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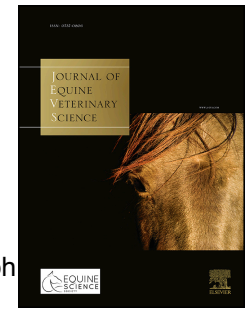

Immunohistochemical analysis of programmed death-ligand 1 expression in equine sarcoids

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PII: S0737-0806(20)30429-9

DOI: https://doi.org/10.1016/j.jevs.2020.103338

Reference: YJEVS 103338

To appear in: *Journal of Equine Veterinary Science*

Received Date: 25 September 2020

Revised Date: 20 November 2020

Accepted Date: 20 November 2020

Please cite this article as: Benvegnen J, De Breuyn B, Gerber V, Rottenberg S, Koch C, Immunohistochemical analysis of programmed death-ligand 1 expression in equine sarcoids, *Journal of Equine Veterinary Science* (2021), doi: https://doi.org/10.1016/j.jevs.2020.103338.

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source: https://doi.org/10.7892/boris.149869 | downloaded: 14.7.2024

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Immunohistochemical analysis of programmed death-ligand 1 expression in equine sarcoids

1853 words, 1 figure, 2 table

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Abstract

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The aim of the study was to assess the expression of the immune checkpoint inhibitor programmed death-ligand 1 (PD-L1) in equine sarcoids (ES). PD-L1 is expressed by various cancer cells to block T cell-mediated elimination of tumor cells.

Antibodies targeting human PD-L1 were tested by immunohistochemistry (IHC) for their cross-reactivity with equine PD-L1 using formalin-fixed, paraffin-embedded (FFPE) tissues.

Our results do not support an important role of PD-L1-mediated immune evasion in ES disease, and hence do not offer a rationale for anti-PD-1/PD-L1-based immunotherapy against ES.

Keywords: Equine sarcoids, immune checkpoint inhibition, immune evasion, immunohistochemistry, PD-L1

1. Introduction

Equine sarcoids (ES) are the most common tumors in equids and account for more than half of all skin tumors in this species [1,2]. Treatment is often challenging due to the notoriously high propensity for tumor recurrence [3], the lack of tissue-sparing treatment options and effective systemic or prophylactic treatment modalities.

In human medical oncology, a novel therapeutic approach has recently achieved remarkable successes: targeting of the immune checkpoint inhibitor programmed death-ligand 1 (PD-L1) or programmed cell death protein 1 (PD-1) [4,5]. It has resulted in tumor eradication in cancer patients previously thought to be incurable (*e.g.* advanced melanoma or lung cancer).

The physiologic role of immune checkpoints is to maintain self-tolerance and protect tissues from self-damage, for instance while responding to an infection [6–8]. The dysregulation of these immune checkpoint proteins can be observed in various cancers, and represents an important mechanism for tumor cells to evade the immune system [6,9]. PD-L1 is expressed

by various cancer cells to block T cell-mediated elimination of the tumor cells by binding to programmed cell death protein 1 (PD-1) at the surface of T lymphocytes. This mechanism of immune evasion can be prevented by using specific antibodies against PD-L1 or PD-1 [6].

The aim of this study was to establish an immunohistochemistry (IHC) staining protocol to assess PD-L1 expression in ES-derived and other equine tissue samples. As an additional control to investigate the cross-reactivity of the antibody for equine PD-L1, we also tested mandibular lymph node as lymph nodal tissue has been described to contain immune cells expressing PD-L1 in humans [10].

We hypothesized that (transformed) equine fibroblasts derived from ES tumors express PD-L1. Substantial expression would support PD-L1 as a mechanism of immune evasion in ES disease and consequently, immunotherapy directed against PD-L1 would be a reasonable approach for the treatment of ES tumors.

2. Material and Methods

Lesional tissue samples from ten horses presented for surgical removal of histologically confirmed ES tumors were investigated. Samples were obtained from seven geldings, two mares and one stallion of different breeds (one Shetland pony, one Rocky mountain horse, one American Quarter horse, one Arabian, one Friesian, one Swiss Warmblood, one Franches-Montagnes and one mixed breed) and included ES tumors of different gross morphology (three fibroblastic, four verrucous, two nodular and one mixed). These samples were retrieved from the ISME (Swiss Institue of Equine Medicine) tumor tissue bank.

Samples of equine placenta from a Franches-Montagnes mare and samples of an equine mandibular lymph node from a Selle-Français mare were collected from patients of the ISME. All owner gave their informed written consent.

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PD-L1 is expressed at the surface of villous syncytiotrophoblasts and cytotrophoblasts of the placenta to confer the fetomaternal tolerance [11]. Human placenta tissue samples, used as positive control, were obtained from the Translational Research Unit (TRU) of the University of Bern.

A rabbit polyclonal antibody (orb158130, Biorbyt, UK) was used, which reacts with human and mouse PD-L1, and is predicted to cross-react with the horse antigen. Comparison of the equine PD-L1 amino acid sequence using BLAST (https://blast.ncbi.nlm.nih.gov) revealed an 80% homology to human PD-L1.

As a positive control for the IHC protocol on FFPE slides the cytokeratine AE1/AE3 antibody (monoclonal mouse α-human, Dako, M3515) was used at a dilution of 1:50. Negative controls were created by omitting the primary antibodies.

Four-µm FFPE tumor sections were dried on silane-coated slides. The slides were subsequently deparaffinizaded and re-hydrated. Antigen retrieval was performed in citrate buffer (pH 6.0), by boiling for 10 minutes in the microwave oven. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 minutes. Bovine serum albumin, also at a concentration of 3%, was subsequently added. The primary antibody (orb158130, Biorbyt, dilution 1:200, incubation at room temperature) was applied overnight. The secondary biotinylated antibody (Dako, K0675) + System HRP (Dako, K0675) were added. Between every step the slides were washed in 0,05% Tween Tris-buffered saline (TBS/Tween) solution at pH 7.6 (Dako, S3006). DAB (3,3‘-diaminobenzidine tetrahydrochloride) was used for the staining. In a final step, slides were counterstained with hematoxylin. IHC protocol is shown in detail in **Table 1**.

The stained slides were assessed by a board-certified pathologist (SR) using light microscopy, inspecting the sections with x4, x10, x20 and x40 objectives. A semiquantitative scoring

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system was applied for the assessment of cell staining. A positive cell was defined as a cell exhibiting a membranous signal alone or a membranous and cytoplasmatic signal. The following scoring system was used: grade 0: 0%, grade 1: <10%, grade 2:10-25%, grade 3: 25-50%, and grade 4: >50% of cells staining positive for PD-L1.

3. Results

Positive (cytokeratin-staining) and negative controls (primary antibody omitted) yielded expected results, thus highlighting the technical accuracy of the experiment (data not shown).

PD-L1-staining of equine placental tissue yielded a membrane and a cytoplasmic signal (**Figure 1**), consistent with the staining of the human placenta samples. Although the signal was somewhat weaker than on the human placenta, the result suggests that the orb158130 antibody does detect PD-L1 in horses. This result was corroborated in the horse lymph node, showing a few clearly positive cells, especially in the area of lymph follicle but also in others areas. The signal was mainly granular and localized in the membrane and the cytoplasm. In 7/9 examined ES tumors, less than 10% of the tumor cells exhibited a positive signal. Only in 2/9 ES tumors 25-50% of the cells showed a positive signal for PD-L1 (score 3+). The results are summarized in **Table 2**.

4. Discussion

Based on the findings of this study, ES-derived, transformed equine fibroblasts do not appear to consistently express PD-L1. In fact, most of the cells of the analyzed ES tumors stained negative for the protein PD-L1. In 7/9 of the examined ES tumor samples, less than 10% of the tumor cells tested positive for PD-L1 expression. In contrast, cells in equine and human placental samples consistently expressed PD-L1. Similarly, cells in lymph tissue showed a granular membranous and cytoplasmic signal.

Although the number of examined tumors is low, this pilot experiment suggests that PD-L1 is not generally expressed by ES-derived transformed equine fibroblasts and therefore PD-1 blockade is unlikely a general mechanism of immune evasion in ES disease. However, PD-L1 is not only expressed by tumor cells but also by T- and B-lymphocytes and dendritic cells that infiltrate the tumor microenvironment [12]. Reportedly, the predominant immune cell populations which infiltrate ES tissues are macrophages and monocytes [13], but also T-cells [14]. In our study, we focused on the quantification of PD-L1-positive transformed fibroblasts that are readily differentiated from immune cells, based on morphological criteria. As a small percentage of immune cells was also PD-L1-positive, it would be interesting to characterize the precise nature of these immune cells in ES in the future.

In human medicine, overexpression of PD-L1 has been observed in some cancers, including melanomas [15]. Three equine melanomas were included in the study and in two lesions about 30-50% of tumor cells stained positive for PD-L1. In this study, human placenta delivered at birth was used as a positive control. In human placentas the expression of PD-L1 is highest in the second and third trimester of pregnancy [16]. In equine placenta, endometrial cups are present in the uterine wall from day 40 and up to day 150 of gestation, but regress after day 70. Given the particular role of endometrial cups in the development of the fetal immune system, staining of the epithelium of endometrial cups may differ in earlier gestation stages in equids compared to humans.

The similarities in the PD-L1 staining pattern between human and equine positive control tissues found in this comparative analysis suggest that the antibody used in the described protocol is indeed valid for PD-L1 detection in equine tissues, albeit it is not an unequivocal proof for PD-L1-specificity of the antibody in the horse. As equine keratinocytes also stained positive in five out of nine slides with ES-derived tissues, unspecific cross-reactivity of the antibody cannot be ruled out and is the most likely explanation for this observation.

Alternatively, BPV-DNA may more consistently lead to the expression of PD-L1 in infected equine keratinocytes compared to BPV-transformed equine fibroblasts. Whereas BPV completes an infectious life-cycle within the epidermal keratinocytes of its natural bovine host and leading to the production of countless infectious virions [17], the BPV-infection in the equine host is generally considered "non-productive" or "abortive" [18,19]. More recently, however, BPV-DNA has also been detected in keratinocytes of ES tissues [20], albeit at much lower levels compared to those found in BPV-transformed equine fibroblasts. Nonetheless, these findings combined with reports of ES disease-transmission in the absence of a bovine source for BPV [21] give reason to belief that the BPV-infection in ES-affected horses may not be entirely "abortive". Likewise, it may be speculated that a BPV-infection may induce PD-L1 expression in equine keratinocytes but not in fibroblasts. Thus, this would be an explanation for the positive staining for PD-L1 of keratinocytes associated with ES-lesions, in the absence of positive staining of fibroblasts, as observed in this study.

Ideally, the specificity of an antibody should be tested in isogenic cell lines or tissues containing a wild-type and a knockout of the gene of interest. Using CRISPR-cas9 technology it would be possible to induce *Pd-l1* knockouts in cultured cells that normally express this receptor and thereby generate such controls. Another approach would be the expression of the equine *Pd-l1* cDNA in cells that are PD-L1 negative. However, a disadvantage of this approach is that non-physiologically high expression levels are reached and that it remains unclear whether the antibody detects physiologically relevant protein levels.

While our results do not support PD-L1 as a potential therapeutic target, it is not the only protein able to downregulate the immune response. Many immunosuppressive mechanisms have been documented in the tumor microenvironment, including the recruitment of regulatory T cells (Tregs) [22] or myeloid derived suppressor cells (MDSC) [23], production of IL-10 [24] and TGFβ [25] or expression of other immune checkpoint regulators, such as

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CTLA-4 (cytotoxic T lymphocyte antigen) [26,27]. In this study, we focused on PD-L1, but further studies are needed to evaluate potential mechanisms of immune evasion and the presence of other negative regulatory molecules in the microenvironment of ES tumors.

5. Conclusion

In this experiment, we observed positive PD-L1 staining in equine placental and lymph follicle cells, but only in low numbers of ES-derived equine fibroblasts. This suggests that this PD-L1 is not regularly expressed in ES and PD-L1 blockade does not serve as an important mechanism of immune evasion in this form of neoplasia. However, these findings need to be confirmed in a greater number of samples and using antibodies that are more thoroughly validated for applications in equine-derived tissues. Nonetheless, PD-L1 is not only expressed by tumor cells but also by immune cells that infiltrate the tumor microenvironment, and future research may reveal the precise nature of the immune cells within ES tissues that express PD-L1 and unravel their role in ES disease. Finally yet importantly, preliminary results obtained on equine melanomas indicate the potential of a targeted therapy of this tumor type with anti-PD-L1 antibodies.

Declarations

- List of abbreviations: PD-L1: programmed death-ligand 1, PD-1: Programmed cell death 1, ES: Equine sarcoid, IHC: Immunohistochemistry, FFPE: Formalin-fixed, paraffin-embedded, DAB: diaminobenzidine tetrahydrochloride, HRP: Horseradish peroxidase, MDSC: myeloid derived suppressor cells, CTLA-4: cytotoxic T lymphocyte antigen, TGFβ : Transforming growth factor beta
- Ethics approval and consent to participate: ethical approval / animal use permission obtained nr. BE 110/15
- Declarations of interest: none

- Availability of data and materials: The data that support the findings of this study are available from the corresponding author upon reasonable request.
- Funding: The authors acknowledge ISMEquine Research (reference nr.36.333) for partial funding of this study.
- Authors 'Contributions: SV and CK conceived this study. JB and BDB carried out the experiment, SV performed the histological examination. JB drafted the manuscripts. CK was a major contributor in writing the manuscript. VG revised the manuscript. All authors read and approved the final manuscript
- Acknowledgements: Not applicable

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Tables

Table 1: Immunohistochemistry protocol used in the study

Deparaffination and Hybridation	
Melting paraffin in the oven at 60°C	➢ 10min
Xylol	➢ 10min
Xylol	➢ 10min
Ethanol 100%	➢ 2min
Ethanol 100%	➢ 2min
Ethanol 95%	➢ 2min
Ethanol 70%	➢ 2min
Distilled water	➢ 2min
Antigen- Retrieval	
10mM Citrate Buffer pH 6.0	➢ 10min boiling in microwave
Room temperature cooling	➢ 5min
Distilled water	➢ 2x 5min
Endogenous peroxidase block	
H_2O_2 3% (in water)	➢ 20min
Distilled water	➢ 2x 5min
Wash with Dako washing buffer (S3006)	➢ 5min
Biotin blot	
Block endogenous protein with bovine serum albumin 3% (BSA) in PBS	➢ 30min
Wash with Dako washing buffer (S3006)	➢ 2x5min
Primary antibody	
PD-L1 in Ab diluent (S0809, Dako)	➢ Dilution 1 :200, overnight incubation
Wash with Dako washing buffer (S3006)	➢ 3x5min
Secondary antibody	
Universal secondary antibody (K0675, Dako)	➢ 30min
Wash with Dako washing buffer (S3006)	➢ 3x5min
HRP-conjugat streptavidin (K0675, Dako)	➢ 30min
Wash with Dako washing buffer (S3006)	➢ 3x5min
DAB	➢ 4min
Distilled water	➢ 5min
Hematoxylin couterstain	➢ Few seconds
Running tap water	➢ 5min
Distilled water	➢ 5min
Mounting	

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278 **Table 2:** Summary of the number of slides per score. One equine sarcoid was excluded of the
279 study because most of the tissue was destroyed after the immunohistochemistry procedure.

280

Score	**0**	**1+**	**2+**	**3+**	**4+**
Human placenta					1
Equine placenta					1
Lymph node		1			
Equine Sarcoids					
• Verrucous	1	1		1	
• Nodular		2			
• Fibroblastic	1	1		1	
• Mixed (verrucous and fibroblastic)	1				

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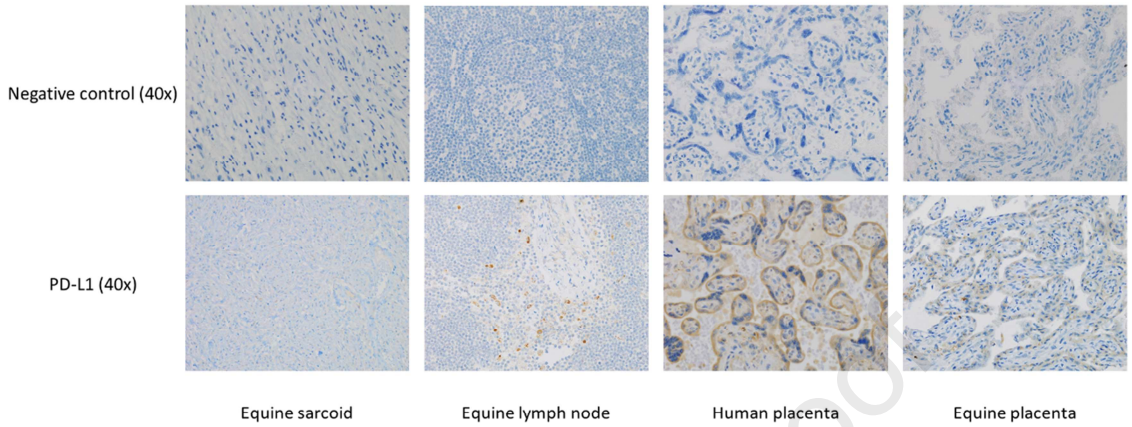
283 **Figure**



284
285 **Figure 1**: Equine sarcoid, negative (no programmed death-ligand 1 staining) and positive
286 controls. Note that the signal is consistently weaker in equine tissues compared to human
287 tissues.

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Highlights

- Establishment of an IHC protocol for equine PD-L1 using FFPE material
- Preliminary finding indicate that equine sarcoid-derived tissues do not frequently express PD-L1
- The low expression levels of PD-L1 in equine sarcoid-derived tissues argue against PD-L1 contribution to immune evasion in this neoplastic disease

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Declarations of interest: none

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Ethical approval / animal use permission obtained nr. BE 110/15

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