J. Comp. Path. 2014, Vol. 150, 198–203

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NEOPLASTIC DISEASE

Multiple Polymerase Chain Reaction Markers for the Differentiation of Canine Cutaneous Peripheral Nerve Sheath Tumours versus Canine Fibrosarcomas

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Summary

Currently canine fibrosarcomas and peripheral nerve sheath tumours (PNSTs) are differentiated by their histopathological phenotype. Preliminary global transcriptomic analysis has identified genes with significant differential expression in both tumour types that may act as potential tumour markers. The aim of the present study was to establish reverse transcriptase polymerase chain reaction (RT-PCR) assays for the differentiation of formalin-fixed and paraffin wax-embedded tumours of both types. Fifty histologically well-defined examples of canine fibrosarcomas and PNSTs were characterized immunohistochemically for the expression of S100, laminin and PGP 9.5. RT-PCR assays for the potential fibrosarcoma markers *FHL2-Ex4* and *FHL2-Ex9* and the PNST markers *GLI1* and *CLEC3B* were established and tested for their specificity and sensitivity to differentiate fibrosarcomas and PNSTs by their mRNA expression. Immunohistochemical analysis challenged the value of S100, laminin and PGP 9.5 for the diagnosis of PNSTs, since both PNSTs and fibrosarcomas showed similar expression of these proteins. In contrast, a combination of the markers *GLI1* and *CLEC3B* differentiated PNSTs from fibrosarcomas with a sensitivity of 89% and a specificity of 87%. The proposed fibrosarcoma markers *FHL2-Ex4* and *FHL2-Ex9* failed to separate PNSTs and fibrosarcomas (sensitivity 50%, specificity 88%). The failure of these markers to unequivocally separate fibrosarcomas and PNSTs raises questions as to whether histologically uniform PNSTs are less uniform at the molecular level than expected or if both tumour types, despite their different morphology, are more closely related in terms of their histogenesis than previously thought.

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Keywords: dog; fibrosarcoma; gene expression; PNST

Peripheral nerve sheath tumours (PNSTs) and fibrosarcomas are part of a large group of soft tissue sarcomas defined by a spindle cell phenotype and similar biological behaviour (Liptak and Forrest, 2007). Differentiation of these soft tissue sarcomas is mainly based on their histopathological phenotype (Hendrick *et al*., 1998), but several immunohistochemical PNST markers have been proposed (Chijiwa *et al*., 2004; Gaitero *et al*., 2008; Dennis *et al*., 2011).

S100 expression has been shown to be a moderately sensitive PNST marker, while the expression of S100 in fibrosarcomas has been investigated less often (Perez *et al*., 1996; Choi and Kusewitt, 2003; Schoniger and Summers, 2009). PGP 9.5 was initially described as a marker of human PNSTs and is also commonly expressed in canine PNSTs (Gaitero *et al*., 2008), while PGP 9.5 expression in canine fibrosarcomas has not been analysed. Finally, laminin expression is thought to be restricted to Schwann cells or neoplastic cells originating from them (Haraida *et al*., 1992) and has been described as commonly expressed in PNSTs (Gaitero *et al*.,

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0021-9975/$ - see front matter
http://dx.doi.org/10.1016/j.jcpa.2013.08.006

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2008). A sensitive and specific immunohistochemical PNST marker is not available and immunohistochemistry (IHC) is unable unequivocally to define a PNST.

A comparison of the global mRNA expression of PNSTs and fibrosarcomas was performed recently using microarray technology in an attempt to identify potential mRNA markers of PNST or fibrosarcoma (Klopfleisch *et al*., 2012). This approach identified 45 genes with significant differences in expression levels between the two tumour types. Moreover, seven of these genes with higher expression in PNSTs are known to be mainly expressed in neuroectodermal tissues (Klopfleisch *et al*., 2012). Microarray analysis thus identified, in a small group of tumours, potential PNST marker genes that may be useful as diagnostic markers.

The aim of the present study was to determine whether the known immunohistochemical markers and the potential mRNA markers identified by comparative transcriptomic analysis correlate with the current diagnostic 'gold standard' of histopathological appearance of these tumour types.

Twenty-six canine cutaneous PNSTs and 24 canine fibrosarcomas were selected from the tissue bank of the Institute of Veterinary Pathology, Freie Universität Berlin. Tumours were selected according to the histological features defined by the World Health Organization (Hendrick *et al*., 1998). Only clear examples of tumours of both types were included in the study. Fibrosarcomas had to be composed entirely of well-differentiated to plump, pleomorphic spindle cells arranged in interwoven fascicles without a concentric, perivascular arrangement in whorls or palisades. In contrast, PNSTs were completely composed of wavy spindle cells arranged in bundles, palisades or whorls.

Tissue specimens were fixed in 4% neutral buffered formalin (Klopfleisch *et al*., 2010a). Expression of S100, laminin and PGP 9.5 was detected by IHC. Antigen retrieval was performed for PGP 9.5 by heating in citrate buffer (pH 6.0) for 12 min and for laminin and S100 by incubation with Fast Enzyme® (Zytomed, Berlin, Germany) for 10 min. Sections were incubated overnight at 4°C with polyclonal rabbit anti-human laminin (Dako, Glostrup, Denmark; diluted 1 in 400), polyclonal rabbit anti-human S100 (Sigma, München, Germany; diluted 1 in 800) or monoclonal mouse anti-human PGP 9.5 (clone 13C4, Abcam, Cambridge, UK; diluted 1 in 25). Secondary antibodies were applied as described by Klopfleisch *et al*. (2010b). Tumours were defined as positive for S100, laminin and PGP 9.5 if >10% of tumour cells had expression of the respective marker.

mRNA extraction from formalin-fixed and paraffin wax-embedded (FFPE) tissue samples was performed according to a modified standard protocol (Weiss *et al*., 2011; Klopfleisch *et al*., 2011b). Presence of amplifiable cDNA was tested by amplification of the housekeeper gene *ATP5B* (Klopfleisch *et al*., 2010a). Primer design was performed using the Primer 3 programme (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) with a target primer length of 80–150 base pairs (bp). Potential polymerase chain reaction (PCR) markers included genes that were significantly regulated in microarray analysis and confirmed with quantitative real-time reverse transcriptase PCR (qRT-PCR) (Klopfleisch *et al*., 2012). From the 45 regulated genes, only those with a high-fold change (>10) or relevant tumour function or previously described neuronal-specific expression were selected as potential markers. Consequently, primer design was performed for potential target genes: *GLI1* (forward CTCCTACGGTCACC TCTCCA, reverse GGTCCCTTTTTGGTGATT CAT), *FHL2-Ex4* (forward GCGAGGAGTGTGG GAAGC, reverse GCGAGCAGTGGAAACAGG), *KIF1B*, *ROBO2*, *DOK4*, *PLAGL1*, *OSTM1*, *FNBP1L* and *CSK*. Additionally, previously designed primers were used for *CLEC3B*, *FHL2-Ex9* (Klopfleisch *et al*., 2012) and *ATP5B* (Klopfleisch *et al*., 2011a). Preliminary conventional RT-PCR assays with five fibrosarcomas and five PNSTs were performed and only those genes that discriminated to at least 40% between the two tumour groups were used for further studies.

CLEC3B and *FHL2-Ex9* were amplified using a GoTaq polymerase system with a total reaction volume of 50 μl containing 5 μl cDNA, 20 pmol of each primer, 1.25 U of GoTaq polymerase (Promega, Madison, Wisconsin, USA), 10 μl of Green GoTaq Flexi Buffer (Promega), 5 μl of 2 mM dNTP-Mix (Fermentas, Burlington, Ontario, Canada) and 3 μl of 25 mM $MgCl_2$ (Promega). *GLI1*, *FHL2-Ex4* and *ATP5B* were amplified with a Maxima Hot Start polymerase system with a total reaction volume of 50 μl containing 5 μl cDNA, 20 pmol of each primer and 25 μl Maxima Hot Start Green PCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA). Cycling conditions were 95°C for 5 min followed by 40 cycles at 95°C for 30 sec, Tm for 30 sec, 72°C for 30 sec and a final extension of 72°C for 5 min. PCR products were fractionated on a 2.5% ethidium bromide-stained agarose gel. Each PCR reaction was performed three times in three independent runs and PCR was recorded as positive when at least two of three runs were positive for the appropriate gene. Significance of differences was analysed by the Mann–Whitney *U* test using SPSS Statistics 20 (IBM, Ehningen, Germany).

All PNSTs were composed of wavy spindle cells arranged in bundles, palisades or whorls. Occasional

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