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# Tetraspanin-enriched microdomains and hepatocellular carcinoma progression

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#### ABSTRACT

As in many tumors, heterogeneity within the cell population is one of the main features of hepatocellular carcinoma (HCC). Heterogeneity results from the ability of tumor to produce multiple subpopulations of cells with diverse genetic, biochemical and immunological characteristics. Little is known about how heterogeneity emerges and how it is maintained. Fluctuations in single cells can be masked or completely misrepresented when cell populations are analyzed. It has become exceedingly apparent that the utility of measurement based on the analysis of bulk specimens is limited by intra-tumor genetic and epigenetic heterogeneity, as characteristics of the most abundant cell type might not necessarily predict the properties of cell populations. Yet, such non-uniformities often unveil molecular patterns that can represent mechanisms of tumor progression. Interestingly, variability among single cells in a population may arise from different responses to intrinsic and extrinsic perturbations mainly mediated by the plasma membrane. The association of certain proteins, including tetraspanins, and lipids in specific location on the plasma membrane constitutes specialized structure called tetraspanin-enriched microdomains (TEMs). TEMs organization in cancer may reveal essential clues for understanding pathogenic mechanisms underlying cancer progression. Along these lines, TEMs and HCC progression represent a valuable paradigm for gaining a deeper understanding of such mechanisms.

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#### Introduction

Hepatocellular carcinoma (HCC) is responsible for 85% of primary liver malignancy with high mortality rate worldwide [1]. Several factors including geographical region, gender, racial and ethnical variation can affect the incidence of HCC. Briefly, sub-Sahara Africa and eastern Asia are documented as the regions with high incidence of HCC. In contrast, America, north Europe and Oceania are considered as low-rate [2,3].

Major risk factors for hepatocellular carcinoma include infection with HBV or HCV, alcoholic liver disease, and probably

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http://dx.doi.org/10.1016/j.canlet.2014.05.016 0304-3835/© 2014 Elsevier Ireland Ltd. All rights reserved. nonalcoholic fatty liver disease. Less common causes include hereditary hemochromatosis, alpha1-antitrypsin deficiency, autoimmune hepatitis, some porphyrias, and Wilson's disease. The distribution of these risk factors among patients with hepatocellular carcinoma is highly variable, depending on geographic region and race or ethnic group [4–9]. The most part of these risk factors lead to the formation and progression of cirrhosis, which is present in 80–90% of patients with hepatocellular carcinoma. Cirrhosis from any cause predisposes to HCC and hence can be considered a premalignant condition. Indeed, the majority of patients worldwide with HCC have underlying cirrhosis, it is uncommon to find HCC in the absence of cirrhosis [10]. Liver cirrhosis is characterized by destruction of the hepatic lobular architecture and its replacement by nodules containing proliferative hepatocytes, in the presence of chronic inflammation and fibrosis [11].

Another outstanding and important question in HCC pathogenesis involves the role of chromosomal abnormalities that are present almost universally in HCC and frequently detected in hyperplastic and/or dysplastic nodules of cirrhotic livers [12].

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; TEMs, tetraspanin-enriched microdomains; PI4P, phosphatidyl-inositol 4-phosphate; PI(4,5)P2, phosphatidylinositol 4,5 bisphosphate; GFP, Green fluorescent protein; TGF $\beta$ , transforming growth factor  $\beta$ .

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Some chromosomal defects found in the dysplastic nodules in cirrhotic liver are also present in HCC, which suggests that chromosomal defects occur at early stages of tumor development [13–15]. However, the mechanisms by which chromosomal abnormalities occur are still unknown. Neoplastic evolution of HCC proceeds through a multi-step histological process that is less defined than that of other cancer types. Hyperplastic nodules of regenerating hepatocytes have normal cytological features, and represent a potential first step towards HCC. These lesions can progress to pre-malignant dysplastic nodules, which have abnormal cytological features and these lesions are associated with the increased thickening of the trabeculae, which indicates abnormal liver architecture. The dysplastic nodules can evolve to frank HCC, which is endowed with the capacity to invade the surrounding fibrous stroma and vessels and eventually to metastasize [16,17].

# Tumor formation can be switched on from cell membrane domains

It has long been recognized that differences between cancer cells can arise through variation in the extracellular environment or from genomic alteration. It has only recently become clear that plasma membrane protein fluctuations can also have profound effects on the phenotype [18,19]. These fluctuations cause genetically identical cells to vary significantly in their responsiveness to stimuli, for example, those from the fibrotic microenvironment [20–23].

The spatial organization of plasma membrane components in discrete microdomains is thought to be a key factor in the generation of distinct signal inputs or outputs [24]. Dynamic microdomains have important implications for understanding how signaling complexes are assembled and disassembled in response to stimuli; some components of these signaling complexes might reside permanently in these microdomains but others could have extremely transient interactions [25].

This article focuses on the current knowledge regarding the mechanism of neoplastic progression of HCC that can be attributed to the reorganization of the plasma membrane in a functional unit called tetraspanin-enriched microdomains (TEMs). TEMs promote the instability of pre-malignant hepatocytes enhancing their neoplastic progression.

#### Tetraspanins

Tetraspanins are a large family of proteins (33 in mammals), these proteins have two small and large extracellular loops, four putative hydrophobic membrane-spanning domains and short amino- and carboxy-terminal cytoplasmic domains. Hypervariable region in large extracellular domain of each tetraspanin can distinct it from other members in this family [26]. Tetraspanins (e.g. CD9, CD63, CD81, CD82, CD151) are implicated in numerous cellular pathways including cell proliferation, cell differentiation, cell adhesion, cell fusion, proteins trafficking, migration, viral infections and triggering of immune responses [26,27]. Extracellular domains make tetraspanins being capable to associate with wide range of proteins by homo/heterotypic interactions [28]. It seems these combinations depend on the cell type and differentiation. Treatment with cell membrane detergent following immunoprecipitation and mass spectrometry analysis confirmed that tetraspanins are associated with several kinds of partners [29,30]. The most important tetraspanin partners are integrins, particularly  $\alpha 3\beta 1$ ,  $\alpha 4\beta 1$ ,  $\alpha 6\beta 1$  and  $\alpha 6\beta 4$  isoforms, intracellular associated heterotrimeric G proteins, proteases, CD44, EpCAM, immunoglobulin superfamily members and cytosolic signal transduction molecules as well. It is also necessary to mention that different types of

tetraspanins in association with various partners produce the variability and specificity among cell types [30] Tetraspanins form complexes termed tetraspanin-enriched membrane microdomains (TEMs) by interacting with other tetraspanins and with a variety of transmembrane and cytosolic proteins that are required for their function [31–33]. Changing the composition and localization of TEMs in response to external or internal stimuli reveal that TEMs are dynamic and flexible structures [34]. TEMs have been characterized by immunoelectron microscopy as units, with an area of ~200 nm<sup>2</sup> although size heterogeneity can be observed in this territory between different cell types [34]. Although the structure of TEMs is similar to that of the lipid rafts, TEMs are evidently distinct from lipid rafts based on the absence of lipid raft-specific glycoproteins such as glycosylphosphatidylinositol-anchored proteins. The interactions of tetraspanin can be stabilized by several modifications including palmitoylation and glycosylation. CD9, CD53, CD81, CD82, CD63, CD151 and CD37 are covalently palmitoylated in Golgi apparatus. Palmitoylation plays an essential role in tetraspanin-protein or tetraspanin-lipid interactions and protect tetraspanins from protease attack [28,35]. Since tetraspanins, as well as most of their partners, are highly palmitoylated membrane proteins, the interactions between proteins and lipids are an important component in the function of TEMs. The characterization of the protein compositions of TEMs has revealed different categories of proteins in these microdomains and the recent advent of the proteomic analysis has allowed the characterization of TEMs in different cancers [30,36]. However, much has to be done to functionally characterize the resulting signals from protein-protein and protein-lipid interactions in TEMs. One of the limitations of the experimental approach is due to the fact that anti-tetraspanin antibodies interfere not only with the targeted tetraspanin but also with the entire microdomain. RNA interference of single or multiple genes involved in TEMs, as well as dynamic studies of microscopy such as fluorescence recovery after photobleaching (FRAP) or Förster resonance energy transfer (FRET), may help to overcome this limitation.

#### Tetraspanins in HCC

HCC is a disease characterized by heterogeneous morphological patterns. Since HCC arises in a cirrhotic liver, the most relevant aspect of hepatocarcinogenesis is regeneration of hepatocytes. HCC develops more frequently in cirrhosis of the macronodular type than in micronodular cirrhosis, and even more when macroregenerative nodules (MRNs) are present. MRNs, particularly atypical MRNs, develop as a result of extensive regeneration in a cirrhotic liver. The development of HCC in a cirrhotic liver underlines the importance of these early events indicative of neoplastic transformation. In this regard, the tetraspanins CD81 and CD151 are by far the most comprehensively studied (Fig. 1).

#### **Tetraspanin CD81**

Tetraspanin CD81 was identified originally as the target of an anti-proliferative antibody (TAPA-1) that inhibited *in vitro* cellular proliferation [37]. Data obtained from monoclonal antibodies have shown that this 26 kDa membrane protein is involved in a broad range of cellular functions. These antibodies evoke their effects by mimicking a natural ligand or by altering the interactions between CD81 and its associated proteins. Although CD81 is widely expressed, its levels of expression within a single tissue vary in response to cellular activation [38]. The ability to associate with itself to form homodimers besides interaction with various other receptors is an important feature of CD81 function [39]. Up-regulation of CD81 in pre-malignant hepatocytes can contribute to

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Fig. 1. Expression and distribution of tetraspanin CD81 and CD151 in normal and hyperplastic adenomatous nodules of human cirrhotic liver. (A) Strong CD81 immunoreactivity is found in sinusoidal cells of normal liver. Hepatocytes display both membrane and cytoplasmic staining although the latter is fainter. (B) In cirrhotic liver, hepatocytes display a strong CD81 staining along the plasma membranes (arrowheads). (C) In normal liver, CD151 is expressed in sinusoidal cells, vessel walls and bile duct epithelia, whereas hepatocytes are negative. (D) In cirrhotic liver, CD151 is expressed by nonparenchymal cells and hepatocytes of regenerating nodules (arrowheads).

reorganize the plasma membrane in domains where signaling proteins can be recruited. The regulation of CD81-mediated proliferation is positively associated with activation of the extracellular signal-regulated kinase 1/2 (ERK1/2)/MAPK pathway [40]. The overexpression of CD81 activates ERK1/2 and promotes cell proliferation (Fig. 2). Importantly, CD81 induces reorganization of the plasma membrane amplifying the instability of premalignant hepatocytes and enhancing their neoplastic progression [40]. Therefore, phenotypic heterogeneity could be influenced primarily by a fluctuation of a single protein and associated factors organized in discrete plasma membrane domains. These membrane microdomains represent versatile devices for compartmentalizing different signaling functions. In the non-activated state they float freely carrying a few passenger proteins, however, upon activation, they can coalesce to form larger platforms where proteins create a network for cellular functions such as signaling, processing and transport [41]. When the hepatocytes progress in their transformation, the tendency of CD81 expression is to be lost as reported in two clinical studies showing a decreased or absent expression of CD81, particularly in metastatic lesions [42,43]. Re-expression of CD81 in HCC cells rescues the ability of growing and producing the primary tumor when the cells are injected into the liver of nude rats even though a defective ability to generate satellite nodules in distant parts of the liver (i.e. intrahepatic metastases) is observed [44]. These findings strengthen the vision that CD81 can act either as facilitator of cell proliferation or negative regulator of movement when expressed by cells. This is supported by the view that cell growth and the ability to metastasize are two conditions of malignancy not necessarily overlapping [23,45].

#### **CD151 and additional tetraspanins**

Tetraspanin CD151, whose gene is located on chromosome 11 in human, is a 230 amino acid protein that undergoes N-glycosylation and cysteine palmitoylation [46]. CD151 is noticeably expressed in endothelial, epithelial, Schwann, dendritic and muscle cells (skeletal, smooth and cardiac). Tetraspanin CD151 is also involved in cancer progression and the overexpressed gene has been detected in many types of tumors. For example, in breast, pancreatic, colorectal and non-small-cell lung cancer, elevated expression levels of CD151 are associated with a poor prognosis [46]. The initial evidence that CD151 promotes metastasis came from a study showing that a monoclonal antibody with unknown specificity inhibited metastasis by a human epidermoid carcinoma line in vivo. This antibody inhibited cell migration without affecting adhesion or proliferation [47,48]. Overexpression of CD151 has also been associated with poor prognosis in HCC and potentiates the metastatic behaviour of cancerous cells [49]. It has been proposed that CD151 induces HCC progression via MMP9, PI3k, Akt, GSK3β and the Snail signaling pathways [50]. Additionally, some studies have indicated that CD151 overexpression promotes the invasive/metastatic phenotype of cancer cells by mediating integrin signals, while others have argued that an increased expression of CD151 contributes to activate ERK1/2 [36]. Indeed, elevated expression levels of CD151 and  $\alpha 6$  integrin largely contribute to the motile phenotype of HCC [51]. In spite of CD81, the contribution of CD151 to HCC metastasis/invasion provides an example of the facilitator role of tetraspanins. Tetraspanin TSPAN8 (previously known as CO-029, TM4SF3) and CD82/KAI1, a metastasis suppressor gene, have also been associated with HCC progression. Overexpression of TSPAN8 is described in HCC that are poorly differentiated and prone to intrahepatic spreading [52]. Conversely, downregulation of tetraspanin CD82/KAI1 was observed at the levels of both mRNA and protein in HCC [53]. This is particularly pronounced in poorly differentiated HCC. Notably, the expression levels of CD82/KAI1 are inversely correlated with intrahepatic metastases [54-57].

#### Phosphoinositide 4-kinases and HCC

Type II phosphoinositide 4-kinase (PI4KII) is one of the signaling proteins associated with different tetraspanins including CD81 and CD151 to create functional complexes [58–60]. PI4KII catalyzes the phosphorylation of phosphatidylinositol on the D-4 position of the inositol ring [61–64]. The product of this reaction, phosphatidylinositol 4-phosphate (PI4P) is a precursor in the synthesis of PI3,4P2, PI4,5P2, and PI3,4,5P3, but PI4P itself participates in signal transduction, membrane trafficking, and cytoskeletal reorganization (Fig. 3) [65–69]. A significantly larger fraction of PI4KIIβ in

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**Fig. 2.** CD81 and CD151 signaling in HCC cells. (A) CD81 associates with phosphatidylinositol 4-kinase type II (Pl4KII), which locally produces phosphoinositides such as phosphatidylinositol-4,5-bisphosphate, Pl(4,5)P2. This causes the recruitment and activation of adapter protein Shc. Subsequent Ras-mediated activation of extracellular signal-regulated kinase (ERK/MAPK) pathway leads to proliferation. (B) CD151-associated  $\alpha$ 6 $\beta$ 1 integrin and Pl4KII co-operate to activate the downstream signal of phosphatidylinositol 3-kinase (PI3K)-Akt in a cell adhesion-dependent manner. CD151 (together with associated integrins and EWI-2 proteins) also activates Rac leading to regulation of the actin cytoskeleton, cell spreading and motility.



Fig. 3. Co-localization of CD81-GFP with endogenous PI4P in HCC cells. (A) The cellular distribution and pattern of expression of CD81 fused to GFP in HepG2 cells. (B) Endogenous expression of PI4P in the same cell of panel A is shown. (C) A robust co-localization of CD81 with PI4P is shown here by the merged image.

comparison with PI4KIIα is cytosolic in spite of the high degree of conservation within their cysteine-rich domains [70–74]. HCC cells express two isoforms of PI4KII termed PI4KIIα and PI4KIIβ. PI4KIIβ is strongly expressed in the liver and an anti-metastatic role for this isoform has emerged in HCC [44]. Instead, PI4KIIα expression is upregulated in many human cancers, particularly in malignant melanoma, fibrosarcoma, breast cancer, bladder transitional cell carcinoma and thyroid papillary carcinoma. PI4KIIα is a novel regulator of tumor growth through its action on angiogenesis

and HIF-1 $\alpha$  regulation [75–77]. Overexpression of PI4KII in liver cells leads to enhanced trafficking of the tetraspanin CD81 away from the plasma membrane into intracellular vesicles, which in turn bind to and sequester the cytoskeletal protein  $\alpha$ -actinins. This results in the remodeling of actin cytoskeleton and inhibition of cell migration [44]. Conversely, reduced PI4KII expression leads to an increase in cell motility, which is a crucial step in the development of invasive and metastatic HCC. However, little is known about the role of PI4KII in the regulation of intracellular vesicles

trafficking from the plasma membrane and further work is required to unravel the mechanism by which PI4KII may potentially maintain the levels of tetraspanins on the cell surface [78].

#### Integrins and tetraspanins interactions within HCC

The pattern of integrins expressed by human hepatocytes is strikingly different from most other epithelial cells. Normal adult hepatocytes barely express three integrins such as  $\alpha 1\beta 1$ , a collagen and laminin receptor;  $\alpha 5\beta 1$ , a fibronectin receptor; and  $\alpha 9\beta 1$ , a tenascin receptor. In contrast, other integrin receptors such as  $\alpha 2\beta 1$ ,  $\alpha 3\beta 1$ ,  $\alpha 6\beta 1$ , and  $\alpha 6\beta 4$  are undetectable in normal hepatocytes. One of the most frequent alterations during liver carcinogenesis is *de novo* expression of the integrin,  $\alpha 6\beta 1$  [79]. HCC patients characterized by the presence of multiple tumors and vascular invasion as well as by the absence of encapsulation exhibit increased expression levels of  $\alpha 6\beta 1$ . In fact, the induction of  $\alpha 6\beta 1$  is an early event in hepatocellular carcinogenesis, and it is reasonable to posit that  $\alpha 6\beta 1$  contributes to hepatocarcinogenesis and tumor progression [80–82].  $\alpha 6\beta 1$  forms a complex with endogenous CD151 in HCC cells, but the complex is less stable in comparison with integrin  $\alpha 3\beta 1$ . CD151 mostly functions by modulating several activities. Particularly, CD151 could restrict the mode of  $\alpha 6$  integrin diffusion in the plasma membrane, thereby making the integrins more available for adhesion, migration, and other functions. The effects of CD151 on  $\alpha 6$  integrin distribution may contribute to modulate activation of focal adhesion kinase (FAK) and PI3 kinase (PI3K) signaling pathways important for HCC function [83]. Both FAK and PI3K are of interest not only because they are regulated by integrin-mediated attachment to extracellular matrix components, but also because they are activated by growth factors, thus controlling important functions of tumor cells such as growth and migration (Fig. 2). Another possibility suggests that overexpression of  $\alpha 6\beta 1$  could offer a ligand-independent growth advantage by modulating the cellular architecture or the intracellular signaling pathway required for cell growth. Although  $\alpha 3\beta 1$ integrin associates stably with CD151 in HCC cells [40,51], more controversial appears its role in hepatocarcinogenesis. In fact, a previous study indicated that TGF-B1 was able to induce a significant increase in the expression level of  $\alpha 3\beta 1$ , which in turn cooperated with TGF- $\beta$ 1 to induce a motile phenotype in HCC cells [84]. In a recent study, performed in HCC cells and tissue appraised from patients with varying concentrations of serum TGF-B1, the authors did not detect any distinct up-regulation of  $\alpha 3\beta 1$  in HCC cells after 24 or 48 h of TGF-B1 stimulation and also observed no up-regulation in human HCC specimens showing a high concentration of serum TGF- $\beta$ 1. In this study they have found that the high expression of CD151- $\alpha$ 6 $\beta$ 1 complex promotes invasiveness of HCC cells [51].

#### **Conclusions and perspectives**

A seminal feature of HCC is the ability to produce multiple subpopulations of cells with diverse genetic, biochemical and immunological characteristics. How this heterogeneity emerges and how it is maintained is still unclear. Fluctuations in single tumor cells can be hidden or completely misinterpreted when cell populations are analyzed. Therefore, intra-tumor heterogeneity may foster tumor evolution and adaptation and hinder personalized-medicine strategies that depend on results from imaging procedures or single tumor-biopsy samples. Along these lines, it has become exceedingly apparent that the utility of measurements based on the analysis of bulk tumors is limited by intra-tumor genetic and epigenetic heterogeneity, as characteristics of the most abundant cell type might not necessarily predict the properties of the whole cell populations. This aspect is supported by a recent report describing the presence of distinct diagnostic signatures derived from different biopsies of the same tumor [85]. The dominance of gene-centric views has been challenged with the rapid development of research establishing that because tumors contain phenotypically distinct populations of both tumor and stromal cells that interact in a dynamic and reciprocal manner, these interactions are likely to fuel the emergence of different proteome profiling, including those from microdomains such as TEMs. Nevertheless, many unanswered questions remain regarding TEMs organization in HCC cells. Importantly, several studies deserve to further characterize the basic unit of these structures and whether random expression levels of tetraspanins and their engaging with other tetraspanins or plasma membrane molecular partners contribute to fuel phenotype heterogeneity. Assessment of the dynamics of TEMs composition and stoichiometry in living HCC cells is needed to ascertain whether specific physiologic stimuli can alter the balance between confined and diffusing tetraspanins and their partners.

#### **Conflicts of Interest**

The authors declare no conflict of interest related to this work.

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