340 new cases of lung tumors and 10277 deaths (MANEA et al., 2019). Animal models that more accurately reflect certain human pathologies are of major importance for treatment-related and prognostic medical advances (GRAY et al., 2019).

THE STRUCTURE OF THE THESIS

In the literature there is few data regarding ovine pulmonary adenocarcinoma in Romania.

The thesis entitled "Ovine pulmonary adenocarcinoma - epidemiology, diagnosis methods and mechanisms of oncogenesis" contains 121 pages and is written according to the suggested norms, being structured in two parts.

The first part, represented by the review of the literature, is structured in 7 chapters and comprises 21 pages. In this part of the thesis it was synthesized the current general knowledge related to the main respiratory diseases in sheep, but focused on ovine pulmonary adenocarcinoma, including clinical, epidemiological, etiology, anatomical and histological classification, diagnostic methods, mechanisms of oncogenesis and the possible zoonotic character.

In the second part, extended on 63 pages and structured in 5 chapters, we have detailed the personal researches carried out between 2017 and 2020. Each chapter is divided into sub-chapters that present the aim and objectives, materials and methods used, the obtained results associated to discussions regarding their novelty and compared to other studies, and the partial conclusions made after each individual study. The results of the research were illustrated in a number of 28 figures and summarized in 10 tables. The end of the thesis is represented by the references that have been cited (284 titles).

RESULTS OF RESEARCH

In the second part of this paper, we studied primary lung tumors in sheep, collected between November 2017-May 2019 from two slaughterhouses in the Transylvania region (Romania): I.F. Fluieraş (Bungard locality, Sibiu county) and Agro-Invest Prod (Şieu Măgheruş locality, Bistriţa-Năsăud county). The main objectives of the study were:

• conducting an epidemiological study regarding the situation of ovine pulmonary adenocarcinoma and other pulmonary pathologies in Transylvania (Romania)

• grossly, cytological and histological evaluation of the identified pulmonary neoplasms, and immunohistochemical characterization

• identification of Jaagsiekte sheep reetrovirus (JSRV) using modern diagnostic methods, such as immunocytochemistry, immunohistochemistry, transmission electron microscopy, molecular (PCR) examination and phylogenetic analysis.

• immunohistochemical expression of K-RAS protein and IL6-STAT3 oncogenesis pathway in ovine pulmonary adenocarcinomas

Chapter 9, entitled "Non-neoplastic respiratory diseases of ovine in Transylvania (Romania): epidemiology and morphological features" aimed to carry out

an epidemiological study within two main slaughterhouses in the Transylvania region (Bistrița-Năsăud and Sibiu counties), by grossly and histological evaluation of the pulmonary lesions foound in the ovine species. 2693 cases (lungs) were post-mortem analyzed for this study. The samples were represented by pulmonary affected tissues collected in 10% formalin (pH 7), for 24-72 hours. The tissues were subsequently processed for histopathological examination, being embedded in paraffin and stained using the usual Hematoxylin-Eosin technique. Of this total number (n = 2693), the predominant non-tumoral lesions were parasitic pneumonia, diagnosed in 80.80% of the cases. These in turn were mostly represented by verminous pneumonias, with a prevalence of 55.29%. The gross lesions characteristic for interstitial pneumonia, associated with *Protostrongylus rufescens* infestation were predominately located in the diaphragmatic lobes as brownish-green plaques or as miliary nodules. The infestation associated with *Muellerius capillaris* was characterized by the presence of numerous miliary nodules, of 2-4 mm, located predominantly in the diaphragmatic lobes. *Echinococcus granulosus* infestation was associated with numerous granulomas ranging in size from 1 to 8 cm, randomly distributed in the lung parenchyma in 10.59% of cases. Bacterial pneumonias with features characteristic of caseous lymphadenitis of sheep and goats and chronic manheimiosis had a reduced prevalence, being identified in 2.67% of cases. Non-tumor viral infections associated with lentivirus (progressive pneumonia or Maedi-Visna) were grossly and histological confirmed with a prevalence of 0.14%. Of the total number of evaluated cases, only 15.21% had no gross lesions.

Chapter 10, entitled "Ovine pulmonary adenocarcinoma in Transylvania (Romania): epidemiology and morphological features" aimed to perform an epidemiological regarding the neoplastic pulmonary pathology in sheep. The study was performed in two slaughterhouses in the Transylvania region by grossly evaluation of 2693 cases (lungs) and histological characterization of the identified tumors. Thirty-four pulmonary neoplasms were identified, obtaining a prevalence of 1.18%, predominantly affecting a single pulmonary lobe (58.82%). Grossly, 30 cases (88.23%) were classified as the classical form of ovine pulmonary adenocarcinoma, and 4 cases (11.77%) were classified in the atypical form of the disease. The classical form is characterized by the presence of large grey neoplastic masses, with a homogeneous appearance on cross section, while the atypical form is characterized by small nodular structures predominantly located subpleural and well delimited of the adjacent pulmonary parenchyma. The predominant histological subtype, both within the classical and atypical forms, was acinar pulmonary adenocarcinoma. Within the atypical form, a distinctive morphological appearance was observed in two cases, represented by well delimited solitary masses, subpleurally located, of about 2-5 cm, white-pinkish color, with irregular surface, and a multilobular, gelatinous appearance on cross-section. Histologically, this neoplasms coresponded to mesenchymal growths (MGs), being poorly cellularized, with elongated or stellate cells arranged in short streams and bundles, embedded in an abundant AB-PAS positive extracellular matrix. Moreover, in the classical form MGs were also identified, associated with epithelial proliferation in 6 cases. Thus, MGs were identified in 8/34 cases (23.53%).

Chapter 11, entitled "Immunohistochemical evaluation and characterization of primary pulmonary tumors in ovine ", aimed to describe the neoplastic cells within ovine pulmonary adenocarcinoma using markers for epithelial (MCK, TTF-1) and mesenchymal origin (vimentin, desmin, SMA and S100), and at the same time evaluation of Ki67 proliferation index and expression of major histocompatibility complex II (MHC II) in pulmonary tumors of sheep. The neoplastic epithelial component of ovine pulmonary adenocarcinoma was positive in all evaluated cases for MCK expression and negative for vimentin. Nuclear expression of TTF-1 in epithelial neoplastic cells was observed in all examined cases, suggesting the primary pulmonary origin of the evaluated lung tumors. MGs were positive for vimentin, characterized by intense cytoplasm marking. Expression of MCK, TTF-1 and S100 in MGs was absent. Desmin was intense and diffuse expressed in the MGs, while SMA showed moderate and selective cytoplasmic expression, thus suggesting the myofibroblastic origin of the cells. The classical form of ovine pulmonary adenocarcinoma showed a mean Ki67 proliferation index of 10.87%, while the mean Ki67 proliferation index in the atypical form was 4.54% (3.93%-5.15%). The MGs, unassociated with neoplastic epithelial proliferation had a mean Ki67 index of 2.48% (1.63% -3.27%), and in those associated with epithelial neoplasia, the mesenchymal component recorded an average of Ki67-labeled nuclei of 1.61% (1.44% -1.78%). MHC II expression was absent in the atypical form, and in the classical form it was observed at 12.5% of the evaluated cases.

Chapter 12, entitled "Detection of Jaagsiekte sheep retrovirus (JSRV)" aimed to identify the etiologic agent of ovine pulmonary adenocarcinoma by PCR, IHC, ICC and morphological characterization by transmission electron microscopy. By ICC, JSRV-MA was identified in neoplastic epithelial cells in all evaluated samples. Immunohistochemistry, in both the classical and the atypical form showed a diffuse positive reaction for JSRV-MA, observed in neoplastic cells in 32 cases (94.11%). Neoplastic mesenchymal cells were also labeled for JSRV-MA. PCR examination revealed sequences of 128 pairs of nucleotides of the exogenous form of the Jaagsiekte virus (ExJSRV) obtained in 33/34 (96.85%) of the examined samples. Phylogenetic analysis of the LTR (U3) sequences showed nucleotide homology of 94.87% with the isolate strain from the United Kingdom (GenBank AF105220.1). Ultrastructurally, ovine pulmonary adenocarcinoma is a heterogeneous tumor, composed of neoplastic epithelial cells originating from Clara cells and type II pneumocytes. JSRV was identified by electron microscopy in 3/5 evaluated samples characterized by round / oval structures, of 80-120 nm, arranged individually or in groups of 4-6 viral particles. The viral particles have a moderately electron-dense central area represented by the nucleoid, surrounded by a double membrane with numerous spicules disposed on the outer membrane.

Chapter 13, entitled "Evaluation of two oncogenic pathways with potential involvement in ovine pulmonary adenocarcinoma: KRAS expression and IL-6-STAT3 pathway" aimed to determine the potential role of new oncogenesis mechanisms by immunohistochemical examination and Western blot technique. The samples used were classified in one of the 3 groups: 1) OPA (pulmonary adenocarcinomas - classical form)

- 20 samples; 2) BAH (broncho-alveolar epithelial hyperplasia, from the periphery of the neoplastic process) - 9 samples; 3) M (negative controls-unaffected lung tissue) - 10 samples. KRAS immunoexpression showed no differences between the 3 studied groups. STAT3 was expressed in neoplastic epithelial and mesenchymal cells and also in the broncho-alveolar hyperplasia but with a statistically significant increase in the OPA group compared with BAH ($U = 0$, $p = 0.001$, the non-parametric Mann-Whitney U test). For IL6, the intensity of the reaction was statistically significantly higher in the OPA group, compared to the BAH group ($U = 16.5$, $p = 0.019$, the non-parametric Mann-Whitney U test), and also the number of labelled cells in the OPA group was statistically significant higher ($U = 18.5$, $p = 0.023$, Mann-Whitney U non-parametric test). By the simultaneous evaluation of IL-6 expression, respectively STAT3 in the two groups, no statistical correlation could be established, either regarding the BAH group ($r = 0.224$, $p = 0.602$), or the OPA group ($r = 1$, $p = 0.391$) (The correlation coefficient Kendall's tau). The Western Blot method revealed a statistically significantly higher ($p < 0.05$) amount of IL-6 in the OPA group compared to the M group. The quantitative analysis for STAT3 in the OPA group, showed a significantly higher quantity ($p < 0.05$) in neoplastic lesions, compared to the M group. The BAH group showed a statistical correlation ($p < 0.01$) only regarding the lower quantity of IL-6, compared to the control group.

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