Delladetsima I, Sakellariou S, Govaere O, Poulaki E, Felekouras E, Tiniakos D.

[Hepatic Progenitor Cells in Metastatic Liver Carcinomas.](#)

Histopathology 2018

DOI: <https://doi.org/10.1111/his.13450>

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Date deposited:

18/01/2018

Embargo release date:

08 December 2018

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Article type : Short Report

Hepatic Progenitor Cells in Metastatic Liver Carcinomas

Running title: Hepatic Progenitor Cells in Liver Metastasis

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/his.13450

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Conflict of interest:

All authors declare no competing financial interests.

Abstract

Aims: Hepatic progenitor cells (HPCs) are activated in various liver diseases but their role in carcinomatous environment remains unknown. We aimed to identify the possible presence and topography of HPCs in liver metastases.

Methods: We examined 14 liver resection specimens for colorectal adenocarcinoma (CRC) (n=13) and anal squamous cell carcinoma (SCC) (n=1) metastases. Immunohistochemical markers of colonic (keratin 20-K20, CDX2) and squamous cell origin (p63), HPC (K19, CD56) and stem cell (CD44) markers, and the biliary marker K7 which may also highlight HPCs were applied on routinely processed tissue sections. Double immunohistochemistry/immunofluorescence (K7/CDX2) and confocal microscopy were used on selected sections.

Results: K7-, K19- and CD56-positive ductular structures were encountered within the metastatic tumour (tumour interior and periphery), and in the immediate peritumoural area. Hybrid structures composed of HPCs and metastatic adenocarcinoma cells were recognised and confirmed by double immunostaining (K7/CDX2). Carcinoma cells were also observed singly or in groups within the epithelium of interlobular bile ducts and/or ductules in portal tracts without evidence of carcinomatous infiltration and at a distance from the metastatic foci.

Conclusions: HPCs are observed at the periphery and the interior of liver metastatic carcinomas. Bile ductules and small interlobular bile ducts may attract carcinoma cells serving as potential “metastatic niche”, in line with their recognised role as HPC niches in non-neoplastic liver.

Key words: hepatic progenitor cells, liver metastasis, ductular reaction, metastatic niche, metastatic carcinoma

Introduction

The hepatic progenitor cells (HPC) compartment comprises a spectrum of subtypes of variable differentiation including isolated progenitor cells and string-like ductular structures (DS) at the portal/parenchymal interface known as ductular reaction.^{1,2} Intermediate hepatocytes, keratin 7 (K7)-positive hepatocytes and bile ductules are more differentiated forms of increasing maturity. The HPC immunophenotype includes hepatocytic, cholangiocellular, hepatoblast, and hematopoietic stem cell markers.³⁻⁶

Sufficient data suggest that adult HPCs may also derive from mature hepatocytes through a dedifferentiation process in the context of acute and chronic liver injury.⁷⁻⁹ Hepatocyte loss in association with impaired hepatocyte regenerative capacity, replicative exhaustion and senescence are triggering factors for an alternative regeneration pathway involving HPCs.^{1,5,7} In contrast, the functional role of HPCs in primary and metastatic liver carcinoma still remains unknown. We aimed to explore the presence and topography of HPCs in metastatic liver carcinomas.

Patients and Methods

The presence of HPCs was examined in 13 specimens of liver metastasectomies (segmental hepatectomies n=10, left hepatectomies n=2, right hepatectomy n=1) for colorectal adenocarcinoma (CRC) and a metachronous solitary liver metastasis of anal squamous cell carcinoma (SCC) which was removed by segmental hepatectomy.

Metastatic adenocarcinomas were selected from a total of 60 cases of liver CRC metastasis resected between 2007 and 2014 according to the following inclusion criteria: a) metastatic CRC excised concurrently with the resection of the primary tumour, b) no pre-operative neo-adjuvant chemotherapy, c) primary CRC immunophenotype different from that of HPC, biliary epithelial cells and hepatocytes [CDX2/keratin 20 (K20) positive and keratin 7(K7)/CD56/HepPar-1 negative]. All primary carcinomas and their metastatic foci were of moderate histological differentiation and four showed extensive necrosis. Informed consent was obtained from each patient prior to surgery for use of surplus diagnostic material for research purposes. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Immunohistochemical examination of serial paraffin sections included stains for K20 and CDX2 (CRC markers); K19 and CD56 (HPC markers); CD44 (stem cell marker) and K7 (biliary marker that also highlights HPCs). In the metastatic anal SCC, p63 was used as nuclear marker for the identification of SCC tumour cells. Double immunostaining was performed using K7 and CDX2 in CRC metastases. Additionally, double immunofluorescence for K7 and CDX2 was performed in selected sections evaluated by confocal microscopy. Keratin 19 was not used as HPC marker in the double immunostaining experiments because it may be expressed by CRC cells.

The presence of HPC subtypes was examined within the metastatic tumour (tumour interior and periphery), in the immediate peritumoural area (one low power field x10) and in the adjacent non-neoplastic liver tissue (tumour periphery and peritumoural area are depicted in Supplementary Figure 1). One representative section from each case was examined and the density of HPC subtypes was assessed semi-quantitatively. Ductular reaction at the portal/parenchymal interface of non-neoplastic liver tissue was recorded.

Details on antibodies and methodology can be found in Supporting Information file 1.

Results

In all cases, a variable number of K7-positive and CD56-positive DS and mature ductules were encountered in the immediate peritumoural area, while ductular reaction constituted a rather common finding in the neighbouring non-neoplastic liver. DS were also discernible at the periphery and less frequently in the interior of carcinomatous foci both in the stroma and the epithelial component (Figure 1A). K20, CDX2 and p63 were negative in DS. In contrast, carcinoma cells did not exhibit HPC markers and were immunostained diffusely for K20 and CDX2 in metastatic CRC foci and for p63 in metastatic SCC. CD44-positive cells were rarely encountered. HepPar-1-positive hepatocytes were visible, usually in small groups, in the peritumoural stroma while only few entrapped atrophic HepPar-1-positive hepatocytes were still visible at the tumour periphery.

In all metastatic CRC cases, examination of immunostained serial sections at high magnification revealed HPCs adherent to carcinoma cells, creating hybrid structures of combined HPC/carcinomatous composition. This phenomenon was evident by the differences in cell morphology and immunophenotype between HPCs

and carcinoma cells (Figures 1B, 1C, 1D). HPCs were small-sized, flat with oval nuclei without atypia while the carcinoma cells were larger, cuboidal or cylindrical with atypical nuclei. HPCs exhibited a typical immunohistochemical profile (K7, CD56 positive and K20, CDX2 negative) in contrast to carcinoma cells which stained positively only for CRC markers (K20 and CDX2) or p63. Immunohistochemical investigation also revealed solitary HPCs among carcinoma cells.

The aforementioned observations were confirmed by double K7/CDX2 immunofluorescence assessed by confocal microscopy (Figures 1E & 1F and Supplementary Figure 2) and double K7/CDX2 immunostaining (Supplementary Figure 3) that highlighted the dual nature of the hybrid tubular structures and the structural co-existence of the different cell types. No cells were identified co-expressing K7/CDX2.

In the single anal SCC metastasis case, intratumoural HPCs were encountered as isolated cells within carcinoma sheets recognized by their typical immunoprofile in contrast to p63-positive anal SCC cells.

In six CRC cases and in the anal SCC case, K20- and p63-positive carcinoma cells, single or in small groups, were seen in interlobular ducts and ductules located in portal tracts at a distance from the periphery of the tumours (Figure 2). There were no signs of invasion of the intervening non-neoplastic liver tissue or in the portal tract stroma, neither disruption of the basement membrane of the involved ducts and ductules.

Supporting Information file 2 shows detailed immunohistochemical results for each hepatic CRC metastasis case.

Discussion

HPCs disclose features of stemness represented by their multilineage differentiation capacity, their ability to migrate and their involvement in carcinogenesis.¹⁰

Hepatic stem/progenitor cells give rise to carcinomas with stem cell features and their subtypes,¹¹ while retrodifferentiation of hepatocellular carcinoma (HCC) cells induced *in vitro* by inflammatory cytokines is implicated as an additional carcinogenic pathway.¹² *In vitro* and *in vivo* models in a single study¹³ suggest that HPCs may have a marked effect on HCC cells and appear to stimulate the growth and malignant potential of experimental HCC tumours. In respect to the spatial relationship of HPCs and tumour tissue, oval cells localized inside and outside the carcinomatous foci have been observed in rats,¹⁴ while HPCs have very occasionally been reported in human hepatocellular tumours.^{15,16}

Our study is the first to highlight the existence of HPCs within metastatic liver carcinomas, usually in the form of DS. They were encountered in the newly formed peritumoural and intratumoural stroma, as well as within the carcinomatous epithelial component. The most impressive finding was their presence in hybrid tubular structures of mixed HPC/carcinoma cell composition. The detection of HPCs was facilitated by their specific morphology and immunoprofile in combination with the absence of CRC cell markers K20, CDX2 and the SCC marker p63, and was confirmed by double immunostaining (K7 and CDX2) and double immunofluorescence (K7 and CDX2). Various hypotheses may explain this phenomenon. Activation of HPCs originating from entrapped non-neoplastic ductules and small ducts or HPC migration in the metastatic foci under the influence of chemoattractants are plausible explanations. Regarding the hybrid structures, they may present the end-product of DS contact with carcinomatous elements by creating new

HPC-carcinoma cell adhesions or may derive from DS infiltration by carcinoma cells.

Another unique observation was the detection of carcinoma cells singly or in groups within interlobular bile ducts and/or ductules at a distance from the main tumour mass in the absence of portal tract invasion. Colonisation of major and minor bile ducts and intrabiliary growth has long been recognised.^{17,18} However, it has not been clarified whether this phenomenon may be attributed to bile duct invasion from a parenchymal metastatic focus or whether it represents a genuine intrabiliary metastasis. The presence of single carcinoma cells within the ductular epithelium is indicative of a metastatic process.

What seems to be undisputable in our study is the occasional symbiosis of benign HPCs and biliary epithelial cells with malignant cells, in metastatic liver carcinomas. This phenomenon can be ascribed to the stem cell properties shared by both HPCs and possible cancer stem cells. Endowed with growth factors and cytokines (e.g. lymphotoxin-b, MCP-1, PDGF, TGF- β),¹⁹⁻²³ HPCs participate in fibrogenesis and liver cell regeneration and may potentially contribute to tumour growth and stroma formation adapting a role similar to that of bone marrow stem cells in the expansion of primary and metastatic carcinomas.²⁴ On the other hand, it is reasonable to assume that cytokines, growth factors and chemokines produced by tumour stroma and carcinoma cells may activate and attract HPCs. It is well known, that HPCs require essential pro-inflammatory cytokines and growth factors, analogous to those produced by carcinoma cells to proliferate and differentiate.²⁵ Lastly, the colonisation of small bile ducts and ductules by carcinoma cells highlights a potential metastatic niche that already functions as a suitable microenvironment for non-neoplastic HPCs.

Limitations of our study include the small but representative number of cases and the examination of only two types of metastatic liver cancer in the context of an observational study. It may be argued that the individual immunohistochemical markers used to support or exclude CRC, SCC or HPC origin are not 100% specific or sensitive. However, when these markers are jointly assessed in conjunction with careful morphological examination their application is appropriate for the purpose of our study.

In conclusion, our morphological and immunohistochemical study provides strong indications of HPC presence within metastatic liver carcinomas, usually in the form of immature DS. Bile ductules and small interlobular bile ducts may attract carcinoma cells serving as a possible “metastatic niche” in line with their recognised role as HPC niche in non-neoplastic liver. The role and fate of intracancerous HPCs remains unknown requiring clarification in future in-depth investigations at a molecular level.

Acknowledgements

The authors are grateful to Professor Valeer Desmet (Leuven, Belgium) for his insightful comments and critical review of the manuscript.

Author Contributions

ID designed the study, interpreted immunohistochemical data, drafted and critically revised the manuscript for important intellectual content

SS interpreted immunohistochemical data, drafted and critically revised the manuscript for important intellectual content

OG acquired and interpreted double immunofluorescence/confocal microscopy data and reviewed the manuscript

EP acquired and interpreted immunohistochemical data

EF provided surgical specimens and clinical data

DT designed the study, drafted and critically revised the manuscript for important intellectual content.

All authors approved the final version of the manuscript.

Financial support:

The authors declare no financial support.

List of abbreviations

HPC/HPCs: Hepatic progenitor cell/s

DS: Ductular structures

K: Keratin

CRC: Colorectal adenocarcinoma

SCC: Squamous cell carcinoma

HCC: Hepatocellular carcinoma

List of online Supporting Information

Supporting Information file 1

Details on immunohistochemical/immunofluorescence methodology and antibodies applied

Supporting Information file 2

Table with detailed immunohistochemical results for the 13 CRC cases

Supporting Information file 3

Supplementary Figure 1

Supporting Information file 4

Supplementary Figure 2

Supporting Information file 5

Supplementary Figure 3

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Figure legends

Figure 1: A. Entrapment of ductular structures (DS) within the tumour tissue (K7 immunostain, DAB chromogen, x200). **B.** Hybrid structures composed of CRC cells (K7-) and HPCs (K7+) (K7 immunostain, DAB chromogen, x400). **C.** Hybrid structures composed of K20+ CRC cells and K20- HPC (K20 immunostain, DAB chromogen, x400). **D.** Hybrid structures composed of CRC cells (CD56-) and HPCs (CD56+) (CD56 immunostain, DAB chromogen, x400). **E, F.** Double K7/CDX2 immunofluorescence assessed by confocal microscopy highlights hybrid structures composed of K7+ (green) HPC and

CDX2+ (red) CRC cells (merged with nuclear DAPI counterstaining (blue) E x540, F x920).

Figure 2: Carcinoma cells within the epithelium of interlobular bile ducts and ductules (circles) in portal tracts at a distance from the periphery of the tumour mass. **A.** K20+ CRC tumour cells (K20 immunostain, DAB chromogen, x400). **B.** K7+ bile ductal epithelium, K7- CRC tumour cells (K7 immunostain, DAB chromogen, x200). **C.** p63+ SCC tumour cells (p63 immunostaining, DAB chromogen, x100).

Supplementary Figure legends:

Supplementary Figure 1: Tumor periphery (encircled) and immediate peritumoural area (K7 immunostain, DAB chromogen, x200).

Supplementary Figure 2: Decomposed Figures 1E and 1F showing merged K7 (green)/CDX2 (red) and non-merged K7 (green), CDX2 (red) and DAPI (blue) immunofluorescence.

Supplementary Figure 3: Hybrid structures (arrows) composed of K7+/CDX2- HPC and K7-/CDX2+ CRC cells [double K7/CDX2 immunostaining with cytoplasmic K7 (AEC chromogen) and nuclear CDX2 (DAB chromogen), x400].