Aberrant Expression of Cell Cycle Regulator 14-3-3-σ and E-Cadherin in a Metastatic Cholangiocarcinoma in a Vervet Monkey (*Chlorocebus pygerythrus*)

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Summary

We present a unique case of metastatic cholangiocarcinoma with concurrent abdominal cestodiasis in an African green monkey (*Chlorocebus pygerythrus*) which presented with respiratory insufficiency and abdominal discomfort. There were multiple white-grey masses in the liver and colonic serosa alongside intra-abdominal parasitic cysts. Histopathologically, the liver masses were composed of poorly-differentiated epithelial cells that formed densely cellular solid areas and trabeculae. The neoplastic cells were strongly immunopositive for CK7 but negative for Hep-Par1 antigen which confirmed a diagnosis of cholangiocarcinoma. Interestingly there was strong and diffuse neo-expression in the tumour of the cell cycle regulator 14-3-3σ which is not constitutively expressed in normal liver. There was aberrantly strong expression of E-cadherin, a key cell-cell adhesion protein, in neoplastic cells with evidence of cytoplasmic internalization. This is the first immunohistochemical analysis of 14-3-3σ and E-cadherin in a liver neoplasm in an animal species and the use of these markers requires further investigation in animal liver tumours.

Keywords: cholangiocarcinoma; E-cadherin; 14-3-3σ; non-human primate

The name ´African green monkey´ (AGM) is used by primatologists to designate non-human primates (NHP) of the genus *Chlorocebus* which comprises the six species *C. sabaeus*, *C. aethiops* (grivet), *C. cynosuros*, *C. djamdjamensis*, *C. tantalus* and *C. pygerythrus* (vervet monkey) (Matz-Rensing and Lowenstine, 2018). Vervets and grivets are among the most studied NHP and are crucial models in biomedical research. *C. pygerythrus* is well represented in most zoological institutions and a significant number are kept in captivity in the main primate research centres (Jasinka *et al*., 2013). Vervet monkeys are the best characterized NHP model of study human immunodeficiency virus infection, neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, and various endocrine diseases (Jasinka *et al*., 2013).

Primary liver neoplasms in NHP are uncommon and only a handful of cases have been reported including haemangioma, cystadenoma, hepatocellular carcinoma (HCC), hepatic anaplastic carcinoma, cholangiocarcinoma (CCA) and hepatocholangiolar carcinoma. A literature review reveals only five CCA in NHP but no record of spontaneous cholangiocarcinoma in a vervet monkey (Reindel *et al*., 2000; Miller, 2012; Matz-Rensing and Lowenstine, 2018).

Risk factors for liver neoplasia in human beings include hepatitis B (HBV) and C (HCV) virus infections, parasites, chemical carcinogens and other causes of cirrhosis e.g. alcoholic and non-alcoholic steatohepatitis (Razumilava and Gores, 2014; Squadroni *et al*., 2017). Liver neoplasms in NHP are most frequently associated with experimental HBV inoculation and chemical carcinogens including nitrosamines and aflatoxin. No naturally occurring predisposing causes have been definitively associated with liver neoplasia in NHP (Miller, 2012).

There is a strong interest in the identification of cell biomarkers of proliferative liver lesions in human beings. The cell-cycle regulator protein 14-3-3σ has become a very promising human liver tumour biomarker but, in animal species, has only been investigated in normal canine liver where it is not constitutively expressed (Suarez-Bonnet *et al*., 2010; Padden et al., 2014). E-cadherin, the main cell-adhesion protein, regulates cell-differentiation, maintains cell structure and its loss is associated with tumour invasiveness, metastasis and a poor prognosis (Berreta *et al*., 2017). To date, neither 14-3-3σ nor E-cadherin expression have been investigated in liver neoplasms in any animal species.

In this report we describe the histopathological and immunohistochemical features of a metastatic cholangiocarcinoma in a vervet monkey, with a particular focus on 14-3-3σ and E-cadherin expression in the neoplastic cells.

A 28-year-old, female entire, vervet monkey (*Chlorocebus pygerythrus)* from a zoological facility presented with respiratory insufficiency and abdominal discomfort. An exploratory laparotomy revealed a poorly demarcated white-grey, multilobulated mass replacing approximately 40% of the liver parenchyma. Parasitic-like, white, translucent, 1 x 1 cm intraabdominal cysts were also found attached to the greater omentum. Intraoperative euthanasia was performed on welfare grounds.

Samples of heart, lung, liver, kidney, uterus, intestine and brain tissues were fixed in 10% formalin and submitted for histopathological analysis at the Royal Veterinary College. Tissues were processed routinely, embedded in paraffin-wax, and sections cut (4μm) and stained with haematoxylin and eosin (HE), Perl's Prussian blue or by the periodic acid-Schiff (PAS) method.

Expanding and effacing liver sections was a well-demarcated, unencapsulated, multilobulated, infiltrative, densely cellular, malignant epithelial neoplasm. Approximately 90% of neoplastic cells formed trabeculae and cords that varied from two to eight cells thick and only occasionally formed densely packed, patternless solid areas. Trabeculae formed multiple large lobules separated by fine fibrovascular connective tissue septa lined by compressed hepatocytes. Neoplastic cells were polyhedral, with large amounts of brightly eosinophilic cytoplasm and contained one or multiple (up to five) large nuclei. Cell nuclei were round to oval with coarsely clumped chromatin and one or two prominent nucleoli. Anisocytosis, megalocytosis, anisokaryosis and macrokaryosis were frequent. There were 15 mitoses in 10 x400 fields (2.37 mm^2). The boundary between the neoplasm and normal liver tissue was variably outlined by a rim of lymphocytes and plasma cells (Fig. 1). Unaffected liver parenchyma had multifocal areas of lymphoplasmacytic pericholangitis, bile duct reduplication and mild portal fibrosis. The use of Perl´s Prussian blue stain did not reveal excessive iron accumulation. The PAS method highlighted thin basement membranes supporting neoplastic trabeculae. PAS-positive secretion was not observed within the neoplasm. Effacing and infiltrating the lung (Supplementary Fig. 1) and the colonic serosa and muscularis (Supplementary Fig. 2) were multiple metastatic foci of similar histological appearance. The grossly observed omental cysts contained cestode larvae that measured 4 x 0.8 mm (Supplementary Fig. 3). They had a ridged eosinophilic tegument and solid body cavity with numerous calcareous corpuscles. The anterior end had muscular suckers and within the parenchyma, thin muscles separated the medullary and cortical regions (Supplementary Fig. 4). Histologically, the parasites were consistent with *Mesocestoides* sp. which was confirmed by PCR analysis with amplification of the *ITS2* gene (Crosbie *et al*., 2000). Additional tissues were histopathologically unremarkable except for kidney in which tubular loss, fibrosis and mild, multifocal lymphoplasmacytic interstitial nephritis were considered incidental age-related changes.

Serial (3μm) sections of liver, colon and lung were immunohistochemically analysed for expression of cytokeratin (CK) AE1/AE3, CK8/18, CK7, CK20, HepPar1, vimentin, COX-2, E-Cadherin and 14-3-3σ antigens (Supplementary Table 1). Canine liver (previously used for antibody optimisation in the authors' laboratory; Suarez-Bonnet et al., 2010) and NHP kidney were used as positive controls. As negative controls, primary antibodies were replaced by homologous non-immune serum (Suarez-Bonnet *et al.*, 2017). Within regions of non-neoplastic primate liver, normal hepatocytes were strongly positive for anti-AE1/AE3, CK 8/18 and Hep-Par1 antigens. Normal biliary epithelium was positive for AE1/AE3, CK7, CK8/18 and negative for Hep-Par1 antigens. Vimentin was expressed only in mesenchymal cells (including Kupffer cells) in normal and neoplastic liver. In non-neoplastic primate liver, E-cadherin expression was weak and membranous in both hepatocytes and bile ducts. Neoplastic cells were negative for Hep-Par1 (Fig. 2) but strongly positive for anti-AE1/AE3, CK8/18, CK7 (Fig. 2; Supplementary Fig. 5). E-Cadherin immunolabelling was positive with both membrane localization and cytoplasmic internalization of antigen (Fig. 3). Immunolabelling of 14-3-3σ was strong in the cytoplasm and nuclei of neoplastic cells while non-neoplastic hepatocytes and bile ducts were negative (Fig. 4). COX-2 was variably expressed in hepatocyte cytoplasm in periportal regions of non-neoplastic liver, corresponding to regions of mild pericholangitis. Neoplastic cells and bile ducts within non-neoplastic liver were diffusely negative for COX-2. The histopathological, histochemical and immunohistochemical results were consistent with a diagnosis of CCA with colonic and lung metastases.

To the best of our knowledge, this is the first description of 14-3-3σ and E-cadherin expression in a hepatic neoplasm in an animal species and of spontaneous metastatic cholangiocarcinoma in a vervet monkey. A single combined hepatocellular-cholangiocellular carcinoma was found

in a survey of 1065 NHP necropsies (Seibold and Wolf, 1973). A case report of a CCA in a capuchin monkey and rare old reports of cholangiomas in other cercopithecus monkeys lacked immunohistochemical confirmation or characterisation (Brown *et al*., 1980).

Cholangiocarcinoma is an uncommon malignancy, arising from any point in the biliary tree but characterised by expression of cholangiocyte differentiation markers. The incidence in human beings varies geographically, presumably reflecting differences in local risk factors and genetics (Squadroni *et al*., 2017). In NHP, the relatively few necropsies performed in zoo facilities is a limiting factor in obtaining an approximate incidence (Matz-Rensing and Lowenstine, 2018).

Most human CCA arise *de novo*, although recently, cirrhosis and HBV and HCV infections have been recognised as risk factors. The contribution of HBV and HCV in tumour development differs in western countries, where hepatitis C is more prevalent, compared to Asian countries, where hepatitis B is endemic. The HBV and HCV status of this monkey is unknown. Interestingly, chronic lymphoplasmacytic cholangitis with bile duct hyperplasia and portal fibrosis was present in areas of non-tumoral liver. These changes are similar to those described in human beings with sclerosing cholangitis (Razumilava and Gores, 2014; Squadroni *et al*, 2017). The persistent release of pro-inflammatory cytokines which accompanies degenerative, necrotic and regenerative changes may have favoured tumorigenesis in this case (Fava *et al*., 2007).

Hepatobiliary trematodiasis has been associated with cholangiocarcinoma and less often with hepatocellular carcinoma in humans and NHP (Razumilava and Gores, 2014, Squadroni *et al*., 2017; Díaz-Delgado *et al*., 2018). Although intraabdominal *Mesocestoides* sp. were identified

in this case, as cestodes do not follow the same intracanalicular migration route as trematodes, an association with CCA seems less likely. No other cases of concurrent abdominal mesocestodiasis and neoplasia have been reported.

The immunohistochemical profile of this neoplasm is similar to that reported for human CCA (Berreta *et al*., 2017). However, our results vary slightly from previous reports in NHP. Reindel *et al*. (2000) and Laing *et al*. (2013) described cases of HCC that expressed both CK7 and CK8/18. In normal liver, CK7 is restricted to biliary epithelium and thus liver tumours expressing CK7 are probably cholangiocarcinomas unless they also express Hep-Par1, in which case a diagnosis of HCC would be more appropriate (Porter *et al*., 2004). Our case was diffusely positive for CK7 and CK8/18 but negative for Hep-Par1, which confirms the diagnosis of cholangiocarcinoma. It is likely that the HCC reported by Reindel *et al*. (2000) and Laing *et al*. (2013) were in fact CCA or combined hepatocellular-cholangiocellular carcinoma.

E-Cadherin is a cell-surface protein that has a prominent role in cell-cell adhesion and a well-established tumour suppressor function. The protein is normally expressed on the cell membrane with loss from that location frequent in CCA (Vaquero *et al*., 2017). Loss of E-cadherin expression from the cell membrane is often accompanied by its detection within the cytoplasm which can even be aberrantly upregulated (Jones *et al*. 2020). Cytoplasmic internalization and aberrant overexpression were present in this CCA compared with the weak membranous expression observed in normal bile ducts and hepatocytes. The role of E-cadherin in tumour progression has been extensively studied. E-cadherin facilitates vascular invasion in human inflammatory breast cancer in which the chemoresistance of tumour emboli is associated with the cohesive network provided by E-cadherin overexpression (Rodriguez *et al*.

2012; Vaquero *et al*., 2017). Aberrant cytoplasmic E-cadherin expression has also been observed in neoplastic emboli in canine and equine squamous cell carcinoma (Belluco *et al*., 2013, Suarez-Bonnet *et al*., 2018). Additional mechanisms such as promotion of EGFR-mediated PI3K activation leading to pro-survival, pro-migratory AKT signalling and enhancement of anti-apoptotic proteins Bcl-2 and anoikis resistance of neoplastic cells have also been reported (Rodriguez *et al*. 2012).

14-3-3σ is a cell-cycle regulator that functions as either a tumour suppressor or an oncoprotein in a tumour-dependent manner. Both overexpression, neo-expression and loss have been reported in a range of neoplasms (Yang *et al*., 2017). There is neo-expression of 14-3-3σ in human CCA and HCC which is in agreement with our findings of absence of 14-3-3σ in normal hepatocytes and bile ducts but strong and homogeneous neo-expression in the CCA. Several groups recommend the use of 14-3-3σ as a novel and reliable biomarker for liver neoplasia (Wu et al., 2012; Padden *et al*., 2014; Reis *et al*., 2015) but the utility of this marker in NHP has not previously been explored. Although, the underlying mechanism of action is not well understood, neoplastic cell migration, invasion and anoikis resistance were reduced in 14-3-3σ knock-out CCA cell lines, suggesting that this protein could be a promising therapeutic target (Khongmanee *et al*., 2013; Yang *et al*., 2017). Furthermore, 14-3-3σ is normally expressed in the cytoplasm. When nuclear translocation occurs, as in this case, it is associated with highly aggressive biological behaviour in carcinomas in other animal species (Suarez-Bonnet *et al*., 2018).

Normal human biliary epithelium is COX-2 negative but neoplastic cells in some human CCA cases have variable expression (Motiño *et al*., 2016). COX-2 is induced by several transcription factors and modulated by the balance between oncogenes and tumour suppressor genes. The

negative expression of COX-2 in this neoplasm suggests that it does not play a role in oncogenesis or that its expression is being suppressed or down-regulated.

In summary, we have characterized a metastatic CCA with concurrent abdominal mesocestodiasis, in a species of NHP in which this neoplasm has not been previously reported. Furthermore, the expression of 14-3-3σ and E-cadherin in this case highlights interesting comparative features with human CCA. Further investigation of these markers in liver neoplasms in other animal species is warranted.

Acknowledgments

We would like to dedicate this manuscript to the late Prof Michael Day whose global contributions to this journal and the field of veterinary and comparative pathology are unmatched. The authors did not receive direct funding to undertake this case report.

Conflict of Interest Statement

The authors declared no potential conflicts of interest with respect to the research and/or publication of this article.

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Figure legends

Fig. 1. Neoplastic cells form thick, short trabeculae and nests (lower left) and have marked anisocytosis, anisokaryosis and occasional macrokaryosis. Neoplasm is bordered by dense rim of lymphocytes that blend with non-neoplastic hepatic parenchyma (top right). HE. Bar, 50 μm.

Fig. 2. Cholangiocarcinoma cells strongly express CK7 antigen as do normal bile ducts (top right). IHC. Bar, 100 μm. Inset: Non-neoplastic hepatocytes express Hep-Par1 while cholangiocarcinoma cells are consistently negative (asterisks). IHC. Bar, 50 μm.

Fig. 3. Neoplastic cells exhibit strong membranous (arrows) and cytoplasmic E-Cadherin expression. Note bizarre trinucleated cells (arrowheads). IHC. Bar, 100 μm.

Fig. 4. Neoplastic cells are diffusely and strongly immunopositive for 14-3-3σ. Non-neoplastic hepatocytes are negative (asterisk). IHC. IHC. Bar, 100 μm. Inset: Strong cytoplasmic and nuclear expression of 14-3-3σ in neoplastic cells. IHC. Bar, 20 μm.

Please supply legends for Supplementary figures- add here

Supplementary Fig. 1. Lung. An extensive area of tumour metastasis infiltrates and effaces alveolar spaces. HE. Bar, 200 μm.

Supplementary Fig. 2 Colon. The tunica muscularis is infiltrated and effaced by tumour metastasis. HE. Bar, 200 μm.

Supplementary Fig. 3 *Mesocestoides* sp. larvae (tetrathyridium). HE. Bar, 500 μm.

Supplementary Fig. 4 *Mesocestoides* sp. larvae (tetrathyridium). There is an invaginated scolex with two pairs of suckers (arrows) and calcareous corpuscles (arrowheads) embedded within the parenchyma. HE. Bar, 50 μm.

Supplementary Fig. 5 Cholangiocarcinoma cells strongly express CK7 antigen as do normal bile ducts (arrows). IHC. Bar, 100 μm.