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Nuclear pleomorphism: Role in grading and prognosis of canine mammary carcinomas

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ABSTRACT

Canine mammary tumours are highly heterogeneous in morphology and behaviour and successful clinical management requires robust prognostic factors. Histological grade, determined by the Nottingham nuclear pleomorphism scoring method, has been considered one of these factors. Despite the adoption of this method, it is unknown whether inter-observer agreement exists regarding the assessment of its parameters in canine mammary carcinomas (CMC). In this study, the agreement between two observers in scoring nuclear pleomorphism using the Nottingham method was evaluated in 89 cases of CMC. Histological evidence of vascular invasion and/or lymph node metastases (both early signs of tumour aggressiveness) was recorded. For 48 animals, two years of follow-up data were available. Nuclear pleomorphism was quantitatively assessed using a stereological method that allowed for an unbiased estimation of nuclear size and its variability by determining the volume-weighted mean nuclear volume ($\overline{v}_v$). Differences between the $\overline{v}_v$ estimations and nuclear pleomorphism scores were evaluated. Additionally, the prognostic significance of clinicopathological features including nuclear score and $\overline{v}_v$ was evaluated.

A poor agreement between the two observers was obtained (κ value 0.46). Tumours scored as 1 and 2 presented similar $\overline{v}_v$ values and only tumours that scored 3 presented significantly higher estimates. The $\overline{v}_v$ value was not associated with vascular invasion and/or lymph node metastases, but was higher in tumours that progressed during follow-up. In multivariable analysis, only tumour size was an independent factor regarding evidence of aggressiveness and an optimal cut-off of 2.9 cm was defined.

Introduction

Mammary gland tumours are the most frequent cancer reported in female dogs, particularly in countries where spaying is not performed routinely early in life (Sorenmo, 2003). Approximately half of the affected dogs have malignant disease based on histopathological examination (Lana et al., 2007). Metastases to distant organs are the most common cause of morbidity and mortality associated with canine mammary tumours (Lana et al., 2007).

Mammary neoplasia is a complex disease characterized by a heterogeneous clinical and biological behaviour that probably contributes to the limited therapeutic options that are currently available (Goldschmidt et al., 2011; Klopfleisch et al., 2011; Sorenmo et al., 2011). Searching for new prognostic parameters is of paramount importance (Philibert et al., 2003; Chang et al., 2005; Santos et al., 2013). Histological grading is well established as an important prognostic factor in human breast cancer (Rakha et al., 2010). In veterinary pathology, different grading systems have been used and claimed to have prognostic value, but such a variety of methods is itself a drawback (Goldschmidt et al., 2011; Matos et al., 2012).

One of the most frequently used methods for histological grading of canine mammary tumours is the Nottingham method, originally developed for human breast cancer (Elston and Ellis, 1998). Karayannopoulou et al. (2005) provided some evidence that this method had advantages when compared to previous methods (that only use nuclear and cellular pleomorphism as parameters) for

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prognostic purposes in mammary tumours of dogs. The Nottingham grade method is based on the semi-quantitative assessment of three morphological features, namely, tubule formation, mitotic counts and nuclear pleomorphism. The latter should mainly be evaluated by comparing the nuclear size and variation with normal mammary epithelial cells or lymphocytes (Elston and Ellis, 1998). Nuclear pleomorphism is considered a hallmark of malignant transformation and increases in the absence of cell differentiation.

Irrespective of the method, grading is usually based on a subjective, experience-dependent judgment by a pathologist, and on qualitative or semi-quantitative evaluations of morphologic and cytological features (Sørensen, 1992; Artacho-Pérula and Roldán-Villalobos, 1997). Not only the observer, but also the selection of the tumour areas to assess grade can significantly influence the scoring of the parameters. This is especially relevant for canine mammary tumours, which are intrinsically very heterogeneous (Klopfleisch et al., 2011).

The subjective nature of morphological evaluation is associated with a high risk of inter- and intra-observer variations that can affect reproducibility and accuracy and limit comparative analysis between studies (Artacho-Pérula and Roldán-Villalobos, 1997). A simple way to overcome such subjectivity is to use unbiased quantitative parameters for scoring neoplasms by applying appropriate stereological methods. This has been used in various tumour types, including human breast cancer (Sørensen, 1992; Ladekarl and Sørensen, 1993; Steiner et al., 1994; Artacho-Pérula and Roldán-Villalobos, 1997; Ladekarl, 1998; Yörükoglu et al., 1998; Soda et al., 1999; Fujikawa et al., 2000). The design-based stereological estimates are precise, shape-independent, and allow for an objective evaluation of several cytological features, including nuclear pleomorphism (Ladekarl, 1998). However, to the best of our knowledge, stereological methods have not been applied for studying canine mammary carcinomas (CMC).

The point-sampled intercepts (PSI) method is considered the easiest way to objectively quantify nuclear pleomorphism (Gundersen and Jensen, 1985; Ladekarl, 1998). The PSI generates estimations of the volume-weighted mean nuclear volume, $\overline{v}_v$ (Sørensen, 1992). The $\overline{v}_v$ is not an intuitive parameter and involves sampling the nuclei in proportion to their volumes, therefore reflecting nuclear size variation and pleomorphism (Sørensen, 1992). The $\overline{v}_v$ increases as the nucleus enlarges, and is further augmented when a substantial variation in nuclear size exists. This parameter is mostly used in histopathology, and the $\overline{v}_v$ estimate has been shown to provide relevant information in diagnosis and grading of breast tumours (Ladekarl and Sørensen, 1993; Artacho-Pérula and Roldán-Villalobos, 1997).

The aims of this study were (1) to estimate the inter-observer variability in scoring nuclear pleomorphism using the criteria of the Nottingham grading system; (2) to assess nuclear pleomorphism of CMC using stereological tools, namely the PSI method; (3) to compare the stereological estimation with the histological score of nuclear pleomorphism; and (4) to verify the prognostic significance of the nuclear pleomorphism estimations by using univariable and multivariable analyses.

Materials and methods

Clinical cases and histological analysis

Eighty-nine spontaneous CMC (47 simple carcinomas and 42 complex carcinomas) from 56 female dogs that underwent surgical removal were analysed retrospectively. Owners gave informed consent for the surgery (with curative intents) and follow-up after declining postoperative adjuvant therapy. The protocol was performed in compliance with the European Union Directives for the protection of animals used for scientific purposes (1999/575/CE and 2010/63/UE) and approved by the Ethics Committee of the Institute of Biomedical Sciences Abel Salazar, University of Porto. Tumours were accurately measured in the largest diameter with a calliper before surgery by the clinician (AdM or AS). Surgical techniques were decided on an individual basis and resulted in 47% radical, 26% regional and 17% local mastectomies.

Histological and stereological evaluations were performed in all of the slides resulting from one slab of the tumour, sectioned following the largest cross diameter of the tumour. The histological diagnosis was performed by two observers (MS and PDP) using the criteria of the World Health Organization classification (Misdorp et al., 1999). The presence of peritumoural vascular invasion and regional lymph node metastases was recorded. The lymph node status was evaluated in routine slides and after immunolabelling with pancytokeratin AE1/AE3 and cytokeratin 14, as previously described by Matos et al. (2006).

The histological grading of nuclear pleomorphism was performed according to the Nottingham method criteria considering luminal epithelial cells present in all the tumoural area. Briefly, nuclei were scored as follows: (1) no visible increase in size and shape variability compared to normal surrounding mammary epithelial cells; (2) moderate variation in size and shape (nuclei generally larger than normal ones); (3) marked increased variation in size and shape, with very large and bizarre forms observed in at least one-quarter of the tumour area (Fig. 1). Two observers (MS and PDP) performed the nuclear grading independently in order to determine the inter-observer agreement. For cases with scoring discrepancy, a consensus was subsequently reached by reviewing the slides using a multi-head microscope.

Stereological analysis

For the stereological analysis, all the tumour area of the slides was considered. As tumour size varied from 0.3 to 15 cm; an entire slab across the largest cross diameter resulted in one to nine slides. A systematic random sampling approach for the selection of the fields was used in each slide, meaning that the first field of sampling was randomly selected and thereafter fields were sampled systematically by adjusting the distance between individual fields of vision roughly proportional to the overall area of the tumoural tissue. The stereological analysis was performed with a workstation comprising a microscope (BX-50 Olympus) equipped with a 100x oil immersion objective (Olympus Uplan), a CCD video camera (Sony) connected to a PC monitor, and a motorized stage (Prior) for stepwise displacements in x-y directions; the workstation was controlled by the software CAST-Grid (v1.5, Olympus).

The $\overline{v}_v$ was estimated by the PSI method (Gundersen and Jensen, 1985). This parameter quantifies the nuclear size and pleomorphism, estimated with a test-system made of parallel lines bearing a systematic pattern of points (Fig. 2). Only the nuclear profiles of epithelial cells hit by one of these points were sampled. On these profiles, the line segments overlying the nucleus were measured (from boundary to boundary) (Fig. 2); this resulted in a length (l) that was used to estimate the $\overline{v}_v$ (Gundersen and Jensen, 1985). For estimating the $\overline{v}_v$ value, the average of total counted intercepts in each slide was 166 (±69 standard deviation [SD]).

Formula to estimate the volume-weighted mean nuclear volume, $\overline{v}_v$:

$$\overline{v}_v(nucleus) = (\pi/3)\cdot \overline{l}_0^3$$

Follow-up and survival study

The follow-up study was performed as described by Santos et al. (2011). Briefly, each female dog was evaluated prior to surgery, 3 weeks after the procedure and every 3 months, thereafter for a 2-year period. Each evaluation included a complete physical examination, thoracic radiographs (three views) and an abdominal ultrasound. Any clinical signs or lesions that could be related to tumour progression, either in the scheduled evaluations or in between them was thoroughly investigated (e.g. fine-needle aspirate, ultrasound-guided biopsy, and skeletal radiography). A complete necropsy was performed when the animals died spontaneously or were euthanised in search for evidence of subclinical local recurrence or metastatic disease. All suspected lesions were confirmed by histopathology.

The survival analysis was performed following the most recent recommendations for prognostic studies in CMC (Matos et al., 2012), using only the cases presented as one malignant tumour per animal ($n = 40$ animals) and cases of multiple malignant tumours per animal with no evidence of disease progression (recurrence and/or metastases) during the follow-up period (8 animals; 18 tumours). Overall survival (OS) was calculated from the date of surgery to the date of animal death/euthanasia due to tumour metastasis. Disease-free interval (DFI) was calculated from the date of surgery to the date of detection of tumour progression, i.e., confirmed local recurrence and/or metastases. In the survival study, cases were censored (1) if animals died due to causes unrelated to tumour; (2) if they were lost to follow-up; and (3) if they were alive and free of distant metastases 24 months after surgery.

Statistical analysis

Cohen's κ statistic was used to assess the inter-observer agreement for the histological scoring of nuclear pleomorphism. An 'almost perfect' and 'substantial agreement' exists when the κ value is between 0.81 to 1 and 0.61 to 0.8, respectively. In contrast, when κ is < 0.6, the agreement is considered 'moderate to poor' (Vieira and Garrett, 2005). Additionally, an asymptotic marginal homogeneity test was used, to evaluate if statistical differences between the observers existed. ANOVA test and Tukey's post-hoc multicomparison were used to assess for differences between the