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Immunohistochemical analysis of metastasising hepatocellular carcinomas in dogs

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ABSTRACT: In this study, the immunohistochemical features of primary hepatocellular carcinomas and their metastases in visceral organs, including the lungs, spleen and kidneys were examined using antibodies against carcino-embryonic antigen (CEA) cytokeratin (CK) 7 and 20, CD4, CD8, minichromosome maintenance protein 3 (MCM3), vimentin, and alpha 1 foetoprotein (AFP). In addition, Mallory's connective tissue stain, van Gieson's stain and Gomori methenamine silver stain were used. The study was performed on liver samples collected *post mortem* from five mixed-breed dogs aged 9–12 years. The tumours were classified according to the World Health Organization Classification of Tumours. Strong expression of MCM3 and AFP was found in the hepatic cancer cells and in the metastases to the lungs, spleen and kidneys. The primary tumours and metastatic foci did not react positively with the anti-CD4, anti-CD8, CEA, CK7 and CK20 antibodies. The connective tissue in the primary tumour and the metastases showed a positive reaction to vimentin. Canine hepatocellular carcinomas that metastasise are highly-malignant well-differentiated tumours that produce AFP and trace amounts of both carcinoembryonic antigen and cytokeratin. Therefore, the metastasis resembles the primary tumour and has a common phenotype and genotype with the primary tumour.

Keywords: cell markers; expression; CEA; CK7; CK20; CD4; CD8; vimentin; AFP; immunohistochemistry; pleomorphic cells; liver

Liver tumours constitute 0.6 to 12.5% of all tumours in dogs, and are usually malignant. They usually affect 10- to 12-year-old dogs although liver tumours in younger dogs have also been reported (Patnaik et al. 1981; Center et al. 1992; Post and Patnaik 1992; Nyland et al. 1999; Withrow 2001; Liptak et al. 2004). Our previous studies, carried out between 2000 and 2011, revealed that the highest incidence of liver tumours was found in 7- to 11-year-old dogs (Nowak and Madej 2006; Nowak et al. 2010).

Liver tumours may develop from hepatocytes, bile ducts or any other liver tissue. Primary liver tumours occur much less frequently than metastases of malignant tumours in the liver. In humans, metastases in the liver occur approximately twenty times more frequently than primary liver tumours. Gastrointestinal carcinomas, gall bladder carcinomas, pancreatic cancers, lung cancers, breast cancers, kidney cancers and malignant melanomas most commonly metastasise to the liver (Mulligan and Mulligan 1949; Kapatkin et al. 1992; Popp 1992; Withrow 2001; Leibman et al. 2003; Liptak et al. 2004). Liver tumours are also associated with malignant neoplasias of the lymphatic/haematopoietic system. Additionally, all systemic malignant tumours may metastasise to the liver.

Canine hepatocellular carcinomas are macroscopically polymorphic and appear either as small, round, well-demarcated foci or as large, diffuse, infiltrating lesions (Mulligan 1949; Mulligan and Mulligan 1949; Monlux et al. 1956; Center et al. 1992; Krus and Skrzypek-Fakhoury 1996; Withrow 2001). Although hepatocellular carcinoma (HCC)

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can be found in all hepatic lobes, it is observed most frequently in the left, lateral lobe. This could be due to the fact that the lobe constitutes one third of total liver weight (Lahat et al. 2010). HCCs are more likely to protrude above the surface of the organ than be embedded in the liver stroma (Kapatkin et al. 1992).

The microscopic appearance of HCC cells in tissue samples may vary. The growth pattern of the cells may be lamellar, trabecular or pseudoglandular. Necrotic foci and blood-filled cavities may be present between the strands of the HCC cells (Kapatkin et al. 1992; Schlageter et al. 2014).

Pleomorphic HCC cells are similar to hepatocytes. They have a large, round, nucleus located centrally and a slightly eosinophilic cytoplasm. Giant cells with a scant, basophilic cytoplasm with fat and glycogen vacuoles may also be present (Popp 1992; Krus and Skrzypek-Fakhoury 1996; Masserdotti and Drigo 2012). Mitotic figures appear more frequently than in adenomas (Ramaiah and Alleman 2002). The stroma of the tumour is formed from poorly vascularised connective tissue (Popp 1992; Krus and Skrzypek-Fakhoury 1996; van Sprundel et al. 2013).

The aim of this study was to carry out a complex histological (Mallory's connective tissue stain, van Gieson's stain and Gomori methenamine silver stain) and immunohistochemical analysis (with the use of carcino-embryonic antigen (CEA), cytokeratin (CK) 7 and CK20, anti-CD4, anti-CD8, minichromosome maintenance protein 3 (MCM3), vimentin and alpha 1 foetoprotein (AFP) antibodies) of canine metastatic hepatocellular carcinoma. In addition, the expression levels of the cell markers in the primary and metastatic tumours were compared.

MATERIAL AND METHODS

The study was carried out on liver samples collected *post mortem* from five mixed-breed dogs (9- to 12-years-old) euthanized due to extensive liver tumours. The samples were fixed in 7% buffered formalin for 24 h. They were then embedded in paraffin, and the blocks were cut into 4 µm sections. The haematoxylin and eosin stained sections were classified according to the World Health Organization Classification of Tumours (Hamilton and Aaltonen 2000). Mallory's connective tissue (Sigma-Aldrich, St Louis, USA), van Gieson's (Sigma-Aldrich), and the Gomori methenamine silver stains were also used (Sigma-Aldrich).

The immunohistochemical studies were carried out on 4 µm-thick paraffin sections, which were placed on silanised glass slides (Menzel Glaser, Braunschweig, Germany). The sections were then deparaffinised with xylene and passed through a series of decreasing alcohol concentrations to water. The tissue antigens fixed in formalin were retrieved using the EnVision™ FLEX Target Retrieval Solution High pH (50×) (DakoCytomation, Glostrup, Denmark) by heating the sections in a 96 °C water bath for 20 min. The endogenous peroxidase was blocked by applying the EnVision™ FLEX Peroxidase-Blocking Reagent for 10 min (LSAB2, HRP, DakoCytomation). Next, monoclonal mouse anti-vimentin (1 : 100; DakoCytomation), monoclonal mouse anti-human carcinoembryonic antigen (1 : 100; DakoCytomation), monoclonal mouse anti-human cytokeratin 7 (Clone OV-TL 12/30, 1 : 100; DakoCytomation), monoclonal mouse anti-human cytokeratin 20 (Clone Ks20.8, 1 : 100; DakoCytomation), polyclonal rabbit anti-human alpha-1-foetoprotein (1 : 100; DakoCytomation), and the monoclonal mouse anti-human minichromosome maintenance protein 3 (clone 101, 1 : 50; Novocastra, Dublin, USA) antibodies were applied. The specimens were incubated at room temperature for 20 min. They were then washed 20 times with the EnVision™ FLEX Wash Buffer (DakoCytomation). Next, the EnVision™ (FLEX/HR SM802, DakoCytomation) visualisation system was placed on the sections, and they were incubated at room temperature for 20 min. The immunocytochemical reaction was elicited by 3,3'-diaminobenzidine tetrahydrochloride (DAB) EnVision™ (FLEX DAB$^+$ Chromoge, DakoCytomation). The sections were then washed with distilled water. All the sections were counterstained with Meyer's haematoxylin. In all the cases, controls in which the specific antibody was substituted with the Primary Negative Control (DakoCytomation), were included.

Microphotographs of all the studied tumours were subjected to a computer-aided image analysis via a computer coupled to a BX53 optical microscope (Olympus, Tokyo, Japan). This set-up allowed recording as well as digital analysis of the images. The measurements were carried out using the Cell software (Olympus Soft Imaging Solutions GmbH, Germany).

The expression of CEA, CK7, CK20, CD4, CD8, vimentin and AFP was evaluated using the modi-

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fied semi-quantitative immunoreactive score scale according to Remmele and Stegner (1987). The method takes into account both the proportion of positively stained cells and the intensity of the reaction colour. The final results represent the product of both parameters, with values ranging from 0 to 12 points (no reaction = 0 points (–); weak reaction = 1–2 points (+), moderate reaction = 3–4 points (++), intense reaction = 6–12 points (+++)). The expression of MCM3 was evaluated quantitatively by estimating the percentage of positive cells (0–5% = no reaction (–), 6–25% = weak reaction (+), 26–50% = moderate reaction (++), above 50% = intense reaction (+++)).

RESULTS

The histological analysis of the liver tumours using the World Health Organization Classification of Tumours (Hamilton and Aaltonen 2000) confirmed that all the samples were HCCs. In the analysed tumours, pleomorphic cells were either arranged in strands resembling the organ structure or were irregular (Figures 1A, 1B and 1C). The cells containing abundant cytoplasm had nuclei located centrally with clearly visible nucleoli (Figure 1A). Mitotic figures were also present (Figure 1B). Necrotic foci, blood stasis and fatty degeneration of hepatocytes were seen within the proliferations (Figure 1B). The tumours gave metastases to the kidneys, spleen and lungs.

Abnormal hepatocytes in the metastatic foci in the kidneys, spleen and lungs resembled the structure of the cells present in the primary tumour foci in the liver. They formed foci surrounded by a connective tissue capsule, which in turn was surrounded by an inflammatory infiltrate (Figures 1E and 1F).

The connective tissue stroma and mild liver fibrosis was visualised with Mallory's connective tissue stain, van Gieson's stain and the Gomori methenamine silver stain (Figure 2).

The anti-CD4 and anti-CD8 antibodies did not give a positive reaction in the primary tumour or in the metastases. There was no expression of CEA, CK7 and CK20 in the primary or metasta-

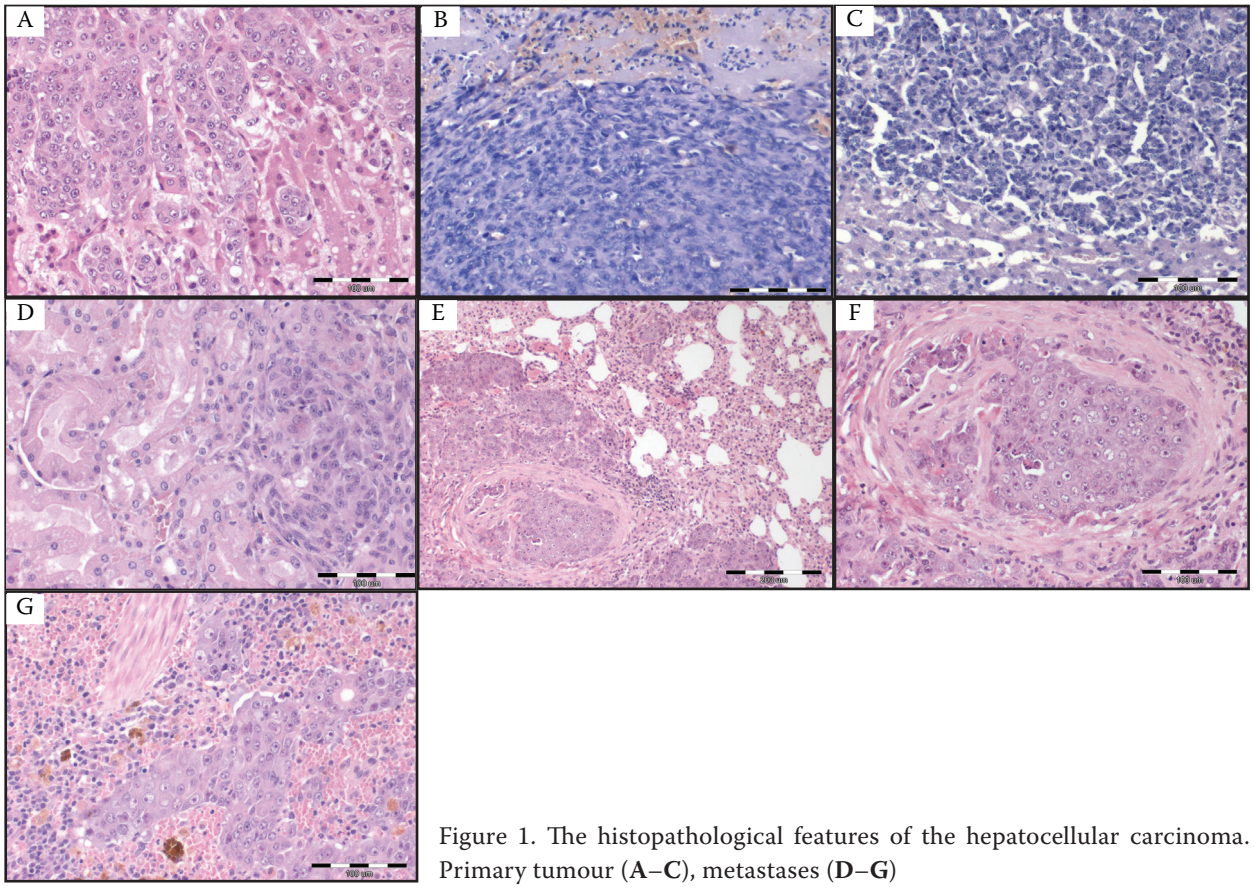

Figure 1. The histopathological features of the hepatocellular carcinoma. Primary tumour (**A**–**C**), metastases (**D**–**G**)

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sised tumours. Vimentin reacted positively in the connective tissue of the primary tumour and in the metastases. The proliferation marker MCM3 was expressed in 65 to 80% of the positively reacting cells in the primary tumours. The expression of MCM3 varied in the metastatic foci. In the spleen, the expression level of MCM3 in the tumour cells was 40%, while in tumour cells of the lungs and kidney the expression level reached 60%. The expression of AFP was assessed as eight points on the Remmel scale in the primary tumour and metastases. The obtained results are presented in Table 1.

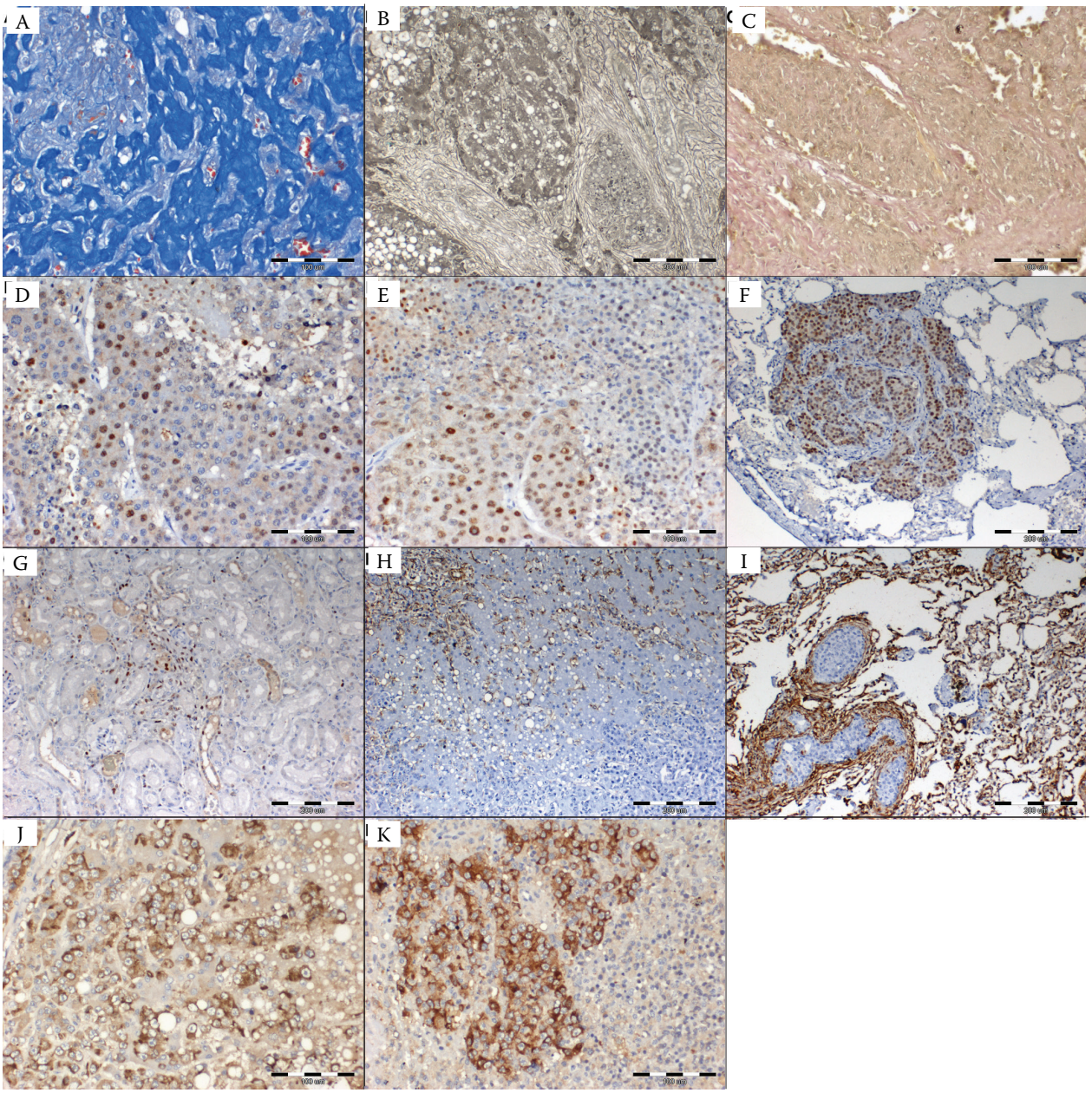

Figure 2. The histopathological staining and immunoexpression of selected cell markers in hepatocellular carcinomas in dogs. Primary tumour: Mallory's connective tissue stain (**A**), Gomori methenamine silver stain (**B**), the van Gieson's stain (**C**), the expression of MCM3 in the nucleus of cells in the primary tumour (**D**), the expression of MCM3 in the nucleus of cells in the metastatic foci-spleen (**E**), the expression of MCM3 in the nucleus of the cells in the metastatic foci-lung (**F**), the expression of MCM3 in the nucleus of the cells in the metastatic foci-kidney (**G**), lack of vimentin expression in the cancer cells in the primary tumour (**H**), lack of vimentin expression in the cancer cells in the metastasis (**I**), cytoplasmic expression of AFP in the primary tumour (**J**), cytoplasmic expression of AFP in the metastasis in the spleen (**K**)

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Table 1. Comparison of the results of the immunohistochemical studies in hepatocellular carcinomas and their metastases in dogs

Staining method	Primary tumour	Metastases
Mallory's staining	+/–	+/–
van Gieson's staining	+/–	+/–
Gomori staining	+/–	+/–
CEA	–	–
CK7/CK20	–	–
Anti-CD4/Anti-CD8	–	–
MCM3	+++	++
Vimentin	–	–
AFP	+++	+++

+++ = positive reaction in more than 65% of the studied samples, ++ = positive reaction in 40–60% of the cases, + = positive reaction in fewer than 40% of the cases, +/– = slight positive reaction or no reaction, – = no reaction in any of the samples

DISCUSSION

In dogs, hepatocellular carcinoma occurs very infrequently and accounts for only 0.6 to 1.3% of all tumours in dogs (Teshima et al. 2013; van Sprundel et al. 2014). In most cases, this cancer occurs in older dogs (80% of cases are in dogs ten years or older), although it was also reported in very young animals (Patnaik et al. 1980; Hamilton and Aaltonen 2000). Hepatocellular carcinoma is difficult to clinically diagnose due to the fact that it presents with nonspecific symptoms. Immunohistochemical examination is becoming increasingly useful in the histopathological diagnosis of HCC (Shu-Qin et al. 2013; Teshima et al. 2013; van Sprundel et al. 2013).

In humans, HCC is usually associated with preexisting liver lesions, such as cirrhosis, inflammation or viral infections (Clayton et al. 2012; EASL-EORTC 2012; Teshima et al. 2013). We did not detect significant hyperplasia of the connective tissue in the studied HCC samples. Therefore, it can be assumed that the connective tissue was not associated with the tumour growth and development. Hyperplasia of connective tissue in liver occurs as a side-effect of the presence of lesions and damage of hepatocytes and in dogs is not directly associated with carcinogenesis in this organ. In humans, hyperplasia is an indicator of cirrhosis, which often precedes development of HCC.

The cancer foci were not present as an inflammatory reaction and did not induce an inflammatory response, which was confirmed by the lack of a reaction with the anti-CD4 and CD8 antibodies. Inflammatory reactions were found only in the metastatic foci in the lungs, where a lymphocytic and histiocytic infiltrate was observed.

Carcino-embryonic antigen is physiologically present in cells of the foetal intestine, pancreas and liver. It is present in trace amounts in adults (Maliszewski et al. 2008). In the course of HCC in humans, there is a slight increase in CEA, CK7 and CK20 expression (Song et al. 1982; Shu-Qin et al. 2013). We did not find these proteins in our samples, suggesting that the cancer cells we analysed may have contained only trace amounts of those proteins, undetectable by immunohistochemistry.

AFP is a protein with a molecular weight of 70 kDa, produced by foetal liver cells and cells of the yolk sac (Mizejewski 1997; Leong et al. 1999). In our study, we found strong cytoplasmic expression of this protein in liver cancer cells both in the primary tumour and in the metastases. Our findings are in accordance with the results of de las Mulas et al. (1995), who found this marker only in hepatocellular carcinomas. Similarly, Kitao et al. (2006) found the presence of AFP in highly differentiated hepatocellular carcinomas.

Vimentin is a mesenchymal cell marker. Physiological foetal hepatocytes have a weakly-positive reaction to vimentin since its expression decreases during development (Dellagi et al. 1983; Nakajima et al. 2004; Handra-Luca et al. 2011; Ji-Woon et al. 2014). A directly proportional relationship between the expression of vimentin in both sarcoma and carcinoma cell lines and a decrease in tumour growth and the induction of apoptosis in those cells was found by Lahat et al. (2010). The fact that there was no expression of vimentin in our samples may indicate that the HCC cells were highly differentiated.

We used MCM3 to assess the proliferative potential and the degree of malignancy of the tumour and its metastases. MCM3 staining was strong in the nuclei of cells. This protein is part of the minichromosomal maintenance complex that plays a key role in DNA replication during S phase (Musahl et al. 1998; Endl et al. 2001; Nowak et al. 2008). Our results may indicate a high grade of malignancy of the studied hepatocellular carcinomas. The fast and infiltrative tumour growth and the presence of metastases in the studied organs, which also

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showed MCM3 expression, seem to further support that idea.

Cirrhosis did not accompany hepatocellular carcinomas in our study, which distinguishes our findings from human studies. We found little fibrous tissue in the samples, which also differs from the situation in fibrolamellar carcinomas in humans. That type of tumour is not accompanied by cirrhosis and is not associated with hepatitis B virus infections. However, in fibrolamellar carcinomas, cancer hepatocytes are separated by collagen lamellae. HCC is highly differentiated and highly malignant, which makes it difficult to diagnose using immunohistochemistry. We also found that cells in the lung, spleen and kidney metastases stained in a similar way to the cancer liver cells. The metastasis resembled the primary tumour and had a common phenotype and genotype with the primary tumour. The immunohistochemical techniques we used may be routinely used in the diagnosis of metastatic liver cancer in order to accurately diagnose this type of tumour in dogs. They may also have some prognostic value.

In summary, metastasising hepatocellular carcinoma is a highly differentiated (no vimentin expression) and highly malignant (MCM3 strong expression) tumour that produces AFP and trace amounts of CEA and CK7 and CK20. Cells in metastatic foci have similar properties to the primary tumour. They also have a common phenotype and genotype with the primary tumour.

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