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NEOPLASTIC DISEASE

Immunohistochemical Labelling for Cyclo-oxygenase-2: Does the Positive Control Guarantee Standardized Results?

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Summary

Since the identification of cyclo-oxygenase-2 as a potentially important therapeutic target in veterinary oncology, numerous studies on its expression have been conducted. Unfortunately, results have been heterogeneous and conclusions are difficult to draw. We tested the ability of a defined positive control to guarantee reproducibility of results among different laboratories. Valid positive controls were defined by positivity of the renal macula densa without background labelling. Fifteen colorectal tumours and 15 oral squamous cell carcinomas were labelled immunohistochemically by six European laboratories. Slides were evaluated in blinded fashion for percentage of positive cells and labelling intensity by three pathologists, and results were analyzed statistically for reproducibility and inter-reader variability. Macula densa positivity was an insufficiently sensitive control to guarantee reproducible results for percentage of positive cells and labelling intensity. Inter-reader variability was proven statistically, making the case for image analysis or other automated quantitative evaluation techniques.

Keywords: cyclo-oxygenase-2; immunohistochemistry; inter-reader variability; reproducibility

Introduction

Cyclo-oxygenase (COX) is an enzyme that takes part in the conversion of arachidonic acid to proinflammatory prostaglandins (Dore, 2011). The COX enzyme system comprises three isoforms: COX-1, which is constitutively expressed in most tissues and participates in several normal physiological activities; COX-2, which is normally absent (except in a small number of tissues such as placenta, brain and kidney); and COX-3, apparently expressed in brain and spinal cord and whose existence and functions remain to be elucidated (Dore, 2011). Specific mechanisms whereby COX-2 contributes to tumour development and progression are inhibition of cellular apoptosis and stimulation of cell proliferation, cell survival, tumour invasion and angiogenesis (Sobolewski *et al.*, 2010; Dore, 2011). In human medicine, its prognostic significance is well established for several tumours, where higher levels

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of COX-2 expression correlate with a shorter survival time and the long-term use of non-steroidal anti-inflammatory drugs has been shown to reduce the incidence of tumour development (Sobolewski *et al.*, 2010; Dore, 2011). In dogs, COX-2 expression has been reported in several tumours, including intestinal adenocarcinomas (McEntee *et al.*, 2002; Knottenbelt *et al.*, 2006), oral squamous cell carcinomas (Pestili de Almeida *et al.*, 2001; Mohammed *et al.*, 2004; Pires *et al.*, 2010), bladder tumours (Knottenbelt *et al.*, 2006; Sledge *et al.*, 2015), haemangiosarcomas (Heller *et al.*, 2005a), histiocytic tumours (Heller *et al.*, 2005a), mammary tumours (Dore *et al.*, 2003; Millanta *et al.*, 2006, 2014; Dias Pereira *et al.*, 2009; Lavalle *et al.*, 2009; Queiroga *et al.*, 2010; Clemente *et al.*, 2013), mast cell tumours (Heller *et al.*, 2005a; Prada *et al.*, 2012), ovarian carcinomas (Borzacchiello *et al.*, 2007), nasal carcinomas (Impellizeri and Esplin, 2008; Belshaw *et al.*, 2011) and renal carcinomas (Khan *et al.*, 2001). Unfortunately, the immunohistochemical findings with regard to COX-2 expression vary significantly, even for a single tumour type, posing a problem of interpretation for clinicians and researchers.

Taking mammary tumours as an example, depending on the study, the percentage of immunohistochemically-positive samples ranged from 0% to 100% in normal tissue, from 20% to 100% in adenomas and from 56% to 100% in adenocarcinomas (Dore *et al.*, 2003; Heller *et al.*, 2005b; Millanta *et al.*, 2006, 2014; Queiroga *et al.*, 2007; Dias Pereira *et al.*, 2009; Lavalle *et al.*, 2009).

In an attempt to understand this discrepancy between results for different COX-2 expression studies performed with canine tumour specimens, we examined the reproducibility (no inter-laboratory variability) of COX-2 immunohistochemistry (IHC), using a defined positive control as the only parameter to guarantee inter-laboratory standardization and analyzing inter-observer variability of the obtained labelling.

Materials and Methods

Since COX-2 is expressed in the renal macula densa of man and several animals, fetal or neonatal kidneys are considered an appropriate positive control for the immunohistochemical study of expression of this antigen (Radi, 2009; Smith *et al.*, 2012). In the present study, the positive control consisted of sections of the kidney of a 1-day-old puppy. The organ was collected during a necropsy examination, which was performed <12 h after death. The specimen was fixed in 10% neutral buffered formalin for approximately 48 h prior to routine processing and embedding in paraffin wax.

Six independent European veterinary pathology laboratories received sets of almost identical slides containing serial sections (4 μm) from archived paraffin wax-embedded samples. Each slide set included one section each of 15 colorectal adenocarcinomas and of 15 oral squamous cell carcinomas, two sections from the positive control and one slide containing sections of normal organs as negative controls (small and large intestine and pancreas). The protocols applied by each laboratory are provided in Table 1. To further reduce potential variation, the same antibody was used throughout the study (anti COX-2, clone 33, Transduction Laboratories, Lexington, Kentucky, USA).

The positive reaction in the positive control was defined as intense labelling of the macula densa, without any background labelling. Each tumour section was analyzed for the proportion of positively labelled cells and the labelling intensity. The former was scored as: 0%, 1%, 2–9%, 10–30%, 31–60% or 61–100% of labelled cells within the tumour. Intensity was recorded as: −, no labelling; +, weak labelling; ++, moderate labelling; or +++, strong labelling (Queiroga *et al.*, 2007). The reproducibility of the technique and the inter-observer variability were tested by comparing the results obtained by three pathologists (MC, SB and TS), who each read all slides in the six slide sets produced and labelled at each of the six participating laboratories. These slides were encoded and mixed randomly so that the pathologists were blinded to the origin of each slide. To reproduce general research conditions, the three pathologists agreed in advance on the scoring system, but they had not been trained for consensual evaluation of the slides. During the study, they met three times and spent 1 h on each occasion using a multi-headed microscope to agree on the scoring system.

In order to test inter-laboratory variability, simple Kappa and weighted Kappa statistics were calculated. The simple Kappa statistic was accompanied by the corresponding Kappa exact test and weighted Kappa with confidence intervals, in order to assess the agreement of both scores. In order to test inter-observer variability, Cochran–Mantel–Haenszel (CMH) statistics were calculated associated with CMH tests, which assess the general association between laboratories and response adjusted for samples. The statistical analyses, conducted using SAS 9.2 software (SAS, Cary, North Carolina, USA), were carried out for the percentage of positively labelled cells and the intensity of the labelling on identical sets of slides. Statistical significance was based on two-tailed tests of the null hypothesis resulting in a P value of 0.05 or less. A quantification of inter-reader variability was studied on two identical sets

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