Case Report

Spontaneous pulmonary co-metastasis of hepatoblastoma arising within a hepatocellular carcinoma in an aged C57BL/6J mouse

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Abstract: Murine hepatoblastoma (HB) is a rare spontaneous tumor with controversial histogenesis. It mainly occurs in aged males, frequently in close association with preexisting hepatocellular neoplasms. The present work describes a spontaneous HB arising within a hepatocellular carcinoma (HCC) in a 22-month-old male C57BL/6J mouse. The mouse also developed pulmonary co-metastases with either tumor components physically associated within the same metastatic foci. Microscopically, the HB consisted of a densely cellular neoplastic growth composed of palisades and perivascular pseudorosettes of poorly differentiated primitive cells, with a scant amount of cytoplasm, elongated hyperchromatic nuclei, and a high mitotic rate, whereas the hepatocellular carcinoma was composed of solid areas of neoplastic hepatocytes. Both in primary tumors and their metastases, β-catenin immunohistochemistry revealed a strong nucleocytoplasmic signal in HB cells, while neoplastic hepatocytes displayed a delicate membranous staining pattern. These findings suggest that the Wnt/β-catenin oncogenic pathway is upregulated in murine HB but not in the co-existing HCC, thus providing some insights into their divergent pathogenesis. Coexisting murine HB and HCC have been demonstrated to be completely distinct entities including origin, mutational landscape, and molecular profile. In this context, they might be regarded as collision tumors because of their intimate association, unique histologic features, and distinct immunohistochemical patterns. Nevertheless, the nature of their coevolution and progression to a co-metastatic phenotype reflects a close interdependence and support the overall idea that HB's origin and progression might be promoted by not otherwise specified paracrine stimuli provided by the concurrent hepatocellular tumor (the so called "interaction theory"). (DOI: 10.1293/tox.2017-0067; J Toxicol Pathol 2018; 31: 195–199)

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Hepatoblastoma (HB) is a primitive, poorly differentiated, embryonal hepatic neoplasm. In mice, HBs are rare spontaneous entities that mainly occur in aged male animals[1] and are usually seen within or adjacent to preexisting hepatocellular adenomas (HCAs) or carcinomas (HCCs)[2, 3]. The cell of origin of HBs remains unknown, although liver blastemal cells, neoplastic hepatocytes, oval cells, and biliary epithelial cells have been proposed[3]. Previously considered to be the result of malignant progression of benign or low-grade hepatocellular tumors, it has been recently documented that murine hepatoblastoma most likely represents a distinct entity displaying a completely different mutational spectrum and transcriptome if compared with hepatocellular tumors[4].

In humans, HBs are mainly observed in young children below the age of 5 years and are exceptionally rare in adults[5, 6]. In contrast to their murine counterpart, human HBs are always solitary tumors unassociated with preexisting hepatocellular neoplasias and are thought to arise *de novo* from hepatoblast remnants of the embryonic liver[2, 7, 8]. On the other hand, HBs in both mice and humans share similarities, such as primitive morphologic features, predominance in the male gender, and a comparable metastatic rate with lungs as the most common metastatic site[2, 9]. Furthermore, genetic alterations have been associated with this malignancy, with human and mouse HBs both harboring oncogenic mutations in *Ctnnb1*[10, 11] leading to constitutive activation of the Wnt/β-catenin pathway.

Considering nonhuman species, hepatoblastomas have been described in equine fetuses, neonates, and occasionally young adult horses [12–14], in young and adult sheep[15], and in a cat[16], llama[17] and dog[18]. In all these species, HBs have been mainly reported in young animals, paralleling the human counterpart, with only a minority of cases occurring in adults. With reference to laboratory animals, there are rare reports of this lesion in rats[3]. A single case was described

in a 10-day-preterm fetus of a male cynomolgus macaque (*Macaca fascicularis*)[19].

This study aimed to describe pathological features of a spontaneous murine condition characterized by the co-occurrence of HB and HCC and development of pulmonary co-metastases consisting of closely associated neoplastic cells from both the hepatic cancers.

The animal considered in this study was an experimentally naïve 22-month-old male C57BL/6J mouse included as control in an aging study. The mouse was sacrificed because of generalized deterioration of clinical conditions associated with marked abdominal enlargement and dyspnea. A complete necropsy and histopathological examination were performed following the same protocols and procedures as described by Castiglioni *et al.*[20]. For immunohistochemistry (IHC), 4 μm formalin-fixed paraffin-embedded sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by incubating sections in 3% H_2O_2 for 15 min. For antigen retrieval, sections were immersed in citrate buffer (pH 6.0), heated, and cooled at room temperature. β-catenin (rabbit monoclonal, clone 6B3, dilution 1:150, Cell Signaling) specific antibody was applied for 1.5 hours at room temperature. The reaction was amplified with an avidin-biotin method (Vectastain Elite ABC Kit PK-6100, Vector Laboratories Inc., Burlingame, CA, USA) and visualized with 3,3′-diaminobenzidine. Sections were counterstained with hematoxylin. A negative immunohistochemical control was prepared by replacing the primary antibody with an irrelevant one, and a known positive control section was included in the immunolabeling assay. Procedures involving animals were performed in accordance with the guidelines of the Catholic University of Leuven (KU Leuven) Animal Care and Use Ethical Committee, which specifically approved this study (reference number 072/2015).

At necropsy, ascites was detected, and the entire left hepatic lobe and part of the median were markedly enlarged by a large, mottled gray-brown multinodular mass measuring approximately $1.5 \times 1.5 \times 1$ cm. The lungs were characterized by multiple small brownish nodules (measuring approximately 1 to 3 mm in diameter) that were particularly evident along the margins of the lobes.

Histologically, the affected hepatic parenchyma was almost completely effaced by two distinct neoplastic growths (Fig. 1). The first one was characterized by multiple coalescing HCAs consisting of expansile masses that were moderately cellular, unencapsulated, relatively well demarcated and composed of well-differentiated neoplastic hepatocytes arranged in cords or thin trabeculae separated by a delicate sinusoidal vascular network. Scattered throughout the HCAs there were foci of increased atypia with higher N/C ratios, hyperchromatic nuclei, with neoplastic hepatocytes arranged in thicker trabeculae (more than 3 cells in thickness) separated by irregularly distended sinusoids often forming pseudocystic spaces, and with foci of coagulative necrosis. These were interpreted as foci of progression suggesting a morphological diagnosis of trabecular type HCC arising within an HCA (Fig. 2). The second neoplastic entity arose within the previously described hepatocellular tumor as a multicentric process. These growths were infiltrating, unencapsulated, and composed of dense palisades and perivascular pseudorosettes of poorly differentiated primitive cells, with a scant amount of cytoplasm, elongated hyperchromatic nuclei, and a high mitotic rate (Fig. 3). Scattered throughout there were foci of coagulative necrosis and hemorrhages and few foci of squamous differentiation (Fig. 3, inset). Morphologically, the tumor was consistent with a diagnosis of HB. Histopathology of the pulmonary lesions revealed disseminated neoplastic aggregates multifocally plugging small and mid-sized pulmonary vessels (tumor emboli) and invading the surrounding parenchyma (tumor metastases). Pulmonary lesions recapitulated the two different neoplastic growths observed within the liver, with neoplastic hepatocytes closely associated with primitive, poorly differentiated neoplastic cells (interpreted as pulmonary metastases of HB arising within an HCC) (Fig. 4).

Immunohistochemistry of β-catenin revealed that neoplastic hepatocytes in both hepatic and pulmonary locations showed mild to moderate membranous immunoreactivity, whereas a strong nucleocytoplasmic signal was evident in HB tumor cells (Fig. 5 and 6).

The complex hepatic changes observed in this aged C57BL/6J mouse recapitulate the well-characterized scenario in which HB is usually seen within or adjacent to preexisting hepatocellular tumors[1]. The presence of areas of HCC within HCA supports the overall idea that hepatocellular carcinogenesis in the mouse often follows the preneoplastic pathway, with preneoplastic lesions evolving into adenomas and later on into foci of carcinomatous progression arising within adenomas (hence the diagnostic designation of HCC arising within HCA)[3]. The hepatoblastoma here examined showed foci of squamous differentiation, in agreement with current literature that frequently reports small areas of bone and squamous differentiation along with foci of extramedullary hematopoiesis[3]. Both murine HCC and HB exhibit metastatic potential, with the lung being the most frequently involved distant organ[2]. To the authors' knowledge, evidence of pulmonary co-metastases has been reported only once prior to this report[2].

The different patterns of β-catenin expression observed in both hepatic and pulmonary locations, with a strong nucleocytoplasmic signal in HB and delicate membranous immunoreactivity displayed by the hepatocellular proliferations, are in line with the well-established notion that the Wnt/β-catenin oncogenic pathway drives murine HB but not HCC tumorigenesis[4]. The divergent oncogenic mechanisms activated by these two tumors further support the hypothesis that HB does not progress from the concurrent hepatocellular tumor and that it should rather be regarded as a separate neoplasm. Comparative transcriptomic analysis demonstrated a high degree of concordance among murine HB, embryonic mouse hepatoblasts, and hepatic pluripotent stem cells, suggesting that these poorly differentiated
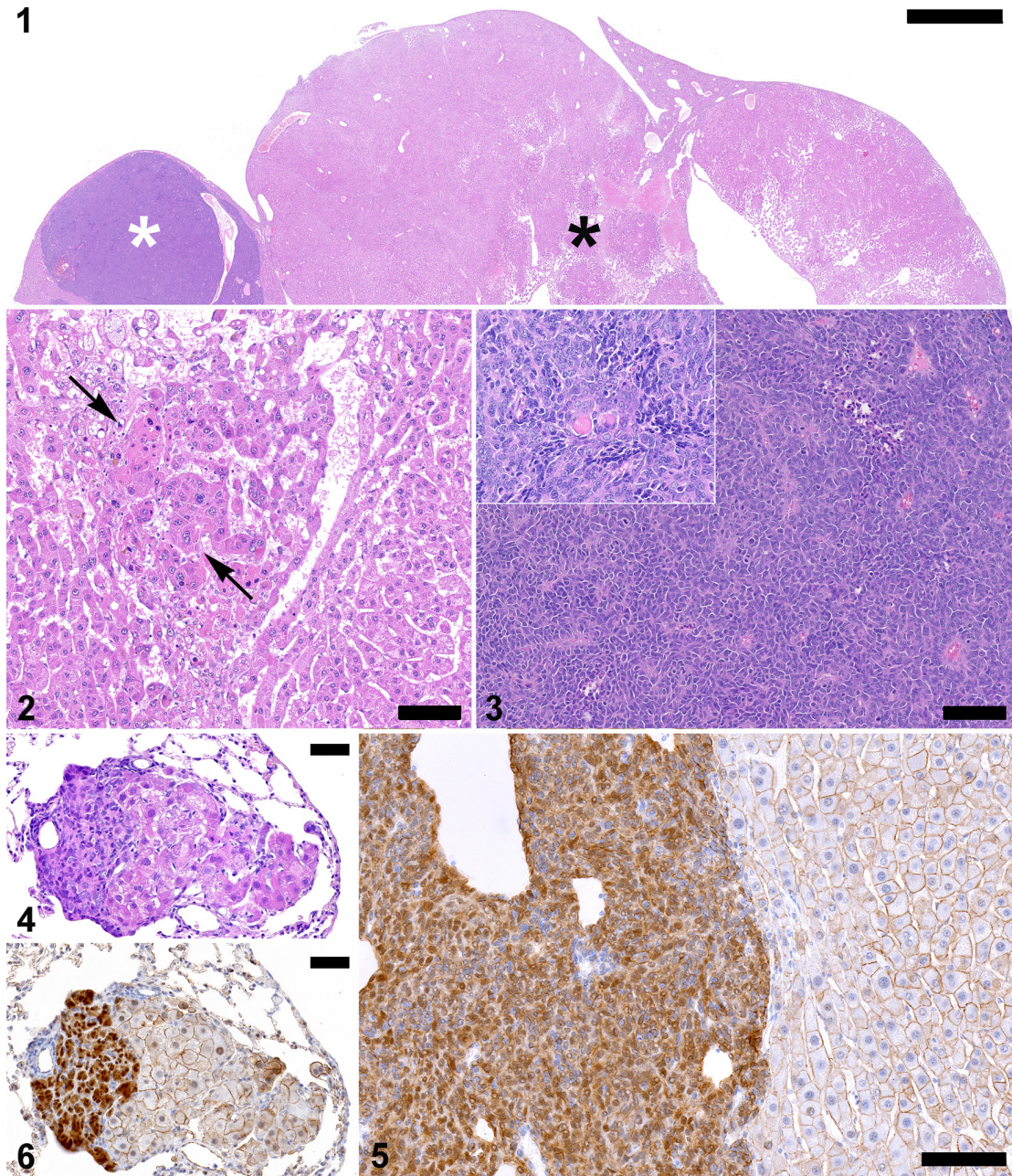
Fig. 1. Liver, 22-month-old male C57BL/6J mouse. The hepatic parenchyma is expanded and effaced by two different neoplastic growths that are in close proximity: a hepatocellular carcinoma (black asterisk) and a hepatoblastoma (white asterisk). Hematoxylin and eosin. Scale bar: 2 mm.

Fig. 2. Liver, 22-month-old male C57BL/6J mouse. Hepatocellular carcinoma arising within a hepatocellular adenoma. Note the carcinomatous focus (arrows) characterized by increased trabecular thickness and findings of atypia in tumor cells including a higher N/C ratio and hyperchromasia. Hematoxylin and eosin. Scale bar: 100 μm.

Fig. 3. Liver, 22-month-old male C57BL/6J mouse. The hepatoblastoma consists of poorly differentiated primitive cells with elongated hyperchromatic nuclei, scant cytoplasm, and a high mitotic rate. Tumor cells are typically arranged in dense palisades and perivascular pseudorosettes. Small scattered foci of squamous differentiation (inset) are also evident. Hematoxylin and eosin. Scale bar: 100 μm.

Fig. 4. Lung, 22-month-old male C57BL/6J mouse. Metastatic focus consisting of combined hepatoblastoma and hepatocellular carcinoma. Hematoxylin and eosin. Scale bar: 50 μm.

Fig. 5. Liver, 22-month-old male C57BL/6J mouse. Hepatoblastoma (left) and adjacent hepatocellular adenoma (right). β-catenin immunohistochemistry reveals a strong nucleocytoplasmic signal in hepatoblastoma tumor cells, while neoplastic hepatocytes in the surrounding adenoma display a delicate membranous staining pattern. β-catenin immunohistochemistry (IHC); hematoxylin counterstain. Scale bar: 100 μm.

Fig. 6. Lung, 22-month-old male C57BL/6J mouse. The same metastatic focus is depicted as in Fig. 4. β-catenin immunohistochemistry reveals a strong nucleocytoplasmic signal in the hepatoblastoma fraction of the metastatic lesion, while the hepatocarcinomatous component maintains a delicate membranous staining pattern. β-catenin immunohistochemistry (IHC); hematoxylin counterstain. Scale bar: 50 μm.

and primitive tumors likely originate from a primordial hepatic precursor cell[4, 21]. Recent studies also showed that coexisting HCC and HB do not share the same mutational spectrum, thus providing compelling evidence that they are indeed distinct tumor entities[4, 10]. In light of these considerations, we propose to consider the entity here described as a collision tumor, that is defined as two distinct neoplasms with diverse origins and phenotypes coinciding at the same location[22]. Furthermore, as suggested by the frequent formation of HB within (or near) a well-developed hepatocellular neoplasm, the molecular events that lead to hepatic precursor cell transformation and HB formation are facilitated by not otherwise specified paracrine stimuli provided by the preexisting neoplastic hepatocytes. In this context, we support the idea that the microenvironment created by the hepatocellular tumors promotes the development of HB via a still unclear paracrine mechanism. Such a type of interaction, known as the "interaction theory", has already been proposed for a series of co-developing neoplasms in humans[23, 24]. In this context, the evidence of co-metastases, in which both tumor cell types are physically associated within the same metastatic focus, possibly indicates that the close biological interdependence between HB and HCC also contributes to tumor progression and combined metastatic spread.

In conclusion, to the authors' knowledge, this is the first report characterizing immunohistochemically both hepatic and pulmonary co-metastasis and gives potentially interesting insights into the pathogenesis and coevolution of these frequently associated cancers, raising attention concerning whether they might be regarded as collision tumors, although they have not yet been classified as such academically.

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References

1. Chhabra RS, Eustis S, Haseman JK, Kurtz PJ, and Carlton BD. Comparative carcinogenicity of ethylene thiourea with or without perinatal exposure in rats and mice. Fundam Appl Toxicol. **18**: 405–417. 1992. [Medline] [CrossRef]

2. Turusov VS, Torii M, Sills RC, Willson GA, Herbert RA, Hailey JR, Haseman JK, and Boorman GA. Hepatoblastomas in mice in the US National Toxicology Program (NTP) studies. Toxicol Pathol. **30**: 580–591. 2002. [Medline] [CrossRef]

3. Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Küttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, and Ward JM. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. Toxi-

4. Bhusari S, Pandiri AR, Nagai H, Wang Y, Foley J, Hong HH, Ton TV, DeVito M, Shockley KR, Peddada SD, Gerrish KE, Malarkey DE, Hooth MJ, Sills RC, and Hoenerhoff MJ. Genomic profiling reveals unique molecular alterations in hepatoblastomas and adjacent hepatocellular carcinomas in B6C3F1 mice. Toxicol Pathol. **43**: 1114–1126. 2015. [Medline] [CrossRef]

5. Spector LG, and Birch J. The epidemiology of hepatoblastoma. Pediatr Blood Cancer. **59**: 776–779. 2012. [Medline] [CrossRef]

6. Cruz RJ Jr, Ranganathan S, Mazariegos G, Soltys K, Nayyar N, Sun Q, Bond G, Shaw PH, Haberman K, Krishnamurti L, Marsh JW, Humar A, and Sindhi R. Analysis of national and single-center incidence and survival after liver transplantation for hepatoblastoma: new trends and future opportunities. Surgery. **153**: 150–159. 2013. [Medline] [CrossRef]

7. Lack EE, Neave C, and Vawter GF. Hepatoblastoma. A clinical and pathologic study of 54 cases. Am J Surg Pathol. **6**: 693–705. 1982. [Medline] [CrossRef]

8. von Schweinitz D. Hepatoblastoma: recent developments in research and treatment. Semin Pediatr Surg. **21**: 21–30. 2012. [Medline] [CrossRef]

9. Herzog CE, Andrassy RJ, and Eftekhari F. Childhood cancers: hepatoblastoma. Oncologist. **5**: 445–453. 2000. [Medline] [CrossRef]

10. Anna CH, Sills RC, Foley JF, Stockton PS, Ton TV, and Devereux TR. Beta-catenin mutations and protein accumulation in all hepatoblastomas examined from B6C3F1 mice treated with anthraquinone or oxazepam. Cancer Res. **60**: 2864–2868. 2000. [Medline]

11. Cairo S, Armengol C, De Reyniès A, Wei Y, Thomas E, Renard CA, Goga A, Balakrishnan A, Semeraro M, Gresh L, Pontoglio M, Strick-Marchand H, Levillayer F, Nouet Y, Rickman D, Gauthier F, Branchereau S, Brugières L, Laithier V, Bouvier R, Boman F, Basso G, Michiels JF, Hofman P, Arbez-Gindre F, Jouan H, Rousselet-Chapeau MC, Berrebi D, Marcellin L, Plenat F, Zachar D, Joubert M, Selves J, Pasquier D, Bioulac-Sage P, Grotzer M, Childs M, Fabre M, and Buendia MA. Hepatic stem-like phenotype and interplay of Wnt/beta-catenin and Myc signaling in aggressive childhood liver cancer. Cancer Cell. **14**: 471–484. 2008. [Medline] [CrossRef]

12. Beeler-Marfisi J, Arroyo L, Caswell JL, Delay J, and Bienzle D. Equine primary liver tumors: a case series and review of the literature. J Vet Diagn Invest. **22**: 174–183. 2010. [Medline] [CrossRef]

13. Cantile C, Arispici M, Abramo F, and Campani D. Hepatoblastoma in a foal. Equine Vet J. **33**: 214–216. 2001. [Medline] [CrossRef]

14. Lennox TJ, Wilson JH, Hayden DW, Bouljihad M, Sage AM, Walser MM, and Manivel JC. Hepatoblastoma with erythrocytosis in a young female horse. J Am Vet Med Assoc. **216**: 718–721. 2000. [Medline] [CrossRef]

15. Manktelow BW. Hepatoblastomas in sheep. J Pathol Bacteriol. **89**: 711–714. 1965. [Medline] [CrossRef]

16. Ano N, Ozaki K, Nomura K, and Narama I. Hepatoblastoma in a cat. Vet Pathol. **48**: 1020–1023. 2011. [Medline] [CrossRef]

17. Watt BC, Cooley AJ, and Darien BJ. Congenital hepato-

blastoma in a neonatal alpaca cria. Can Vet J. **42**: 872–874. 2001. [Medline]

18. Shiga A, Shirota K, Shida T, Yamada T, and Nomura Y. Hepatoblastoma in a dog. J Vet Med Sci. **59**: 1167–1170. 1997. [Medline] [CrossRef]

19. Joint Pathology Center Veterinary Pathology Services Wednesday Slide Conference 2016–2017. from Conference 22, Case I, provided by Advanced Molecular Pathology Laboratory Institute of Molecular and Cell Biology Proteos, Singapore website https://www.askjpc.org/wsco/wsc_showcase2.php?id=1050.

20. Castiglioni V, De Maglie M, Queliti R, Rustighi A, Del Sal G, and Radaelli E. Immunohistochemical characterization of a renal nephroblastoma in a Trp53-mutant and prolyl isomerase 1-deficient mouse. J Toxicol Pathol. **26**: 423–427. 2013. [Medline] [CrossRef]

21. Cullen JM. Tumors of the liver and gallbladder. In: Tumors in Domestic Animals, 5th ed. DJ Meuten (ed). Wiley Blackwell, Ames, Iowa. 602–631. 2017.

22. Murthaiah P, Truskinovsky AM, Shah S, and Dudek AZ. Collision tumor versus multiphenotypic differentiation: a case of carcinoma with features of colonic and lung primary tumors. Anticancer Res. **29**: 1495–1497. 2009. [Medline]

23. de Leval L, Hardy N, Deprez M, Delwaide J, Belaïche J, and Boniver J. Gastric collision between a papillotubular adenocarcinoma and a gastrinoma in a patient with Zollinger-Ellison syndrome. Virchows Arch. **441**: 462–465. 2002. [Medline] [CrossRef]

24. Miteva M, Herschthal D, Ricotti C, Kerl H, and Romanelli P. A rare case of a cutaneous squamomelanocytic tumor: revisiting the histogenesis of combined neoplasms. Am J Dermatopathol. **31**: 599–603. 2009. [Medline] [CrossRef]