Research Article

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Laminin-332 sustains chemoresistance and quiescence as part of the human hepatic cancer stem cell niche

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Background & Aims: Cancer stem cells (CSCs) are thought to be persistent in tumours due to their chemoresistance and to cause relapse and metastasis. Hepatic carcinomas displaying hepatic progenitor cell (HPC) features have been associated with a poor prognosis, though it remains unclear how CSCs relate to these different histological subtypes.

Methods: Candidate CSCs were isolated using the side population (SP) technique from primary tissue samples diagnosed as keratin (K)19-negative or -positive hepatocellular carcinoma (HCC) or as combined hepatocellular/cholangiocarcinoma and analysed for gene and protein expression. The effect of laminin-332 was analysed *in vitro* by using HCC cell lines and *in vivo* using a xenograft mouse model.

Results: The size of the SP correlated with the degree of HPC features found in human hepatic cancer, and also showed an elevated mRNA expression of biliary/HPC markers and the extracellular matrix marker *LAMC2*, the gene encoding the laminin γ2-chain. Immunopositivity for the γ2-chain of laminin-332 was seen in the extracellular matrix surrounding small HPC-like tumour cells with a low proliferation rate. *In vitro*, laminin-332 increased K19 expression, phosphorylated mTOR and decreased phospho-histone H3 expression, indicating reduced cell mitosis. The effect of laminin-332 was enhanced upon mTORC1 inhibition and diminished when inhibiting mTORC1+C2. Resistance to doxorubicin and sorafenib treatment, and the SP fraction increased in the coated condition. *In vivo*, laminin-332 reduced tumour growth and sustained K19 expression.

Conclusions: In this study we identified a prominent role for laminin-332 as part of the specialised CSC niche in maintaining and supporting cell 'stemness', which leads to chemoresistance and quiescence.

Keywords: Hepatic cancer; Cancer stem cell; Microenvironment; Niche; Phenotype switching; Extracellular matrix; ATP-binding cassette transporters.
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Abbreviations: ABC, ATP-binding cassette; CC, cholangiocellular carcinoma; CSC, cancer stem cell; FFPE, formalin-fixed paraffin-embedded; FSC-A, Forward Scatter Area; HCC, hepatocellular carcinoma; HPC, hepatic progenitor cell; ITGA6, integrin alpha 6; K, keratin; Ln, laminin; MP, main population; pHH3, phosphorylated histone H3; pmTOR, phosphorylated Mammalian Target of Rapamycin; qPCR, quantitative real-time PCR; SP, side population.

Introduction

Resistance to chemo- and radio-therapeutic treatment is a common phenomenon, especially in liver cancer. One possible explanation for this phenomenon is the cancer stem cell (CSC) concept. CSCs are cancer cells that possess characteristics associated with normal stem cells, in particular their ability to give rise to all tumour-derived cell types found in a cancer sample. Such cells are proposed to be persistent in tumours due to their resistance to classical chemotherapeutics and to cause relapse and metastasis. However, although the concept of CSCs is intriguing and a large number of experimental studies support the CSC hypothesis, there are still open questions and room for caution [1]. One debate concerns the origin of CSCs. Does the CSC derive initially from a normal stem cell or from a dedifferentiated cell during tumour progression?

The vast majority of primary liver cancers arise from liver epithelial cells and is classically subdivided into hepatocellular carcinomas (HCCs), cholangiocellular carcinomas (CCs) or combined HCC/CC. An unresolved question regarding liver cancers is which epithelial cells–hepatocytes, cholangiocytes, hepatic progenitor cells (HPCs) or all three– have to be considered as

Cancer

the cells of origin. Mature hepatocytes and cholangiocytes have an enormous self-renewal capacity and longevity and so meet the requisite to be targets for oncogenesis [2]. HPCs are a target for carcinogenesis as well, since most of the hepatic cancers arise in a background of chronic liver disease where there is an extensive activation of the HPCs (seen as ductular reaction) [3]. In the regenerating liver, HPCs reside in a specialised microenvironment (the so-called niche), where interactions with the extracellular matrix help decide their cell fate: a choice between differentiation, proliferation or maintaining 'stemness'. Laminins (Ln) are a large family of extracellular matrix proteins mainly distributed along the basement membrane and are comprised of three different chains (α, β and γ). They have been described to be part of the specialised HPC niche in both human and mouse, where they are thought to play a crucial role in sustaining HPC features [4,5]. In the last decade, many laboratories have underlined the role of the isoform-5, more recently renamed as Ln-332, in promoting tumour progression and dissemination [6]. Our group previously showed that the γ2-chain of Ln-332 in particular was highly elevated in HCCs expressing the biliary/HPC marker keratin (K)19 [7]. Other reports described the γ2-chain as a poor prognostic factor for patients diagnosed with a HCC or an intrahepatic CC [8,9]. Ln-332 can interact with the integrin α3β1 and/or the integrin α6β4 receptor and has been reported to increase resistance to gefitinib, an inhibitor of epidermal growth factor receptor, in HCC cell lines *in vitro* [10]. Whether Ln-332 is part of the CSC niche in hepatic cancer and whether it can influence the behaviour of CSCs remains unclear. In small cell lung cancer Ln-integrin interactions attenuate cell survival and chemoresistance through activation of the mammalian target of rapamycin (mTOR) axis [11]. mTOR has been described as a central regulator of cell growth, metabolism and survival [12]. We therefore hypothesised that mTOR could be potentially involved in regulating the hepatic CSC phenotype.

In this study, we aim to characterise the CSC niche in different histopathological subtypes of hepatic carcinomas, reflecting their possible cell of origin. To isolate putative CSCs, the side population (SP) technique was used (based on the functional ability of the ATP-binding cassette transporters to efflux the fluorescent dye Hoeschst33342), which has proven to be useful in HCC cell lines to isolate cells with a 'stemness' signature that show an increased ability for tumour-initiation and chemoresistance [13–16]. Identification and characterisation of the SP was performed using qPCR and immunohistochemistry. In addition, the influence of Ln-332 on 'stemness' traits, such as chemoresistance and quiescence, and potential downstream targets, such as HPC markers, was investigated *in vitro* and *in vivo*.

Material and methods

Human tissue samples

Eighteen patients diagnosed with an hepatic carcinoma between 2008 and 2012 at the University Hospitals in Leuven (Belgium) were included in this study. Immediately after surgical removal of the tumour (resection samples), part of the tissue was fixed in 6% formalin and embedded in paraffin, while the other part was stored in Recovery™ Cell Culture Freezing Medium (Invitrogen, Carlsbad, CA, USA) in liquid nitrogen. The histopathological diagnosis of HCCs or combined HCC/CCs was performed according to the World Health Organization criteria. Fibrolamellar HCCs were not included. HCCs were further subdivided into K19-negative and K19-positive HCCs as previously described [17]. The study was approved by the ethical committee of the University Hospitals Leuven, Belgium.

Side population analysis

SP analysis was performed as previously published [7]. A detailed description can be found in the Supplementary material. In short, frozen samples were thawed and dissociated using LiberaseBlendzyme 3 (0.8 Wunsch unit/ml, 1.5 h, 37 °C) (Roche, Basel, Switzerland). Single cell suspension was incubated with 5 μg/ml Hoechst33342 (Sigma-Aldrich, St Louis MO, USA) for 90 min at 37 °C. The ABC-transporter inhibitor Verapamil (100 μM; Sigma-Aldrich) was used to assess the SP phenotype from the rest of the main population (MP); propidium iodide (2 μg/ml; Sigma-Aldrich) to exclude dead cells. Samples were analysed using a FACSAriaII (BD Biosciences) under UV excitation (450/50, 675/20 filter).

The size of the SP was calculated as percentage of the total viable population. Median cell size of SP and MP was analysed based on the Forward Scatter Area (FSC-A). Sorted cells were fixed using BD CytoRich™ System (BD Biosciences) and processed into cytospins for Papanicolaou stainings or immunocytochemistry. Part of the sorted cells out of twelve patient samples (four per histological group) was processed for qPCR analysis. The description of the qPCR method and the list of primers can be found in Supplementary material and Supplementary Table 1.

Immunohistochemistry/immunocytochemistry

Five-micrometre-thick human formalin-fixed paraffin-embedded (FFPE) tissue slides and cytospins were stained with the Leica BOND-MAX™ system (Leica Microsystems GmbH, Wetzlar, Germany), using the Novocastra Bond Polymer Detection Kit (Leica). Visualisation was done using DAB-Chromogen, followed by a haematoxylin counterstaining. Cultured primary cells and cell lines were stained using BrightVision Poly-HRP (Immunologic, Duiven, The Netherlands) and visualised with 3-amino-9-ethylcarbazole. Primary antibodies were directed against ABCB1/MDR1 (1/10 for FFPE slides, 1/100 for cytospins; Monosan, Uden, The Netherlands), ABCG2/BCRP (1/5 for FFPE slides, 1/100 for cytospins; Santa Cruz Biotechnology, Dallas, Texas, USA), K19 (1/25; Dako, Glostrup, Denmark), γ2-chain of Ln-332 (1/500; Sigma-Aldrich), Ln-332 (an antibody recognizing the complete protein, 1/100; Abcam, Cambridge, UK), phospho-mTOR (pmTOR, Ser2481; 1/50; Cell Signaling, Danvers, MA, USA) and phospho-Histone H3 (pHH3; 1/100; Millipore, Billerica, MA, USA).

Immunofluorescence

Human FFPE samples were deparaffinised, processed for epitope retrieval and stained for γ2-chain of Ln-332+K19 or γ2-chain of Ln-332+Ki67 (ready-to-use, Dako) for 45 min. Cell culture samples were fixed in 4% formaldehyde solution and stained for K19+pHH3 for 45 min. Afterwards samples were incubated for 30 min with Alexa Fluor® 568 Goat Anti-Rabbit and Alexa Fluor® 488 Goat Anti-Mouse (1/100, Invitrogen). Samples were counterstained with DAPI (Invitrogen). Fluorescent signals were observed using Zeiss Axioplan (Carl Zeiss, Oberkochen, Germany).

In vitro growth potential of primary samples

To assess the *in vitro* growth potential of primary human hepatic cancer, dissociated primary tissue samples (n = 2 per histological group) were grown in Advanced Dulbecco's Modified Eagle's Medium (Invitrogen), supplemented with 10% heat-inactivated Fetal Bovine Serum and L-Glutamine (Invitrogen); at 37 °C in a humidified chamber supplemented with 5% CO_2. Cells that showed growth potential were passed on and characterised by immunocytochemistry at twelve weeks.

In vitro Ln-332 coating experiments

Characterisation cell lines

Human HCC cell lines PLC/PRF/5, HepG2 (ECACC, Salisbury, UK) and Hep3B2 (ATCC-LGC Standards, Molsheim Cedex, France) were grown in similar conditions as the dissociated human samples. Cells were processed into FFPE tissue blocks using the Cellient Automated Cell Block System (Hologic, Bedford, MA, USA) and stained for K19, γ2-chain of Ln-332 and integrin α6 receptor chain (ITGA6; 1/50; Sigma-Aldrich) expression using immunohistochemistry as described above.

mRNA expression

Six-well cell culture plates were coated with Ln-332 (Biolamina, Stockholm, Sweden) at a concentration of 2 μg/cm^2 (1.5 h, 37 °C) and seeded with 6×10^5 HCC cells (n = 3 per cell line). After 72 h the cells were harvested for mRNA extraction and processed for qPCR analysis (Supplementary material).