The known unknowns of equine mammary neoplasia

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Introduction

Primary equine mammary tumours are rare entities that are considered to have a poor prognosis (Shank, 2009). In the case report associated with this clinical commentary, the authors describe the clinical presentation, management, and histopathological assessment of a comedocarcinoma arising in the mammary gland of a 20 year-old Arabian mare (Sabiza et al., *In press*). In their article, the authors allude to some of the challenges of diagnosis and prognostication in cases of equine mammary neoplasia. In spite of a number of detailed descriptions of individual cases or case series of equine mammary tumours, there are numerous unanswered questions regarding carcinomas arising in the equine mammary gland, and the concept of ‘known unknowns’ springs to mind. This clinical commentary will consider a selection of interesting and challenging ‘known unknowns’ relating to the pathogenesis, diagnosis, and prognosis of equine mammary tumours.

The relationship between mammary tumourigenesis and mare reproductive history is currently undetermined

At the time of presentation for investigation of mammary enlargement and bleeding from the teat, the mare in the associated case report was lactating (Sabiza et al., *In press*). This highlights an interesting question regarding the role, if any, of parity as a risk factor for equine mammary tumour development. In dogs and cats, the currently available data suggest that parity is not a risk factor for mammary tumourigenesis (Taylor et al., 1976; Overley et al., 2005). By contrast, in humans, there is an elevated breast cancer risk associated with lifetime nulliparity, and for women who have had their first childbirth at a more advanced age. Thus, for younger women having a full-term pregnancy has a relative ‘protective’ effect against breast cancer, quantified as a 50% reduced risk for women who had their first full-term pregnancy whilst under 20 years old (Subramani and Lakshmanaswamy, 2017).

Childbirth at any age is associated with a transiently increased breast cancer risk and, in essence, this phase of elevated risk extends for women who are older at the time of their first full-term pregnancy (Borges et al., 2020).

Identification of such patterns requires large and sophisticated epidemiological studies (Lambe et al., 1994), and execution of similar veterinary investigations is often challenging. The associated difficulties are compounded and elevated in the case of rare veterinary tumour types where limited numbers of cases are recorded. Furthermore, in many cases, equine patients will have multiple owners over the course of a lifetime, and a complete reproductive history may be unavailable to the attending veterinarian, and cannot be accurately documented for research purposes. At present, there is therefore insufficient evidence available to interrogate any potential relationship between mammary tumourigenesis and parity in the mare.

Initial clinical distinction between mastitis and mammary neoplasia may be challenging

In the mare, clinical distinction between mastitis and mammary neoplasia may be difficult (Reppas et al., 1996; Kato et al., 1998; Hirayama et al., 2003; Shank, 2009; Hughes et al., 2015; Sabiza et al., *In press*). Indeed, it has been suggested that horse owners and those caring for horses may associate any form of mammary gland enlargement with mastitis (Canisso et al., 2019). The presence of discharge from mammary tumours, potentially associated with draining sinus tracts, necrosis, and/or secondary infection, may complicate the clinical picture. Whilst non-specific clinical signs such as ventral or hind limb oedema are unhelpful and may be associated with a diagnosis of a mammary tumour (Munson, 1987) or mastitis (McCue and Wilson, 1989), some authors have postulated that ulceration of the overlying skin may be more indicative of a mammary tumour than of mastitis (Prendergast et

al., 1999). The two conditions may also occur in tandem, with mastitis arising as a consequence of the presence of mammary neoplasia.

An erroneous diagnosis of primary mastitis may lengthen the time to correct diagnosis of a mammary tumour and may consequently delay surgical removal of the mass, if this treatment is selected as appropriate (Shank, 2009). In this regard, cytology can be a valuable tool in distinguishing some cases of mastitis from cases of mammary neoplasia. Although the secretions from a mastitic mamma have an inconsistent composition and may therefore appear cytologically normal in some cases, in other instances mastitic secretions are characterised by the presence of viable and degenerate neutrophils, necrosis and/or other degenerate debris. By contrast, cytology from neoplastic masses may reveal an abnormal population of cells exhibiting criteria of malignancy although, again, in some cases it is not helpful (Freeman, 2002; Shank, 2009). The distinction is not absolute, and, in general, chronic inflammatory processes may also result in a degree of cytological cellular atypia. Nonetheless, cytology may be a valuable tool in assessment of mammary enlargement and discharge.

A specific framework for phenotyping equine mammary tumours is yet to be established

Surgical excision of mammary masses in mares has been suggested to be the most viable therapeutic modality in appropriately selected surgical candidates (Sysel et al., 1993; Shank, 2009). In the associated clinical report, a radical mastectomy was undertaken and subsequently histological sections from the mass were examined (Sabiza et al., *In press*). Currently, there is no classification system for the morphological diagnosis of equine

mammary tumours, although veterinary pathologists tend to employ morphological diagnoses similar to those used in canine and feline systems (Goldschmidt et al., 2017).

Most primary mammary tumours are carcinomas and adenocarcinomas of epithelial origin, and therefore express pan-cytokeratin, as in the case described in the associated report (Sabiza et al., *In press*). In the non-neoplastic mammary gland, the epithelial component comprises a bilayer (Fig 1 and 2) and the luminal and basal epithelia are distinguished by differing expression patterns of specific cytokeratins. Consequently, expression patterns of specific cytokeratins can potentially be employed to identify carcinomas of luminal and basal origin.

Cytokeratins 8 and 18 are two exclusively luminal markers in the human breast (Dontu and Ince, 2015) and cytokeratin 8 has previously been demonstrated to be similarly expressed in non-neoplastic equine mammary luminal epithelia (Hirayama et al., 2003). Cytokeratin 18 expression has been reported to be negative in frozen sections of normal equine mammary gland (Bussche et al., 2017). We have detected weak cytokeratin 8/18 expression in luminal epithelial cells using sections from formalin-fixed, paraffin-embedded tissue (Fig. 1). An equine mammary carcinoma expressing cytokeratin 18 has been described (Bussche et al., 2017).

Cytokeratin 14 is frequently used as a marker of basal, or myoepithelial, cells in the adult mouse (Gusterson and Eaves, 2018). In humans cytokeratin 14 is not exclusively expressed by basal epithelia, being expressed in basal epithelia in large ducts but in the luminal layer in lobules (Dontu and Ince, 2015). In the horse, cytokeratin 14 appears to be expressed predominantly in the basal compartment (Fig. 2) (Hirayama et al., 2003; Hughes et al., 2015)

and mammary carcinomas expressing cytokeratin 14 have been described (Hughes et al., 2015). Alpha-smooth muscle actin is also expressed by most mammary basal epithelial cells, and it confers the contractile properties required for the expulsion of milk (Gusterson et al., 2005; Stewart et al., 2019; Hitchcock et al., 2020). Alpha-smooth muscle actin expression has previously been demonstrated in normal equine mammary gland (Kato et al., 1998; Hirayama et al., 2003) (Fig. 1).

Taken together, these data suggest that assessment of expression of specific cytokeratins and contractile proteins such as alpha-smooth muscle actin would be a fruitful focus for further investigations delineating the histological phenotype, and cell type of origin, of equine mammary tumours. Given that commercial antibodies are most usually tested against human or murine tissues, it is vital that any such studies follow rigorous protocols and that antibodies are tested and standardised on equine tissues representing appropriate positive and negative controls (Ramos-Vara, 2017).

Prognostic factors for mammary tumours are currently lacking

In their report, Sabiza et al. evaluated tubule formation, nuclear pleomorphism, and mitotic count in histological sections from the mammary mass (Sabiza et al., *In press*). These morphological features reflect degree of differentiation and form the basis of the Elston and Ellis system for grading breast cancer (Elston and Ellis, 1991). However, although the Elston and Ellis system has been applied to veterinary mammary cancers, such as feline mammary neoplasia, authors studying mammary tumours in that species have recently proposed that adaptations to the grading system may provide more accurate prognostic value (Mills et al., 2015; Dagher et al., 2019). This illustrates the difficulties associated with applying a grading scheme designed for one species to the same tumour type occurring in a different species.

Whilst the broad framework may be helpful, parameters, and cut-off points for numerical values such as mitotic count, are likely to require modification in order to offer optimum prognostic value. As noted, currently, no such grading framework exists for mammary tumours arising in mares (Sabiza et al., *In press*).

In addition to morphological assessment, expression of molecular markers may be evaluated in tissue sections to guide prognostication. Currently, robust data regarding such prognostic markers is lacking for equine mammary tumours. We have previously demonstrated that a subset of equine mammary carcinomas exhibit nuclear expression of STAT3 (Hughes et al., 2015), suggesting that this transcription factor, frequently constitutively active in invasive breast cancer (Watson and Miller, 1995), might also be activated in some equine mammary tumours. However, we have yet to evaluate the use of STAT3 immunohistochemical staining as a prognostic marker for equine mammary tumours.

Other authors have utilised quantitative reverse transcription polymerase chain reaction to demonstrate reduction in p53 expression in a single equine mammary tumour compared to adjacent non-neoplastic tissue. This observation indicates that evaluation of p53 expression in a larger set of tumours may be warranted (Bussche et al., 2017).

Conclusions

It has been previously noted that challenges surrounding the diagnosis and management of equine mammary tumours, particularly in remote areas, may constitute a significant psychological burden to the veterinary clinician as well as having deleterious effects on the patient's owner and/or groom (Boyce and Goodwin, 2017). Some of the known unknowns discussed here, particularly those associated with diagnosis and prognostication, contribute to

this burden. This consideration, together with the veterinary community's strong desire to further scientific knowledge and to provide the best outcome for our patients, constitute strong drivers for continued research in this field.

Materials and methods for unpublished experiments

Dual immunohistochemical staining for alpha smooth muscle actin (rabbit monoclonal antibody catalogue number ab124964; 1:2000)¹ and keratin 8/18 (mouse monoclonal antibody catalogue number #4546; 1:50)² was conducted using a standard protocol and an ImmPRESS Duet Double Staining Polymer Kit³. Negative control slides were prepared using species-matched immunoglobulins.

Declarations

Authorship: KH conceived and designed the clinical commentary, performed experiments, and wrote the manuscript.

Authors' declarations of interests: There are no conflicts of interest.

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Ethical Animal Research: Owner informed consent: Sections used for immunohistochemistry were obtained from tissues that were surplus to diagnostic requirements collected from cases examined *post mortem* by the anatomic pathology service of the Department of Veterinary Medicine, University of Cambridge. Consent for retention of tissues for teaching and research purposes was granted at the time of submission for *post mortem* examination.

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Manufacturers' addresses

¹ Abcam, Cambridge, Cambridgeshire, CB2 0AX, United Kingdom.

² Cell Signaling Technology, Danvers, MA, 01923, United States of America.

³ Vector Laboratories, Peterborough, Cambridgeshire, PE2 6XS, United Kingdom.

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Figure legends

Figure 1. Luminal equine mammary epithelial cells weakly express cytokeratin 8/18, and basal mammary epithelial cells express alpha-smooth muscle actin. Dual immunohistochemical staining for cytokeratin 8/18 (brown; arrowhead) and alpha-smooth muscle actin (red; arrow) in non-neoplastic equine mammary tissue. The mammary epithelial bilayer is indicated by a bracket and an asterisk. Haematoxylin counterstain. Scale bar indicates 50 µm.

293

294 **Figure 2. Basal equine mammary epithelial cells express cytokeratin 14.**

295 Immunohistochemical staining for cytokeratin 14 (brown) (Hughes et al., 2015) to delineate
296 the basal epithelial cells (arrow). Non-neoplastic equine mammary tissue. The mammary
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