Cellular characteristics of keratin 19-positive canine hepatocellular tumours explain its aggressive behaviour

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ABSTRACT
The expression of the hepatic progenitor cell marker keratin 19 (K19) in canine hepatocellular carcinomas is linked with a poor prognosis. To better understand this aggressive behaviour, K19-positive hepatocellular carcinomas (n=5) and K19-negative hepatocellular adenomas (n=6) were immunohistochemically stained for proteins involved in malignant tumour development. The K19-positive carcinomas showed marked positivity for platelet-derived growth factor receptor alpha polypeptide (PDGFRα), laminin, integrin beta-1/CD29, B-cell-specific Moloney murine leukaemia virus integration site 1, glypican-3 (GPC-3) and prominin-1/CD133, in contrast with K19-negative hepatocellular adenomas. Conversely, neurofibromatosis type 2 was highly expressed in the hepatocellular adenomas in contrast with the hepatocellular carcinomas. This expression pattern is clearly in line with the observed aggressive behaviour. The presence of the malignancy markers PDGFRα and GPC-3 might make it possible to develop specific strategies to intervene in tumour growth and to devise novel serological tests and personalised treatment methods for canine hepatocellular carcinomas.

INTRODUCTION
Many tumours have been shown to possess characteristics of non-neoplastic stem cells.1 This phenomenon indicates that these tumours have similar features as can be found in stem cells including a marked capacity for proliferation and the capacity to differentiate to various cell types, resulting in a heterogeneous population of neoplastic cells within a tumour.2

Adult stem cells in the liver are called hepatic progenitor cells (HPCs) and are activated in the majority of liver diseases.3–7 HPCs may also be a potential source for carcinogenesis.8 9 One specific marker has been proven to identify neoplastic cells with HPC characteristics in primary hepatic cancers. This marker, keratin 19 (K19), can be used for the identification of neoplasms with HPC characteristics and has resulted in a novel classification scheme for primary hepatic neoplasms in both man,10 11 dog,12 and cats.13 The presence of K19-positivity in human liver tumours has been linked with a poor prognosis.9 14 A comparable finding was made in dogs with regards to prognostic significance of K19-positivity in primary hepatocellular tumours.15 Although K19-positivity is clearly associated with a poor prognosis, a mechanistic explanation for this remains unclear.

To better understand the aggressiveness, the authors investigated cellular characteristics of K19-positive hepatocellular tumours compared with K19-negative canine hepatocellular tumours. In this immunohistochemical study, several malignancy and cell-type-specific markers are evaluated including platelet-derived growth factor receptor alpha polypeptide (PDGFRα),16 17 integrin beta-1 (Itgβ1/CD29),18 laminin,19 polycomb ring finger oncogene (B-cell-specific Moloney murine leukaemia virus integration site 1; Bmi-1),20 glypican-3 (GPC-3),21 22 neurofibromatosis type 2 (merlin/NF2),23 24 macrophage marker MAC87,25 and CD133.26 All these markers play a distinct role in the progression of tumours regarding angiogenesis, invasion, proliferation and increased survival. These cellular characteristics may provide insight into the mechanisms of malignant transformation of the K19-positive hepatocellular tumours and may help to devise novel personalised treatment methods.

MATERIALS AND METHODS
Eleven formalin-fixed paraffin-embedded samples of primary liver tumours were selected from a group of 106 canine primary liver tumours implemented in a previous characterisation study.15 The selection of the 11 hepatocellular tumours was based on K19-staining and comprised 6 out of the
original 62 well-differentiated K19-negative hepatocellular adenomas (HCAs) and 5 out of the original 17 poorly differentiated (>90 per cent of tumour cells positive) hepatocellular carcinomas (HCCs). Representative pictures of the histology and K19 staining are provided in Fig 1.

Immunohistochemistry (IHC) was performed essentially as described previously for PDGFRα, CD29, laminin, Bmi-1, GPC-3, NF2, MAC387 and CD133 (Table 1). Omission of the primary antibody as well as isotype controls served as negative controls (data not shown).

RESULTS

PDGFRα, a tyrosine kinase receptor, showed intense cytoplasmic positivity in 100 per cent of the tumour cells in all HCCs (Fig 2A) while in HCAs this staining was much less marked and was present in fewer cells (Fig 2B). Extracellular matrix component laminin presented a marked

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**FIG 1:** Representative histological characteristics of the selected canine tumours. HE staining of hepatocellular adenoma with well-demarcated tumour and differentiated hepatocytes (A). Keratin 19 (K19) staining of a hepatocellular adenoma (HCA) with negative staining in the neoplastic tissue (B). HE staining of hepatocellular carcinoma (HCC) with trabecular structures of hepatocytes and marked cellular/nuclear pleomorphism and mitotic figures. (C) K19 staining of a HCC with marked cytoplasmic staining of the tumour cells (D).

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**Table 1:** Antibody characteristics and experimental procedures for immunohistochemistry

| Antibody | Manufacturer | Type | Clone | Antigen retrieval | Dilution | Wash buffer | Incubation |
|----------|--------------|------|-------|-------------------|----------|-------------|------------|
| K19      | Novocastra   | Mouse mAb | b170  | Proteinase K      | 1:100    | TBS         | O/N 4°C    |
| PDGFRα   | Abcam        | Rabbit Ab | Polyclonal | TE buffer   | 1:100    | PBS         | O/N 4°C    |
| CD29     | BD Biosciences | Mouse mAb | 18/CD29 | Citrate      | 1:100    | PBS         | O/N 4°C    |
| Laminin  | Abcam        | Rabbit Ab | Polyclonal | Proteinase K | 1:100    | PBS         | O/N 4°C    |
| Bmi-1    | Millipore    | Mouse mAb | F6    | TE buffer      | 1:150    | PBS         | O/N 4°C    |
| Glypican-3 | Biomosaics | Mouse mAb | 1G12  | Citrate      | 1:100    | PBS         | O/N 4°C    |
| NF2      | Sigma        | Rabbit Ab | Polyclonal | Proteinase K | 1:300    | PBS         | 60 min. RT |
| MAC387   | Abcam        | Mouse mAb | MAC387 | Proteinase K | 1:1,000  | PBS         | O/N 4°C    |
| CD133    | eBioscience  | Rat mAb | 13A4  | Pepsin       | 1:25     | PBS         | O/N 4°C    |
| Isotype control | Vector laboratories | Mouse IgG | I-2000 | TE buffer | Adjusted to concentration | PBS | O/N 4°C |
| Isotype control | Vector laboratories | Rabbit IgG | I-1000 | TE buffer | Adjusted to concentration | PBS | O/N 4°C |

Bmi-1, B-cell-specific Moloney murine leukaemia virus integration site 1; K19, keratin 19; mAb, monoclonal antibody; MAC387, macrophage antigen 387; NF2, neurofibromatosis type 2; O/N, overnight; PDGFRα, platelet-derived growth factor receptor alpha polypeptide; RT, room temperature; TBS, Tris-buffered saline; TE, Tris/EDTA buffer.
cytoplasmic positivity in all HCCs (Fig 2C), in HCAs four of six samples showed a slight to moderate cytoplasmic positivity, two adenomas remained negative (Fig 2D). Normal surrounding liver tissue showed marked positivity on cholangiocytes, smooth muscle tissue and portal vein endothelial cells (online supplementary figure 1). CD29, an integrin molecule that connects extracellular matrix (eg, laminin) with the cytoskeleton, exhibited a slight to moderate cytoplasmic positivity in four out of five HCCs (Fig 2E), one carcinoma was negative for CD29. All HCAs were negative for CD29 (Fig 2F). Oncogenic and haematopoietic stem cell self-renewal factor Bmi-1 expressed moderate to marked nuclear staining with negative or slight cytoplasmic positivity in four out of five HCCs (Fig 2G), one HCC was negative. Two HCAs had local slight nuclear positivity and the other four were negative (Fig 2H). Normal surrounding liver tissue showed slight nuclear positivity in cholangiocytes (online supplementary figure 1).
Malignancy marker GPC-3 showed a marked cytoplasmic positivity in 100 per cent of the tumour cells in all five HCCs (Fig 3A). The HCCs were all negative for GPC-3, NF2, a known tumour suppressor gene, expressed a slight cytoplasmic staining in three out of five HCCs, two carcinomas were negative (Fig 3C). All HCAs exhibited a moderate to marked cytoplasmic and/or membranous positivity for NF2 (Fig 3D). The adjacent normal liver tissue showed a slight membranous and cytoplasmic positivity of the hepatocytes and a membranous positivity of the bile ducts. MAC387 is a macrophage marker and expressed moderate positivity in all hepatocellular tumours, macrophages were regularly observed in increased numbers near necrotic areas. There was no difference between the HCCs (Fig 3E) and HCAs (Fig 3F). In the adjacent normal liver tissue surrounding the tumours, a moderate number of macrophages could be found in both tumour types (online supplementary figure 1). For CD133, a classical somatic stem cell marker, a slight cytoplasmic staining in
Whether this decrease is caused by the expression of an NF2 splice variant remains to be determined. The presence of CD133 in the K19-positive canine HCCs demonstrates the stem cell character of these tumours. CD133 expression was previously observed in a small percentage (15 per cent) of canine HCCs. The discrepancy between that study and ours might be related with the selection of the K19-positive (>90 per cent positivity) tumours with proven metastatic potential in this material.

The size of this study is small, which is usually a study limitation. The selection of only 5 out of 17 K19-positive HCCs from the original retrospective characterisation study on canine primary epithelial hepatic tumours was due to insufficient paraffin material to acquire high-quality material for the various IHC stainings and potentially affects the power of this study. The advantage, however, to have this very precise selection based on K19 expression is that the two groups of tumours used in this study were clearly defined, and this resulted in a clear-cut difference between these two groups regarding the angiogenesis, tumour proliferation and malignant transformation markers. In addition, the unexpected negative staining of Bmi-1 and CD29 in some HCCs (see Table 2) was observed in the same samples, indicating a possible fixation or long-term storage effect and can be perceived as a possible false negative staining pattern.

The high expression of PDGFRα offers potential ways for new therapeutic options in the veterinary field with the use of specific PDGFRα antagonists either as small molecules or with receptor-specific blocking antibodies. Targeted therapy in the form of selective tyrosine kinase inhibitors has transformed the management of various human cancers and represents a therapeutic breakthrough. Imatinib, a tyrosine kinase inhibitor specific for PDGF-Rs (including PDGFRα), c-KIT and BCR-ABL, has revolutionised the therapy of specific malignancies including HCCs.

In summary, the present study indicates that the differential expression of malignancy markers explains the malignant phenotype of HCC versus HCA. In addition, the marked expression of PDGFRα and GPC-3 in HCC can be used to develop a specific diagnostic serum test and a (personalised) therapeutic approach to intervene in tumour growth.

**Contributors** RS did the analysis and wrote the manuscript. TI analysed the samples. BA participated in the design of the study and helped write the manuscript. ME helped generate the data. LP and JR helped write the manuscript. BS conceived of the study, helped in the analysis and helped write the manuscript.

**Competing interests** None declared.

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data sharing statement** There are no additional unpublished data.

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### Table 2: Immunohistochemical results on the canine hepatocellular tumours

| Antibody   | HCC (n=5)                                                                 | HCA (n=6)                                                                 |
|------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|
| CD29       | +++ (cytoplasmic)                                                         | + (cytoplasmic)                                                           |
| Laminin    | +++ (cytoplasmic)                                                         | 0 (n=2)                                                                  |
|            | + – + (cytoplasmic; n=4)                                                  | + (cytoplasmic; n=4)                                                      |
| Bmi-1      | 0 (n=1)                                                                  | 0 – + (nuclear)                                                           |
| Glypican-3 | +++                                                                      | 0                                                                         |
| NF2        | 0 (n=2)                                                                  | ++ (cytoplasmic and membranous)                                           |
| MAC387     | + – ++                                                                   | + – ++                                                                   |
| CD133      | + (cytoplasmic)                                                           | 0                                                                         |

Intensity of immunohistochemical staining on the hepatocellular tumours; 0, no staining; +, slight staining; ++, moderate staining; +++, marked staining.

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doi: 10.1136/vetreco-2016-000212

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