Expression of Phosphorylated Signal Transducer and Activator of Transcription 3 (pSTAT3) and its Prognostic Significance in Canine Anal Sac Adenocarcinoma

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Summary

Prognostication in canine anal sac adenocarcinomas (ASACs) is difficult due to conflicting evidence regarding metastatic rates and median survival times (MST). The transcription factor signal transducer and activator of transcription 3 (STAT3) is a prognostic predictor in several human cancers. The aim of this retrospective study was to assess STAT3 expression in ASAC and to explore its association with clinical presentation and outcome. We hypothesized that STAT3 expression would distinguish tumours with early versus late metastasis. Records from The Queen’s Veterinary School Hospital, Cambridge, UK were searched for dogs diagnosed with ASACs from 2008 to 2019. Immunohistochemical expression of phosphorylated STAT3 (pSTAT3) was assessed in primary tumours (n = 57) and metastatic lymph nodes (n = 30) and MST were calculated for cases with low and high pSTAT3 expression. Of the 57 cases assessed, 27 presented with primary tumours but no metastasis and 30 with both primary and local metastatic disease. Most cases (50/57) expressed nuclear pSTAT3 within neoplastic cells in both primary tumour and metastatic lymph nodes. pSTAT3 expression was predominantly observed in neoplastic cells at the edges of neoplastic lobules, suggesting a potential role in invasion. There was no significant difference in
pSTAT3 expression between cases metastatic at presentation and those that did not have detectable metastasis at presentation. There was no significant difference between MST in cases with high and low pSTAT3 expression. Cases that presented with metastatic disease had shorter MST (395 days) than those with primary tumours alone (623 days). Although pSTAT3 is variably expressed in primary and metastatic ASACs cells, pSTAT3 did not provide prognostic information for canine ASACs.

Keywords: anal sac adenocarcinoma, canine, prognosis, STAT3

Introduction

Anal sac adenocarcinoma (ASAC), arising from the apocrine glands of the anal sac, is the most common perineal malignancy in dogs, accounting for 17% of tumours in this area (Berrocal et al, 1989). Canine ASAC is described as a locally invasive and metastatic tumour and can display inconsistent clinical behaviour. Reported rates of metastasis are highly variable, ranging from 26% to 96% at diagnosis (Polton and Brearley, 2007; Barnes and Demetriou, 2017; Pradel et al, 2018; Emms, 2005; Bennett et al, 2002) and are independent of tumour size (Brown et al, 2012). The pattern of metastasis is not well-defined with involvement of regional lymph nodes in 26–86% of cases and spread to distant sites (liver, spleen and lungs) in 0–42% of cases (Bennett et al, 2002; Emms, 2005; Polton and Brearley, 2007; Barnes and Demetriou, 2017; Liptak and Turek, 2019). These authors reported median survival times (MST) ranging from 386 to 960 days. Local recurrence rates also vary widely from 5% to 44% (Potanas et al, 2015; Wouda et al, 2016) and are not associated with completeness of excision (Ross et al, 1991; Turek et al, 2003). Due to this diverse clinical behaviour, it has become important to predict which tumours are more likely to have an aggressive phenotype, so that an optimal treatment plan can be constructed for each patient.
Several factors have been investigated as prognostic indicators of ASAC and survival seems to be influenced mainly by clinical stage (Polton and Brearley, 2007). Histological characteristics have been shown to predict outcome in a study of 39 dogs with a predominantly solid pattern, peripheral infiltration of surrounding tissue, presence of necrosis and lymphovascular invasion associated with a shorter progression-free interval and overall survival (Pradel et al, 2018). Reduced expression of E-cadherin is associated with a worse prognosis in cases of ASAC (Polton et al, 2007).

STATs are a family of transcription factors that regulate cellular growth, survival and differentiation (Darnell, 1997). They are activated by a ligand binding to a STAT receptor and activating a receptor-associated Janus kinase (JAK) which, in turn, activates a STAT protein by phosphorylation. When activated, phosphorylated STATs (pSTATs) dimerise and transcriptionally regulate a diverse set of genes, including some that are implicated in malignant progression (Bromberg et al, 1999). Among the STAT transcription factor family, STAT3 plays a central role in carcinogenesis, is dysregulated in a wide number of human cancers (Yu and Jove, 2004) and constitutes a promising therapeutic target for inhibition in cancers such as breast cancer (Hughes et al, 2018).

There is similar evidence that STAT3 is transcriptionally active in a range of equine (Hughes et al, 2015; Hughes et al, 2018), feline (Petterino et al, 2007) and canine (Petterino et al, 2001; Teng et al, 2012; Lin and Palmieri, 2016; Assumpção et al, 2018; Cletzer et al, 2020) neoplasms. Interestingly, a recent study has demonstrated that components of the JAK-STAT signalling pathway, including pSTAT3, are expressed in canine ASAC. However, this neoplasm was not the primary focus of that study and only nine cases were evaluated, with three dogs excluded from the survival analysis (Cletzer et al, 2020). Given the pressing need for more accurate identification of ASACs likely to exhibit an aggressive clinical phenotype and the promising data
that JAK1/2-STAT3 signalling may be active in canine ASAC, we sought to assess pSTAT3 expression in 57 cases of canine ASAC and to explore its association with clinical presentation and outcome. We hypothesized that STAT3 expression would distinguish tumours with early versus late metastasis.

Material and Methods

Case Selection

Histology specimens of primary and metastatic ASACs were identified using the Cambridge University Veterinary School (CUVS) Pathology Database. All neoplasms had been submitted for histopathological examination after excision of the primary tumours and local lymph node metastases with curative intent at The Queen Veterinary School Hospital (QVSH) between 2008 and 2019. Medical records were reviewed and cases were included if a complete medical history was available. Data collected from the clinical records included signalment (breed, sex, neuter status, age and weight) and primary tumour size. The size of the tumour (longest dimension) was estimated by digital rectal examination and measurement with callipers before removal and by measurement with a ruler after surgical excision in all cases. The results from staging procedures were collected including cytological or histological analyses of suspected metastatic lesions. In cases in which sampling was considered too hazardous or declined by the owner, the suspected lesion was considered metastatic if proven to be progressive on serial monitoring.

Follow-up by abdominal ultrasound imaging was recommended 4–6 weeks after surgery and subsequently every 3 months. The owner was requested to contact the hospital if they were suspicious of local recurrence or metastatic disease or if symptoms appeared before the scheduled follow-up.

Western Blotting

In order to corroborate species cross-reactivity for the pSTAT3 antibody used in the study, western blotting was performed. As no fresh frozen tissue from a canine ASAC was available, a sample of
hyperplastic canine mammary tissue from a surgical biopsy sample submitted to the University of Cambridge, Department of Veterinary Medicine, anatomic pathology service was utilized. At the time of submission of the biopsy sample, a small piece of mammary tissue was immediately frozen at −80°C. Western blotting for pSTAT3 was performed following standard protocols for protein extraction and immunoblotting.

Histology and Immunohistochemistry

At the time of original diagnosis, tumour surgical biopsy specimens were fixed in 10% neutral-buffered formalin, processed in paraffin wax, sectioned and subsequently stained with haematoxylin and eosin (HE) following standard protocols. All slides were re-evaluated by two authors (AM & KH), one an American College of Veterinary Pathologists board-certified pathologist (KH) to confirm the diagnosis and to select the best sections for downstream immunohistochemical (IHC) labelling. For patients which had multiple neoplastic masses removed either simultaneously or concurrently in multiple procedures, one representative slide from each neoplastic site was selected. IHC labelling for pSTAT3 (Tyr 705) (Cell Signaling Technology, Leiden, The Netherlands; dilution 1:200) followed a routine protocol using an automated system (Hughes et al., 2018). Sections were counterstained with haematoxylin. A secondary antibody only negative control was used, and the primary antibody used was also validated on canine tissue with negative control comprising species- and isotype- matched immunoglobulins.

Tissue sections were evaluated by a pathologist (KH) blinded to the clinical data. Evaluation of the percentage of neoplastic cells expressing pSTAT3 was performed in a semi-quantitative manner on a five-point scale as follows: 0, <5%; 1, 5–24%; 2, 25–49%; 3, 50–74%; 4, 75–100%. The overall immunolabelling pattern was categorized using a set of standardized descriptors: ‘edge’, reflecting pSTAT3 expression predominantly at the perimeter of neoplastic lobules; multifocal, reflecting patchy pSTAT3 expression throughout the neoplasm; and lobular reflecting cases in which
some neoplastic lobules were predominantly diffusely positive while others were predominantly negative. Edge labelling denoted pSTAT3 expression in neoplastic cells at the edges of neoplastic lobules rather than at the edges of the tissue section, which can be present artefactually in immunolabelled sections. These descriptors were also used in combination with, for example, ‘edge and lobular’ reflecting cases in which either multifocal lobules were diffusely positive or only cells at the lobular perimeter were labelled. A final category, ‘mixed’, was used to denote cases in which all three labelling patterns were recognized in approximately equal abundance.

Statistical Analysis

Differences in the percentages of labelled cells were evaluated by the non-parametric U test of Mann-Whitney in two groups: group 1, cases metastatic at presentation; group 2, cases that did not have detectable metastasis at presentation. Median survival time (MST) was calculated from the date of histological diagnosis to the date of death. Kaplan-Meier survival curves for MST were generated for dogs with low (0, <5% and 1, 5–24%) and high expression (>2, 25–49%) of STAT3. Dogs were excluded from the survival analysis if they were lost to follow-up, died of causes unrelated to the tumour or were alive at the time of analysis. For all statistical analyses, a P value <0.05 was considered significant. GraphPad Prism version 8.1.2 was used for statistical analysis.

Results

Selected Cases

Archived specimens were retrieved for 60 cases and three dogs were excluded because the clinical records were incomplete. Therefore, 57 patients with a total of 87 biopsy specimens were included in the study, with some patients having had tissue removed from more than one site (primary tumour and one or more draining lymph nodes).
The study population comprised 2 entire males, 32 castrated males, 2 entire females and 21 spayed females with a median age of 9 years at the time of diagnosis (range 5–13 years). The breeds most commonly represented were Cocker Spaniel (18), Labrador Retriever (17) and cross-breed (7); 11 other breeds were also represented (English Setter [2], Springer Spaniel [2], Cavalier King Charles Spaniel [2], Dachshund [2], and one each of Border Collie, Cairn Terrier, Dalmatian, English Setter, German Shepherd Dog, Golden Retriever and Irish Setter). At time of surgery, the primary tumour masses measured <2 cm in 21 cases, 2–5 cm in 30 cases, and more than 5 cm in 6 cases. Fifty-four cases (94%) had abdominal ultrasound and thoracic radiographs at presentation, three cases (6%) had abdominal and thoracic computed tomography (CT) scan. Thirty dogs (53%) had metastatic disease at the level of the local regional lymph nodes on initial presentation and were included in group 1. In these cases, metastatic disease was suspected on cytology of fine-needle aspirates in 19 cases and confirmed on histopathology in all 30 cases. Twenty-seven cases (47%) were presented with primary tumours with no evidence of metastasis and were included in group 2. No cases had distant metastatic disease (to spleen, liver or lungs) on presentation. In group 2, six dogs developed local metastases at 8 months, two at 12 months, one at 15 months, one at 18 months and one at 3 years. The other 16 dogs had no evidence of distant metastatic spread at the time of the last follow-up imaging. Abdominal imaging follow-up at 4 weeks post-surgery was available for 24 dogs, 3- and 6-months imaging follow-up was available for 38 dogs and 47 dogs respectively, and 1-year imaging follow-up was available for 20 dogs. Fourteen dogs had abdominal imaging follow up <1 year after surgery. All dogs had abdominal imaging at least once after 1, 3 or 6 years.

Western Blotting

Western blotting using hyperplastic canine mammary tissue resulted in a band of expected size (79–86 kDa), indicating cross-reactivity of the antibody with canine tissue (Fig. 1).

pSTAT3 Immunohistochemistry
pSTAT3 expression was localized to the nucleus of neoplastic cells (Figs. 2–5) as would be anticipated for the detection of the transcriptionally active phosphorylated protein. pSTAT3 expression is summarized in Table 1. Most primary tumours (50/57; 87%) and 27/30 (90%) lymph node metastases exhibited some degree of nuclear pSTAT3 expression. In cases in which pSTAT3 expression was apparent, the immunolabelling pattern in the main mass was categorized as ‘edge’ in 12 cases (Fig. 2), multifocal in 15 cases, lobular in two cases, ‘edge’ and multifocal in 11 cases, ‘edge and lobular’ in four cases (Fig. 3) and ‘mixed’ in six cases. The immunolabelling pattern in lymph nodes was categorized as ‘edge’ in seven cases, multifocal in six cases (Fig. 4), lobular in two cases, ‘edge’ and multifocal in nine cases, ‘edge’ and lobular’ in one case and “mixed” in two cases (Table 2). A preponderance of cases (33/50 primary masses and 19/27 metastatic lymph node lesions) expressed either a predominantly ‘edge’ pattern or a mixed pattern with a strong component of ‘edge’ expression.

pSTAT3 expression was also detected in tumour-associated immune cells and stromal components. Nuclear pSTAT3 expression was observed in the anal sac epithelium and in immune cells within subepithelial foci (Fig. 5).

Prognostic Significance of pSTAT3 Expression in ASAC

There was no significant difference in the level of expression of pSTAT3 between cases with metastases at presentation and cases that did not have detectable metastasis at presentation (P = 0.8, non-parametric U test of Mann-Whitney).

MST for cases that were metastatic at presentation was 395 days (7–1003 days) and 623 days (30–2707 days) for cases that did not have detectable metastasis at presentation. No significant difference in MST was found between dogs with low (0, <5% and 1, 5–24%) and high expression (>2, 25–49%) of pSTAT3 (P = 0.87, Log-rank test). Eleven dogs were excluded from the Kaplan-Meier analysis.
Nine dogs were alive at the time of writing and two, lost to follow-up after surgery, had metastasis at presentation.

**Discussion**

In this study we firstly report that pSTAT3 is transcriptionally active in most canine ASAC and local lymph node metastases. STAT3 regulates cell growth and survival, and may contribute to malignancy by preventing apoptosis through increasing the expression of anti-apoptotic products of genes involved in cell differentiation and regulation of the cell cycle, including cyclin D1, c-myc, p21, and Bcl family members (Yu and Jove, 2004). A correlation between increased expression of pSTAT3 and grade, metastasis, invasion and angiogenesis has been reported in canine diffuse large B-cell lymphoma, canine osteosarcoma, canine prostatic cancer and canine and feline mammary gland tumours (Petterino *et al*, 2001 and 2007; Fossey *et al*, 2009; Lin and Palmieri, 2016; Assumpção *et al*, 2018). Invasion refers to the direct extension and penetration by cancer cells into neighbouring tissues. The proliferation of transformed cells and the progressive increase in tumour size eventually leads to a breach in the barriers between tissues, resulting in tumour extension into adjacent tissues. Local invasion is also the first stage in the process that leads to the development of secondary tumours or metastases (Krakhmal *et al*, 2015). It is thus particularly interesting that, in this study, most cells expressing pSTAT3 were present at the invasive front or peripheral regions of the primary tumours compared with the superficial and central areas in most of our cases (33/50) as shown by the predominance of edge or mixed immunolabelling patterns with a strong component of edge expression. It is possible that the increased expression of pSTAT3 at the edge of neoplastic lobules may indicate a role in facilitating neoplastic cell invasion. A similar pattern of pSTAT3 expression at the invasive edge has been demonstrated in orthotopic mammary carcinomas arising from implantation of 4T1 cells in mice (Hughes *et al*, 2016). Further studies may focus on evaluation of upstream activators of pSTAT3 (Cletzer *et al*, 2020) and downstream gene expression...
patterns (Qu et al, 2009; Hsieh et al, 2005) in order to better clarify the role of pSTAT3 expression in canine ASAC.

The prognosis of patients with ASAC has conventionally been determined by the clinical stage and the presence or absence of metastasis (Polton and Brearley, 2007). Histological characteristics (Pradel et al, 2018) and E-caderin expression (Polton et al, 2007) have been shown to correlate with prognosis but larger prospective studies are needed to validate these findings.

The mechanism of invasion and metastasis of ASAC has not been fully elucidated and the role of the JAK/STAT pathway in canine ASAC has not been fully clarified (Cletzer et al, 2020), particularly the relationship between expression of pSTAT3 and the clinicopathological features of ASAC. We did not find any correlation between STAT3 activation and the presence of lymph node metastasis. Moreover, pSTAT3 expression was not significantly correlated with MST. pSTAT3 was equally expressed in cases that presented with metastases and cases that did not have detectable metastasis at presentation. Therefore, pSTAT3 labelling might not be a useful diagnostic tool to identify more aggressive ASAC phenotypes. Equally, the distribution of pSTAT3 in dogs with low (category 0, <5% and category 1, 5–24%) and high expression (category 2 and above, 25–49%) was similar between groups, and our results do not support the use of qualitative evaluation of pSTAT3 for prediction of survival. However, given the relatively small group size and the fact that several cases had been excluded from the survival analysis, we cannot definitively assert that there is no statistical difference between the groups. Low case numbers, non-standardized treatment protocols and the unpredictability of euthanasia as an endpoint, are all potential sources of statistical error as in any retrospective study.

References


*Legends to Figures*

Fig. 1. Western blotting on hyperplastic canine mammary tissue demonstrates a band of size 79–86 kDa, indicating cross-reactivity of the pSTAT3 antibody with canine tissue.
Fig. 2. Dog, anal sac adenocarcinoma. Distinctive ‘edge’ pattern of pSTAT3 immunolabelling in neoplastic cells at margins of neoplastic lobules (arrows). IHC. Bar, 200 µm.

Fig. 3. Dog, anal sac adenocarcinoma. Lobular (asterisk) and ‘edge’ (arrows) patterns of pSTAT3 immunolabelling. IHC. Bar, 200 µm.

Fig. 4. Dog, anal sac adenocarcinoma. Multifocal pattern of pSTAT3 immunolabelling. IHC. Bar, 200 µm.

Fig. 5. Immunolabelling of pSTAT3 in anal sac epithelium (asterisk) and in subepithelial immune cells (arrows). IHC. Bar, 80 µm.