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Descripción morfológica e inmunohistoquímica de un linfoma en un cordero de dos meses de edad usando los marcadores MUM1/IRF4, CD79a, CD3, CD10 y Ki67

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Morphological and Immunohistochemical Description of a Lymphoma in a Two-Month-Old Lamb Using MUM1/IRF4, CD79a, CD3, CD10 and Ki67 Markers

Alexander R. Ortloff-Trautmann¹ / Javier A. Neumann-Vásquez² / José Valbuena-Mora³

Abstract

This article contains a morphological description of a case of early presentation lymphoma in a lamb, characterised by its unusual clinical history and advanced lesions. The primary tumour was located in the skin in the carpal region, and metastases were observed in the brain, right eye, all the pulmonary lobes, the myocardium and endocardium of the heart, left adrenal gland, abdominal portion of the vagal nerve, serosa of the rumen, the body of lumbar vertebra L3, and the submandibular, mediastinal and mesenteric lymph nodes. Histopathological examination revealed that it was a large cell lymphoma. Immunohistochemical analysis was carried out with antibodies for MUM1/IRF4, CD79a, CD3, CD10 and Ki67, used here for the first time as markers to characterise a lymphoma in this species. The result revealed that it was a B-cell lymphoma.

Keywords: Lymphoma; lamb; immunohistochemical; metástasis.

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Descripción morfológica e inmunohistoquímica de un linfoma en un cordero de dos meses de edad usando los marcadores MUM1/IRF4, CD79a, CD3, CD10 y Ki67

Resumen

En este artículo se describe morfológicamente un caso de linfoma de presentación temprana en la especie ovina, el cual se caracterizó por lo inusual de su historia clínica y lo avanzado de las lesiones. El tumor primario se localizó en la piel del carpo y se observaron metástasis en el cerebro, el ojo derecho, todos los lóbulos pulmonares, el miocardio y endocardio del corazón, la glándula adrenal izquierda, el nervio vago en su porción abdominal, la serosa del rumen, el cuerpo vertebral lumbar L3, los linfonodos submandibulares, mediastínicos y mesentéricos. El examen histopatológico reveló un linfoma no Hodgkin de células grandes. El análisis inmunohistoquímico se realizó con anticuerpos para MUM-1/IRF-4, CD79a, CD3, CD10 y Ki-67, los cuales se usaron por primera vez como marcadores para caracterizar un linfoma en esta especie. El resultado reveló que se trató de un linfoma de células-B.

Palabras clave: linfoma; cordero; inmunohistoquímica; metástasis.

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Descrição morfológica e imuno-histoquímica de um linfoma em um cordeiro de dois meses de idade usando os marcadores MUM1/IRF4, CD79a, CD3, CD10 e Ki67

Resumo

Neste artigo descreve-se morfológicamente um caso de linfoma de apresentação precoce na espécie ovina, caracterizado pela história clínica incomum e as lesões avançadas. O tumor primário foi encontrado na pele do carpo e metástase foi observada no cérebro, olho direito, todos os lobos pulmonares, miocárdio e endocárdio do coração, a glândula adrenal esquerda, nervo vago na porção abdominal, a serosa do rúmen, o corpo vertebral lombar L3, linfonodos submandibulares, mediastinos e mesentéricos. O exame histopatológico revelou linfoma não-Hodgkin de células grandes. A análise imuno-histoquímica foi realizada com anticorpos para MUM-1/IRF-4, CD79a, CD3, CD10 e Ki-67, os quais usaram-se por primeira vez como marcadores para caracterizar um linfoma nessa espécie. O resultado revelou que se tratou de linfoma de células-B.

Palavras-chave: Linfoma; cordeiro; imuno-histoquímica; metástase.

INTRODUCCIÓN

A lymphoma (previously called “lymphosarcoma”) is a malignant neoplasm of lymphoid origin, which is of great importance in veterinary medicine. It is the most frequent type of neoplasm in cats and the second most frequent in dogs. Although *lymphoma* was reported and described as pathology in sheep, cows and pigs several decades ago by different authors (1,2), the classification used to determine the different types of lymphoma in these species is far removed from the detail in which the disease has been studied in dogs and cats. In these species, the classification of the different types of lymphoma has been homologated with the classification used in humans as described by the World Health Organisation (WHO), using both conventional and immunohistochemical histology (3). Since the 1980s, the National Cancer Institute-Working Formulation (NCI-WF) has been developing the classification of lymphomas in humans in the United States, while the Kiel System is used in Europe. Both systems are based on standard histological techniques, which can easily be used in other animal species. These systems have been improved with the use of immunophenotyping and genotyping (4).

In humans, it has been shown that the response to treatment and prognosis in lymphoma patients can be predicted by determination of the type of lymphoma, and this association between good classification and the correct treatment protocol has been well established. The same association has been established in dogs (4).

Despite these advances in oncological pathologies in pets, where immunophenotyping is a routine procedure in patients with lymphoma, the classification system that is still used in small ruminants refers to the anatomical distribution of the lesions (multi-centre, alimentary, thymic and cutaneous) and the cytopathological characteristics of the neoplasm. The following lymphomas are recognised in sheep: lymphoblastoid, pro-lymphocyte, lymphocyte, and reticular cell; however, there has been little development in immunophenotyping (4). Some contributions to immunophenotyping in sheep were developed by Dixon et al. (5), who proposed a technique for classifying lymphomas according to their origins in B or T lymphocytes; however, it has not been adopted in routine use, and the majority of the specific molecular markers now used in other species are absent.

The characteristics of lymphomas and leukaemias differ with the animal's age when it appears, i.e., between young and adult animals. A congenital lymphoma is a form of this disease, which appears at an early age. It is of particular interest because it is sometimes associated with viral infections. In humans, this pathology is the third most frequent type of malignant neoplasm in children aged up to 10 years; the non-Hodgkins B-cell lymphoma is more common, associated with the Epstein-Barr virus, according to some investigations (6). In kittens aged less than 1 year, lymphoma has been associated with the feline leukaemia virus (FeLV) (4). In bovines, the lymphoma associated with the bovine leukaemia virus (BLV) occurs at a much later stage of the animal's life (over 4 years).

Cases of congenital lymphoma are scarce in domestic animals and even rarer in productive animals. A case of congenital B-cell lymphoma in a calf, classified by immunohistochemical techniques, has recently been described (7). A different situation exists among sheep; Johnstone and Manktelow (8) reported lymphomas in lambs as an unexpected finding in slaughterhouses, classifying them only by distribution of the lesions. The object of the present work was to describe a case of early presentation of lymphoma in a lamb, due to its unusual presentation, morphological characteristic and clinical history. Conventional and immunohistochemical techniques were applied, using some molecular markers that have not been used before in this species but which are applied routinely in human and pet pathologies. This work contributes to the information available on this pathology in small ruminants.

MATERIALS AND METHODS

Clinical history. A 2-month old lamb of the Araucano bio-type was brought into the Large Animals Hospital of the Veterinary Sciences and Public Health Department of Universidad Católica de Temuco. The reason for the consultation was that the lamb had multiple ulcerated cutaneous lesions of 2 to 5 cm in diameter. In the anamnesis, the owner stated that, one month earlier,

the patient had been attacked by feral dogs, and he had associated the skin lesions with this attack. He noted that the lesions did not respond to treatment with antibiotics and non-steroidal anti-inflammatories. Clinical examination showed rectal temperature, heart rate and respiration rate within normal physiological parameters. An increase in the volume of the carpal joints was observed, and multiple tumoral lesions with purulent exudate in the skin of the flanks and back; the largest of such lesions was on the skin of the carpal region, with a diameter of 5 x 5 x 5 cm (Figure 1). Due to the lamb's condition and the costs incurred, the owner decided not to continue treatment. During the following days, the animal's health suffered a marked deterioration, and it was ultimately put down using T61^{®1} euthanasia solution.¹

Figure 1. Sagittal section of the tumoral mass in the carpal skin



*Black asterisks indicate the border of the skin. Black arrows indicate the ulcerated surface. Scale in centimetres.

Source: own elaboration

¹ Intervet International GMBH. Germany.

Necropsy and macroscopic examination. The necropsy protocol was followed, and all the organs were removed for analysis. Samples for histopathology were taken from all the organs presenting lesions (detailed below). The entire remaining part of each organ not used for histopathological analysis was fixed in formalin at 10% for one week in a container. Subsequently, even sections were taken with an anatomical scalpel, leaving the surface of the organs smooth; they were examined digitally with a scanner for macroscopic recording and characterisation.

Histopathology with Hematoxylin and Eosin (H&E) staining. Samples were taken for histopathological analysis from all the organs with tumoral masses. For correct fixing, the maximum thickness of each sample was 0.5 cm; they were kept in buffered formalin at 10% for 48 hours. At the end of this period, the samples were dehydrated in increasing concentrations of ethanol (50°, 70°, 80°, 96°, 100°); they were equilibrated in butanol and embedded in Paraplast® according to conventional protocols. The steps containing Paraplast® were carried out in an oven at 60°C. Sections of 5µm thickness were obtained from all the samples in a ThermoScientific® microtome; they were mounted on slides treated with silane (3-amino-propyl-triethoxy-silane) (Polysciences Inc., USA). The sections mounted on slides were left to dry in a stove at 60°C for at least 2 hours. Once the mounted sections had dried, they were deparafinated in xylol for 15 minutes and then re-hydrated in a reducing battery of alcohols (100°, 96°, 80°, 70°, 50°), finishing in water. Once hydrated, they were stained with Mayer's hematoxylin for 3 to 5 minutes and then washed in abundant water. Finally, they were stained with eosin at 1% for 1 to 2 minutes. The sections were washed with water, dehydrated and mounted for analysis under an optical microscope. Initial diagnosis of the

type of neoplasm was made from the H&E stained samples, and this diagnosis was used to define the group of molecular markers for immunophenotyping.

Immunohistochemical technique. Immunophenotyping of the lymphoma was carried out by immunostaining with different primary antibodies (Table 1), and the antigen-antibody reaction was revealed using a secondary antibody conjugated with biotin. The antigens had to be exposed and/or endogenous peroxidase activity blocked before immunostaining. The antigens were exposed in deparafinated sections of tissue. To do this, the sections were incubated in citrate buffer (20mM pH 6.0) and treated in a microwave at 90°C, with three changes over 5 minutes, renewing the citrate buffer in each heating cycle. After exposure of the antigens, the sections were washed for 15 minutes (three changes of 5 minutes) with Tris-PBS buffer (Tris-HCl 10mM pH7,8., Na₂HPO₄ 8,3mM, KH₂PO₄ 3,5mM, and NaCl 0,12M), and then incubated successively: i) primary antibody for 18hs; 2) secondary antibody conjugated with biotin (1:20) for 30 minutes; and 3) streptavidin conjugated with peroxidase for 10 minutes in the dark. They were washed for 15 minutes (three changes of 5 minutes) with Tris-PBS buffer between each incubation step. The high affinity of the streptavidin for biotin allows efficient amplification of the signal. The sections were incubated with H₂O₂ at 0.007% and 3,3'-diaminobenzidine (DAB at 0.2% in Tris-PBS). After incubation in DAB/H₂O₂, the sections were washed in distilled water, dehydrated and mounted for observation. The control for immunostaining was carried out by omitting incubation with the first antibody. The samples were observed, examined and photographed in a Zeiss Axioskop optical microscope connected to a Canon EOS T3i digital camera.

Table 1. Details of antibodies used in immunohistochemical analysis

Primary Antibody	Origin (species)	Immunogen	Concentration/dilution	Commercial reference
anti-MUM1/IRC4	Mouse (monoclonal) Clon MUMp	MUM1 Human	1:100	Dako (IR 644)
anti-CD10	Mouse (monoclonal) Clon 56C6	CD10 (CALLA) Human	10mg/L (direct use)	Leika (PA0270)
anti-Ki67	Mouse (monoclonal) Clon MM1	Ki67 Human	61mg/L (direct use)	Novocastra (NCL-IL-Ki67-MM1)
anti-CD3	Rabbit (policlonal)	CD3 Human	1:150	Dako (N 1580)
anti-CD79a	Mouse (monoclonal) Clon HM57	CD79a Human	1:100	Invitrogen (MA1-81870)

Source: own elaboration

RESULTS

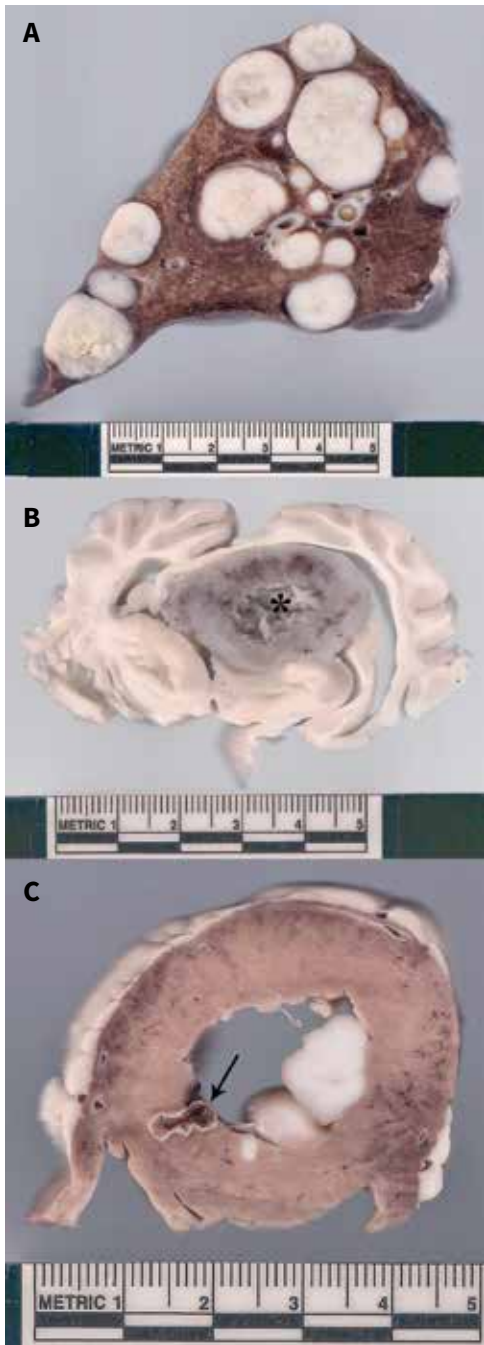
The post-mortem examination revealed the presence of multiple diffuse tumoral masses infiltrating the skin, of irregular shape, with purulent exudate, multifocal necrosis and superficial ulcerated scabs. The largest lesion was in the skin in the left carpal region, measuring 5 cm in length and 5 cm in height, with an irregular shape (Figure 1). Multiple round, white tumoral masses were observed in the brain, right eye, all the pulmonary lobes, myocardium and endocardium of the heart, left adrenal gland, abdominal portion of the vagal nerve, serosa of the rumen, the body of lumbar vertebra L3, and the submandibular, mediastinic and mesenteric lymph nodes. The tumours were up to 2 cm in diameter, soft to cut, some with central necrosis (Figure 2). The liver, kidneys and reproductive organs showed no macroscopic lesions.

Histopathological analysis with H&E of the carpal tumour revealed that it consisted of a circumscribed, non-encapsulated neoplasm, with coagulative necrosis at

the centre and haemorrhaging at the surface; multifocal infiltrate with neutrophils was observed, predominating in superficial zones. The malignant neoplasm cells were characterised as being a polymorphic population of lymphoid origin, distributed diffusely (with no follicular pattern). They were mainly characterised by large lymphocytes (diameter greater than 2 erythrocytes), irregular nucleus, anisokaryosis, fine chromatin, marked nucleolus, and scarce amphophilic cytoplasm. The nucleus/cytoplasm ratio was high. Mitotic cells and apoptotic bodies were frequent, with the presence of small normal lymphocytes among the neoplastic cells. The tumoral masses in the other organs revealed similar cytological and histopathological characteristics, with neoplastic lymphocytes infiltrating the parenchyma; however, inflammatory infiltrate was scarce (Figure 3).

Immunohistochemical analysis revealed that all the cells were positive for MUM/IRF-4 and CD79a, partially for CD10, and negative for CD3 (Figure 4). The Ki-67 marker revealed that 40–50% of the cells were positive (Figure 3).

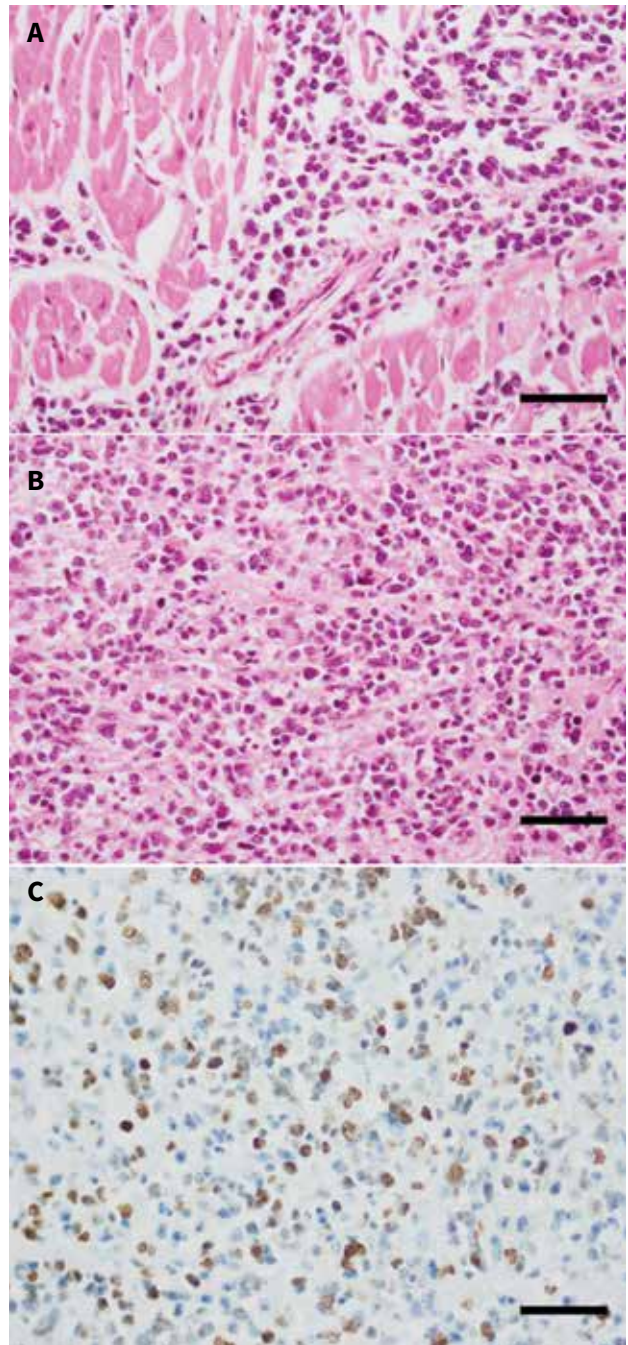
Figure 2. Metastasis of the lymphoma



* **A.** Cross section of the diaphragm lobe of the right lung with multiple metastatic neoplastic foci. **B.** Coronal section of the brain. Metastatic focus in the encephalon with central necrosis (black asterisk). **C.** Cross section of the heart. Metastases are observed in the endocardium and myocardium, with an infarct focus (black arrow). Scale in centimetres.

Source: own elaboration

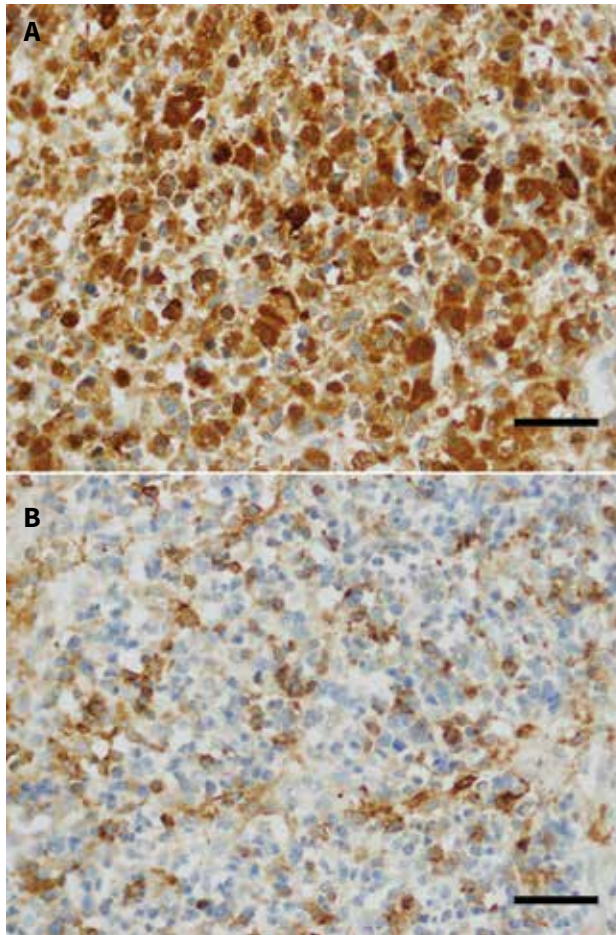
Figure 3. Histological section of a metastasis of the lymphoma.



* **A.** Metastatic infiltration in the myocardium was characterized by a polymorphic population of neoplastic lymphocytes. (H&E). **B.** Metastatic infiltrate in the vagal nerve. **C.** Immunohistochemical analysis with anti-Ki67. Scale: 100 μ m.

Source: own elaboration

Figure 4. Immunohistochemical analysis of histological sections of the primary tumour



* A. All the neoplastic cells are immunopositive for MUM1/IRC4. Anti-MUM1/IRC4. B. Partial expression of CD10. Anti-CD10. Scale: 100 μ m.

Source: own elaboration

DISCUSSION

Presentation of the lymphoma was early in this lamb. The size of the tumours and the animal's age suggest that its origin was probably perinatal or congenital. Neonatal lymphoid neoplasms are rare. Congenital leukaemia is described in humans associated with cytogenetic and molecular abnormalities (t(4;11) (q21.3;q23.3)/KMT2A-AFF1 followed by t(1;22) (p13.3;q13.1)/RBM15-MKL1 and t(8;16)(p11.2;p13.3)/KAT6A-CREBBP

(9); this condition has not been investigated in other domestic species. Some lymphoid neoplasms in other species have been associated with viral etiology. Early presentation of leukaemia/lymphoma in felines (before the age of one year) has been associated with the feline leukaemia virus (FeLV) (10); in bovines, lymphoma has been associated with the bovine leukaemia virus (BLV), but, in this case, it presents in animals older than 4 years (7). In sheep, it has been shown that BLV is able to provoke neoplastic transformation of lymphoid cells (11,12), but, as in bovines, presentation occurs later. One possibility for the origin of this lymphoma is that it was transmitted by the mother's placenta, if she suffered from lymphoma/leukaemia; however, it is unknown whether the ewe had that pathology. Moreover, the first organ of the foetus to receive a neoplastic embolus would be the liver through the placenta, yet this organ was one of the few that did not present tumoral masses, so this possibility was discounted. Judging by the size and shape of the largest tumour, it is possible that the primary tumour was in the carpal skin, and that the tumours in the other organs resulted from metastasis.

According to the results obtained in the immunohistochemical tests, the neoplasm observed in this animal was a lymphoma of the B-lymphocyte lineage. This diagnosis is supported by the immunopositivity of the cells for the anti-CD79a and anti-MUM1/IRF4 antibodies. CD79a is a molecule of the B-cell antigen receptor complex. It is expressed from the pre-pre-B-cell stage up to the differentiated plasmatic cell, making it a good marker for the B-cell lineage; it is expressed in 60% of plasmocytomas in humans and 80% in dogs (13). This marker was positive in a case of congenital lymphoma in a neonatal calf (7). MUM1, also called IRF4, belongs to the family of transcription factors involved in lymph cell differentiation and is needed in the reordering of the immunoglobulin light chain in the pre-B-cell stage during lymphocyte maturation. Although MUM1/IRF4 is not expressed exclusively in the B-cell lineage, but also in activated T-cells, a sub-group of macrophages and dendritic cells, it is used as part of the antibody panel for characterising neoplasms in B-cells in humans (13).

This marker was demonstrated immunohistochemically for the first time in another species than humans by Ramos-Vara et al. (13), and this is the first time that its expression has been shown in sheep tissue.

CD10, also known as CALLA (common acute lymphoblastic leukemia antigen), is a metalloproteinase expressed in pro-B-cells and the cells of germinal centres (14). Although it is used as a marker in the classification and diagnosis of leukaemia/lymphoma, CD10 has also been reported in epithelial neoplasms (in the bladder, liver cells, and kidney carcinomas), mesenchymatic neoplasms (sarcomas), and some occurring in the skin (15). While not an exclusive marker of lymphomas, it is most frequently associated with B-cell lymphomas/leukaemia, so its partial expression in the neoplasm in this lamb could support the findings of the other markers investigated, which point to a diagnosis of B-cell lymphoma. Furthermore, the samples analysed gave negative results for CD3, the marker commonly used for T-cells. It cannot be discounted that this negative response may also be due to a lack of crossed reaction in this species since the anti-CD3 antibody used is designed for human CD3.

Finally, the cells of this lymphoma, which marked positive for the Ki67 marker, were active and proliferating in the cell cycle. Anti-Ki67 recognises the heterodimeric protein Ki67, which reaches peak expression in M phase cells of the cell cycle; tissue where cells are cycling actively, therefore, marks positive, a good marker for proliferating cells (16).

CONCLUSIONS

According to the histological and immunohistochemical description in this case, we concluded that it was a B-cell lymphoma. It should be noted that, apart from the rarity of this type of neoplasm in productive animals, the early presentation and the use of molecular markers routinely used in human medicine—used here for the first time for characterisation in a sheep—provide a ba-

sis for further investigation into the development of the pathology in this species. It is a step towards updating oncological classification systems in veterinary medicine. The antibodies used can be added to the panel of antibodies available for characterising lymphomas in sheep.

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