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ORIGINAL ARTICLE

Expression of the Wilms' tumour gene and its association with *PPARβ/δ* in healthy skin and melanoma of horses

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ABSTRACT

The Wilms' tumour gene (*WT1*) has previously been described as an oncogene in several neoplasms of humans, including melanoma, and its expression increases cancer cell proliferation. Recent reports associate the expression of the *PPARβ/δ* gene (peroxisome proliferator-activated receptor beta/delta) with the downregulation of *WT1* in human melanoma and murine melanoma cell lines. The aim of this work was to analyse the expression of *WT1* and its association with *PPARβ/δ* in samples of healthy and melanoma-affected skin of horses by immunohistochemistry. WT1 protein expression was detected in healthy skin, mainly in the epidermis, hair follicle, sebaceous gland and sweat gland, while no expression was observed in equine melanoma tissues. Moreover, it was observed that *PPARβ/δ* has a basal expression in healthy skin and that it is overexpressed in melanoma. These results were confirmed by a densitometric analysis, where a significant increase of the *WT1*-positive area was observed in healthy skin (128.66 ± 19.84 pixels 10^6) compared with that observed in melanoma (1.94 ± 0.04 pixels 10^6). On the other hand, a positive area with an expression of *PPARβ/δ* in healthy skin (214.94 ± 11.85 pixels 10^6) was significantly decreased compared to melanoma (624.86 ± 181.93 pixels 10^6). These data suggest that there could be a regulation between *WT1* and *PPARβ/δ* in this disease in horses.

KEYWORDS

skin, melanoma, equine, *WT1* gene, *PPARβ/δ* gene

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INTRODUCTION

In equines, tumours of the skin and soft tissues account for 80% of all neoplasms and the three most frequent of them are sarcoids, squamous cell carcinoma and melanoma, the last mentioned having great importance because of its high frequency. These tumours have very

high incidence, compared to other less common tumours present in horses, such as cutaneous lymphoma, mastocytoma and haemangiosarcoma (Schaffer et al., 2012). To facilitate the diagnosis of melanoma in equines, the presence of biomarkers has been investigated, in comparison with those reported for this pathology in humans (Seltenhammer et al., 2014).

A study conducted on 536 cutaneous and mucocutaneous neoplasms including sarcoids, squamous cell carcinoma, melanocytic tumours (melanoma), papillomas and mast cell tumours reported that the most frequent neoplasm found was melanoma, which constituted 87.5% of all cutaneous neoplasms (Valentine, 2006).

In horses, melanoma is differentiated as benign or malignant without giving other categories: unlike melanoma in humans, equine melanoma is not classified into types of aggressiveness. Melanoma usually invades only a localised area and has a slow growth rate; however, when it already causes metastases, they are commonly found under the tail and at the same time over the perianal region, in the jugular region, or around the eyes in the eyelid, where they can be easily identified as they occur in the form of black nodules (Burden, 2011).

On the other hand, it has been highlighted that this type of cancer is most commonly reported in dapple-coloured horses, having a higher prevalence in horses older than 15 years of age, estimated at 70–80% of this species. Furthermore, it has been described that horses of other colour types can also develop melanoma, although this is very rare (Sutton and Coleman, 1997; Fleury et al., 2000; Bellone, 2010; Seltenhammer et al., 2014).

One gene of importance in the study of neoplasms is the Wilms' tumour gene (*WT1*), a transcription factor involved in proliferation, differentiation and cellular apoptosis, which has been detected in a large number of neoplasms including melanoma, so it has been considered to play a relevant oncogenic role (Nakatsuka et al., 2006). In horses, the *WT1* gene has been reported to be located in chromosome 7 and submitted to the GenBank under accession no. Gene ID: 100072704 (NCBI Gene 2017). The predicted sequence for this gene in the horse is of 2,915 bp (accession number XM_005612334.1, NCBI Gene 2013), and its protein has accession number AH008865.2 (NCBI Gene 2016). This protein is found in the SEG locus – AF201736S (Brouillette et al., 2000); however, its biological role has not yet been accurately described in this species.

In molecular studies of human cancers it has been described that the *WT1* gene has a very close association with tumour formation, since a high overexpression of this protein has been demonstrated in a large variety of neoplasms such as leukaemias, lymphomas, melanoma as well as solid tumours of the lungs, thyroid, breast, ovary, uterus and other organs, which suggests that this gene plays a very relevant oncogenic role (Young et al., 2000).

Also, studies have found the expression of the *WT1* gene in more than 80% of melanoma samples; however, no evidence of its expression has been found in healthy (normal) skin, nor in melanocytic benign naevi. For this reason, the possible involvement of *WT1* in healthy skin remains undetermined, and its molecular mechanisms have not yet been described either (Wagner et al., 2008). It has been described that *WT1* expression is increased in advanced stages of melanoma and it is associated with a poor prognosis (Garrido Ruiz et al., 2010).

Our research group observed that the high expression of *WT1* can be silenced with an RNAi that also induces cell death in the murine melanoma cell line B16F10 (Zamora-Avila et al., 2007). Moreover, in an *in vivo* melanoma lung metastatic model, administration of the same RNAi results in a reduction of the tumour mass and increased survival time of mice, suggesting that *WT1* can be considered a good therapeutic target for this neoplasm (Zamora-Avila et al., 2009).

On the other hand, the *PPARβ/δ* genes (Peroxisome Proliferator-Activated Receptors) are ligand-activated transcription factors, which are involved in fatty acid oxidation in the liver, kidney, heart and skeletal muscle, and also participate in the differentiation of adipocytes (Tyagi et al., 2011). The activation of *PPARα* has been shown to inhibit the metastatic potential in melanoma cells, *PPARβ/δ* genes are expressed in human and murine melanoma and their pharmacological role is associated with the inhibition of *WT1* gene expression, while the stimulation of *PPARγ* decreases melanoma cell proliferation (Michiels et al., 2010). These PPARs are activated by a ligand that will strongly influence molecular signalling concerning normal cells and cancer cells (Rodríguez-Martín, 2008). Some evidence suggests that PPARs could play a very important role in skin carcinogenesis; it has been demonstrated that *PPARγ* is expressed in human melanoma tissues and its stimulation decreases the proliferation of melanoma cells (Rodríguez-Martín, 2008). Also, it has been described that the activation of *PPARα* results in the inhibition of potential metastasis of melanoma. The third member of the PPARs family, *PPARβ/δ* is expressed in human melanoma (Rodríguez-Martín, 2008); however, very little is known about the activity or role that it plays in melanocytes and melanoma (Ham et al., 2014). In the equine species, *PPARβ/δ* is located in chromosome 20 with a Gene ID of 100053404 and 1,644 pb (NCBI Gene 2017).

A study has described the interaction of *PPARβ/δ* and *WT1* in human and murine melanoma cells, reporting that the pharmacological activation of *PPARβ/δ* induces inhibition of proliferation without induction of apoptosis, associated with the reduction of *WT1* expression (Michiels et al., 2010).

In a previous study, we detected the expression of the *WT1* gene in the skin, kidney and blood samples of healthy horses, while no *WT1* gene expression was found in biopsies of melanoma, suggesting that *WT1* could act as a tumour suppressor gene in equines (Garza-Rodríguez, 2015). Prompted by the results previously obtained by our working group, and the findings in humans about the interaction that *PPARβ/δ* has in the repression of *WT1*, in the present work we analysed the role of *WT1* and its modulation through association with the expression of *PPARβ/δ* in the healthy skin of horses and in equine melanoma.

MATERIALS AND METHODS

We collected 37 samples of healthy skin and 15 samples of melanoma, as well as kidney and liver samples as positive controls for immunohistochemistry (IHC) analysis. All samples were taken from 53 horses from Gral. Escobedo, Nuevo León, Mexico (25°47′36″ N 100°9′30″ W). Healthy skin and melanoma samples were collected from the inguinal region, the dock of the tail and the perianal region. First, the horses were sedated with a solution of 1.1 mg/kg of xylazine 10% (Procin® Equus, PISA Farmacéutica Agropecuaria, DF México) and 0.01 mg/kg of detomidine (Domosedan®, Pfizer AG, Zurich, Switzerland) through the jugular vein. Later, the area of interest was shaved and asepsis with 70% ethyl alcohol was performed. Local anaesthetic (2% lidocaine) was also applied subcutaneously, and the samples were surgically collected using the standard sampling technique. The tissues were separated into two parts: one was fixed by immersion in 10% buffered formaldehyde for morphological analysis and the other was frozen at −20 °C for molecular analysis. All procedures and the protocol were endorsed by the local committee of bioethics and animal welfare, following the national animal welfare act (NOM-062-ZOO-1999).

Morphological analysis

Samples were fixed with 10% buffered formaldehyde pH 7.2–7.4 for 24 h, then they were processed by conventional histological techniques to obtain paraffin-embedded tissues. Subsequently, 4-µm-thick histological sections were obtained and stained with haematoxylin and eosin (HE) for histological analysis, which was done by three independent certified pathologists. The macroscopic appearance of healthy skin and melanoma was confirmed by histopathological microscopic analysis performed by the certified pathologists.

For IHC analysis, histological sections of 4 µm thickness were deparaffinised with xylene, hydrated gradually with ethanol, and briefly washed with Tris-buffer saline + Tween 20 (TBST) for 3 min each. Antigen retrieval was carried out with a TRS solution (Target Recovery Solution, Dako™) for 60 min at 100 °C, then endogenous peroxidase activity was quenched with 3% H_2O_2 in distilled water and 30-min incubation at room temperature (RT). Sections were rinsed in TBST and proteins were blocked with the Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHQ kit (Abcam, USA) for 30 min at RT. Slices were incubated overnight at 4 °C with specific rabbit polyclonal antibodies anti-WT1 (1:100 dilution, clone 19), and subsequently with the secondary anti-rabbit antibody (diluted 1:50) for 2 h. For the detection of *PPARβ/δ* the first antibody C20 (1:400 dilution) and a secondary anti-goat antibody (diluted 1:200) were used under the above-mentioned conditions. All antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Positivity was visualised with 9-Ethyl-9H-carbazole-3,6-diamine (AEC) and the nuclei were identified using Gill's haematoxylin. Equine kidney and liver were used as positive controls of *WT1* and *PPARβ/δ* expression, respectively; in negative controls, the primary antibody was omitted. Finally, the samples were analysed by light microscopy. Digital high-resolution images were obtained with a Nikon Microscope Eclipse 50i with the image analysis system NIS-Elements software advanced research Digital Sight dDS-2Mu.

Microdensitometric analysis

In this study, a microdensitometric analysis was performed to quantify the positive area for both proteins (WT1 and PPARβ/δ), using the program Image J, v1.51 (National Institute of Health, USA). The background was uniformly eliminated with a digital filter and the staining areas were analysed. The intensity of the immunolabelling is reported as the mean ± SD of the total pixels in the measurement area. This was done in triplicate (3 fields of 3 independent slides, $n = 9$) and the mean value and the standard deviation (SD) were obtained. Results were analysed statistically with Student's *t*-test for a significance value of $P \leq 0.05$. This statistical analysis was performed with the software GraphPad Prism 6.0 for Windows (GraphPad Sofware, Inc., USA).

RESULTS

In the analysis of healthy skin biopsies, it was observed that the epidermis, dermis, hair follicles, sebaceous glands and sweat glands had normal histological characteristics. In contrast, in melanoma samples, many foci of tumour cells were seen, infiltrating especially the epidermis and dermis, although the structure of the epidermis appeared to be normal. Moreover, we also observed an abundant presence of metastatic tumour cells in the lymph nodes in all melanoma samples; these findings confirmed the histopathological diagnosis of melanoma.

In the IHC analysis, positive expression of the WT1 protein was observed in the healthy skin samples, this expression was located mainly in the epidermis, hair follicle, sebaceous gland and sweat gland (Fig. 1A–C). Instead, in skin melanoma, *WT1* expression was not observed, suggesting that *WT1* expression is downregulated in this pathology. Moreover, *WT1* expression could be observed poorly in some foci of infiltrated melanoma tumour cells (Fig. 1D).

On the other hand, *PPARβ/δ* expression in the healthy skin was positive and located in the epidermis, hair follicle, sebaceous gland and sweat glands (Fig. 1E–G). Moreover, in melanoma samples and lymph node metastasis zones, the presence of the PPARβ/δ protein was observed with high positivity (Fig. 1H).

These results were confirmed by densitometric analysis, where we could observe a significant increase ($P = 0.0004$, Student's *t*-test) of the *WT1*-positive area in healthy skin (128.66 ± 19.84 pixels 10^6) compared with that observed in melanoma (1.94 ± 0.04 pixels 10^6). On the other hand, the positive area showing an expression of *PPARβ/δ* in healthy skin (214.94 ± 11.85 pixels 10^6) was significantly decreased ($P = 0.0177$, Student's *t*-test) compared to melanoma (624.86 ± 181.93 pixels 10^6) (Table 1).

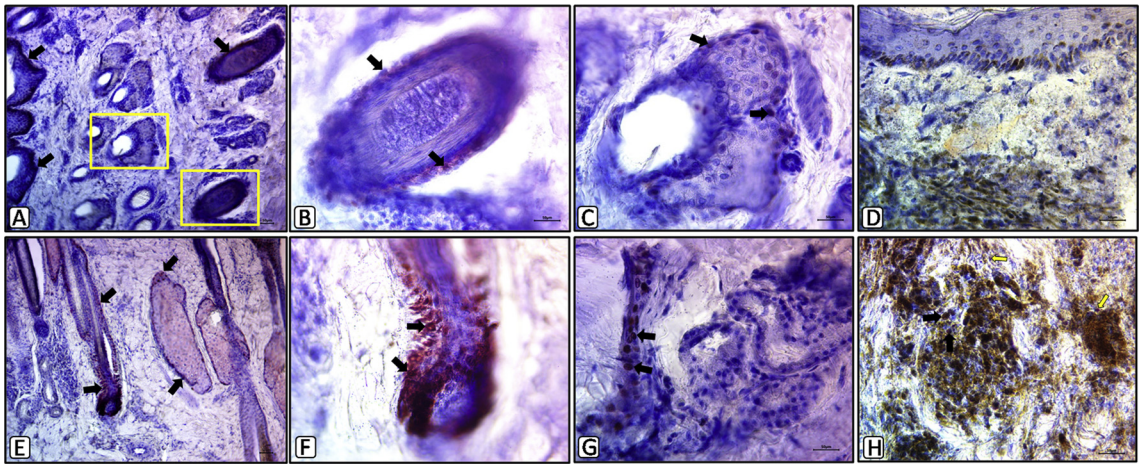

Fig. 1. Immunohistochemistry (IHC) of *WT1* and *PPARβ/δ* in samples of healthy equine skin and equine melanoma. (A–C) IHC micrographs of healthy equine skin: the epidermis can be seen on the left side of the image with positive signal areas (pink-red colour, black arrows) for *WT1* in the basal layer, in the dermis positive areas can be observed in the hair follicles and the sebaceous glands (black arrows). (D) IHC for *WT1* in equine skin with melanoma. The epidermis can be distinguished in the upper part of the micrograph, where the basal layer with abundant hyperplastic melanocytes can be observed. In the dermis, the invasion of melanin-producing tumour cells can be observed throughout the entire length of the tissue. Neither in the epidermis nor in the dermis are there areas positive for *WT1*. (E–G) IHC for *PPARβ/δ* in the healthy equine skin. Only a small fragment of the epidermis can be seen, while the structures of the dermis can be observed, and areas of positivity can be distinguished in the hair follicles, sebaceous glands, and sudoriparous glands (black arrows). (H) IHC for *PPARβ/δ* in equine skin with melanoma. Invasion with tumour cells producing melanin is quite evident, there are abundant cells with a positive signal (red-pink, black arrows), while it is also possible to observe tumour cells with positivity that show a purple colour (yellow arrows). A, E: Bar = 100 μm; B, C, D, F, G and H: Bar = 50 μm

Table 1. Densitometric analysis for cell positivity

	Sample		Student's *t*-test
	Healthy skin	Melanoma	
WT-1	128.66 ± 19.84 pixels 10^6	1.94 ± 0.04 pixels 10^6	$P = 0.0004$
PPARβ/δ	214.94 ± 11.85 pixels 10^6	624.86 ± 181.93 pixels 10^6	$P = 0.0177$

DISCUSSION

In the present work, we analysed for the first time the expression of *WT1* and *PPARβ/δ* in the healthy skin of horses and in equine melanoma to elucidate their possible biological role and their analogy with other species. We analysed these proteins using IHC as a diagnostic technique. The use of AEC as a revealing agent makes detection more accurate for the quantification of proteins from tissues (Hira et al., 2019); in addition, we could readily distinguish positivity because AEC produces a red-pink colour, instead of the black-brown colour that is produced with 3,3′-diaminobenzidine (DAB) or endogenous melanin in melanoma.

In our study, healthy equine skin samples showed basal expression for *WT1* at the level of the hair follicle, in the sebaceous glands and sweat glands, but we did not observe any expression of *WT1* in the skin samples of horses with melanoma, in contrast to previous reports in human melanoma skin where *WT1* is highly expressed in the neoplastic cells.

Previous studies analysing the expression of the WT1 protein by IHC using the polyclonal antibody C-19, demonstrated good quality in distinguishing its increased expression in human melanoma skin, suggesting that *WT1* can be significant in the prognosis of melanoma (Mrowka and Schedl, 2000). In our work, the same WT1 antibody was used in equine melanoma and healthy skin, also using the equine kidney as control, to standardise the label and ensure the reactivity and specificity of the antibody in the equine protein. We observed no expression of *WT1* in melanoma and only basal expression in healthy skin, suggesting that the *WT1* gene does not have the same biological role in horses as in humans, since this gene is usually expressed abundantly in this disease (Wilsher and Cheerala, 2007).

Before this study, there were no reports on the use of the polyclonal antibody C-20 for determining the expression of *PPARβ/δ* in equine melanoma and the healthy skin of horses, and the biological role of *PPARβ/δ* in this type of neoplasia is not known in this species. We found that *PPARβ/δ* has a basal expression in healthy equine skin, mainly in the epidermis, hair follicles and sebaceous glands, as reported in humans (Tyagi et al., 2011), and surprisingly it is overexpressed in equine skin with melanoma.

PPARβ/δ plays a very important role in lipid metabolism in the muscles of horses and humans; it is involved in regulating the metabolism of lipids (Dunning et al., 2014), carbohydrates and proteins (Lee et al., 2006). This could explain its basal expression in healthy skin and the abrupt increase of its expression in melanoma, due to the increased metabolic activity of tumour cells. Moreover, a recent report shows increased expression of *PPARβ* after pharmacological activation in human and murine melanoma cell lines, that induces an inhibition of cell proliferation and low expression of *WT1* due to repression by direct binding of *PPARβ* to the *WT1* promoter (Michiels et al., 2010). From this we inferred that this may cause the decrease in *WT1* expression in equine skin with melanoma; however, it would be necessary to carry out molecular and immunoprecipitation analysis to confirm this interaction and to elucidate the biological role in this equine neoplasia. Currently, in our laboratory, studies are being performed to elucidate other elements directly or indirectly involved in the interaction mechanism between *PPARβ/δ* and *WT1* in samples of healthy skin and melanoma in different regions of the same animal (legs, head, neck, back, perianal region, etc.). Also, the expression of these proteins should be evaluated and compared among different horse breeds to establish if this behaviour is the same or different, since in this study no samples were taken from a particular horse breed.

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