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EDITORIAL

Tumor reactive stroma in cholangiocarcinoma: The fuel behind cancer aggressiveness

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Abstract

Cholangiocarcinoma (CCA) is a highly aggressive epithelial malignancy still carrying a dismal prognosis, owing to early lymph node metastatic dissemination and striking resistance to conventional chemotherapy. Although mechanisms underpinning CCA progression are still a conundrum, it is now increasingly recognized that the desmoplastic microenvironment developing in conjunction with biliary carcinogenesis, recently renamed tumor reactive stroma (TRS), behaves as a paramount tumor-promoting driver. Indeed, once being recruited, activated and dangerously co-opted by neoplastic cells, the cellular components of the TRS (myofibroblasts, macrophages, endothelial cells and mesenchymal stem cells) continuously rekindle malignancy by secreting a huge variety of soluble factors (cyto/chemokines, growth factors, morphogens and proteinases). Furthermore, these factors are long-term stored within an abnormally remodeled extracellular matrix (ECM), which in turn can deleteriously mold cancer cell behavior. In this review, we will highlight evidence for the active role played by reactive stromal cells (as well as by the TRS-associated ECM) in CCA progression, including an overview of the most relevant TRS-derived signals possibly fueling CCA cell aggressiveness. Hopefully, a deeper knowledge of the paracrine communications reciprocally exchanged between cancer and stromal cells will steer the development of innovative, combinatorial therapies, which can finally hinder the progression of CCA, as well as of other cancer types with abundant TRS, such as pancreatic and breast carcinomas.

Key words: Tumor microenvironment; Desmoplasia; Cancer-associated fibroblast; Inflammation; Tumor-

associated macrophage; Lymphatic endothelial cell; Mesenchymal stem cell; Extracellular matrix

Core tip: Cholangiocarcinoma (CCA) is a typically worrisome malignancy, whose incidence has been steadily increasing. In CCA, as cancerous lesions are emerging, the surrounding stroma gradually undergoes a pathological remodeling, eventually becoming a paramount determinant of tumor growth and dissemination. Indeed, the different cell types populating the tumor microenvironment, also referred to as tumor reactive stroma, enable CCA cells to develop an aggressive phenotype, due to the secretion of a multitude of soluble factors. Therefore, functional insights into the harmful relationship between cancer and reactive stromal cells are of utmost importance, in order to unveil novel molecular targets amenable of therapeutic intervention.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a deadly malignancy originating from the epithelial cells lining the biliary tree, including the extrahepatic and intrahepatic portions either. Among primary liver cancers, it represents the second most common type after hepatocellular carcinoma, and its incidence and mortality rate have been steadily increasing for two decades. CCA still carries a very dismal prognosis (less than 5% of patients survives up to 5 years from diagnosis), due to a striking resistance to chemotherapy and a propensity for early intrahepatic or lymph node metastatization, making radical surgery suitable to less than one-third of patients. Furthermore, results from both surgical resection and liver transplant are limited by the high recurrence rates. To date, the pathophysiological mechanisms underlying CCA progression remain largely unknown, and consequently, the development of new effective treatments is a very awkward task[1-3].

Whilst the majority of CCAs are thought to be sporadic, several geographically heterogeneous risk factors have been identified, mostly related to an inflammatory bile duct injury. They include hepatobiliary fluke infestations (*e.g.*, *Opisthorchis viverrini*, *Clonorchis sinensis*), hepatolithiasis, congenital abnormalities of the bile ducts (*e.g.*, Caroli's disease, choledochal cysts), primary sclerosing cholangitis (PSC), viral hepatitis B and C, and exposure to toxic compounds (*e.g.*, thorium dioxide, naphthenic acids). Furthermore, CCA development has been associated with genetic and epigenetic alterations in well-known proto-oncogenes (*e.g.*, KRAS GTPase, isocitrate dehydrogenases 1 and 2) and tumor suppressor genes (*e.g.*, tumor protein p53, cyclin dependent kinase inhibitor 2A). More recent evidence indicates that cholangiocarcinogenesis is driven by chronic deregulation of various signaling pathways deeply involved in cholangiocyte biology, leading to uncontrolled proliferation, evasion of apoptosis, and loss of genome integrity. For instance, increased activity of cytokines and growth factors, such as interleukin (IL)-6, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, and platelet-derived growth factor (PDGF), is a common event in CCA, due to either enhanced production or increased cell responsiveness, and likely contributes to the malignant transformation of cholangiocytes[4,5].

Evidence is mounting that the aggressive behavior of CCA is greatly influenced by paracrine cues released from the inflammatory and mesenchymal cell types populating the tumor microenvironment[6]. Indeed, as cancerous lesions are emerging, the surrounding stroma gradually develops profound and complex changes, undergoing a switch from key player in tissue homeostasis to pathological niche supporting tumor growth and dissemination[7,8]. Therefore, an in-depth insight into the actual contribution of the stromal microenvironment to CCA progression is imperative, with the ultimate goal to pave the way for innovative combinatorial treatments targeting both stromal and cancer cells. Hopefully, this may lead to a more effective management of this devastating malignancy.

THE TUMOR REACTIVE STROMA: A SPECIALIZED COMPARTMENT ORCHESTRATING TUMOR PROGRESSION

Neoplastic bile ducts are tightly enveloped by a striking and diffuse desmoplastic, hypovascularized stroma that is usually referred to as tumor reactive stroma (TRS). This histopathological lesion is made up of a variety of stromal cells embedded in a dense collagenous extracellular matrix (ECM), encompassing myofibroblasts, inflammatory cells, endothelial cells and mesenchymal stem cells (MSCs)[6,9]. Once recruited, activated and co-opted by malignant cholangiocytes, the cellular components of the TRS can diffusely infiltrate the growing tumor and eventually support its progression by secreting a wide range of soluble factors. Indeed, these factors can directly trigger the emergence of malignant phenotypes and/or enhance the migration and aberrant activation of other stromal cells[10,11]. In addition to the plethora of cytokines, chemokines, growth factors and proteinases perpetually released by stromal cells, cell-extrinsic factors, such as hypoxia and abnormally remodeled ECM components, also provide the TRS with invasiveness-promoting properties[12]. Interestingly, it has been proposed that the pro-tumorigenic functions of the TRS could partially rely on the induction of epigenetic, and therefore heritable, changes in cancer cells[13,14]. In fact, gastric, ovarian

and breast cancer cell lines co-cultured with fibroblasts isolated from the tumor milieu, were found to undergo gene-specific DNA hypermethylation events, generally coupled with increased migratory abilities[15-17].

Development of CCA is often associated with inflammation-related alterations, as observed in those cases arising in specific disease settings, such as PSC and congenital hepatic fibrosis. Moreover, recent observations indicate that in the last few years CCA is more often detected on a background of chronic inflammation associated to cirrhosis, regardless of its etiology[18]. As a general concept, the TRS may be regarded as an aberrant, over-healing reparative complex ("wound that does not heal" according to the old Dvorak's paradigm), wherein various types of inflammatory and stromal cells are somehow hijacked by the malignant compartment, whose immunomodulatory functions and metabolic needs eventually prevail over the physiological homeostasis of the normal tissue[6,19]. This behavior also reflects the inherent plasticity of the naïve stroma, which enables it to comply quickly the evolution of the adjacent transformed epithelium, in contrast with the self-limited response occurring in the wound repair[8].

FUNCTIONAL INSIGHTS INTO THE INFLUENCE OF THE TRS ON CCA PROGRESSION

In CCA, increasing evidence has highlighted the prognostic relevance of the molecular alterations related to the generation of the TRS. Indeed, gene expression profiling of microdissected stroma from both tumoral and peritumoral areas of resected human CCA revealed a TRS-specific gene signature, encompassing 1073 genes involved in cell metabolism, cell cycle, cell signaling pathways and ECM biology. In particular, the overexpression of representative genes (namely KIAA0101, TGF-β2, laminin subunit γ2 and osteopontin) was found to significantly correlate, at different levels, with clinicopathological features of CCA patients[20]. Andersen *et al*[21] undertook a similar approach in order to compare the epithelial and stromal transcriptomic profiles in CCA tissues. They identified a stromal gene signature associated with poor clinical outcome. Interestingly, the 1442 differentially expressed genes revealed a stromal dysregulation of both chemokine (CXCR4, CCR7, CCL2, CCL5, CCL19, CCL21) and IL (IL3RA, IL7R, IL10RA, IL18RAP, IL6, IL16, IL33) receptors and ligands. In the next paragraphs, we will discuss in detail how the main components of the TRS are supposed to promote CCA progression.

CANCER-ASSOCIATED FIBROBLASTS

The TRS is predominantly composed by a subpopulation of activated fibroblasts, called cancer-associated fibroblasts (CAFs). In stark contrast with the small number of lowly proliferating fibroblasts populating the naïve stroma, CAFs are present in an exaggerated high number, and exhibit a permanent state of activation, resulting in a broad release of both biochemical signals and ECM components, in particular fibronectin and collagen type I [6,10,12,22]. The main phenotypic markers of CAFs are alpha-smooth muscle actin (α-SMA), vimentin, S100A4 (also called fibroblast specific protein-1) and fibroblast activation protein alpha (FAP)[23]. CAFs are recognized as a heterogeneous population, likely reflecting the variety of cell precursors that they are supposed to originate from, including hepatic stellate cells (HSCs), portal fibroblasts and, to a lesser extent, bone marrow-derived MSCs[10]. The hypothesis that cancer cells themselves may represent an alternative source of CAFs by undergoing epithelial-to-mesenchymal transition (EMT) has gradually waned[14,24]. Nevertheless, neoplastic cells act in concert with inflammatory cells to secrete a vast array of growth factors, cytokines and chemokines ultimately responsible for the recruitment of fibroblasts to the TRS, as well as for their chronic activation state. In this regard, we recently showed that PDGF-DD is overexpressed by CCA cells under the effect of hypoxia, and acts as a key mediator of fibroblast recruitment nearby the tumoral mass. Indeed, PDGF-DD strongly induces fibroblast migration by binding its cognate receptor PDGFRβ (which is extensively expressed by CAFs), thereby activating the Rho GTPases, Rac1 (lamellipodia inducer) and Cdc42 (filopodia inducer), as well as the JNK pathway[24]. Furthermore, conditioned medium from CCA cells sustained the activation of both HSCs and liver myofibroblasts, which actually acquired a more elongated shape and up-regulated the expression of α-SMA, *in vitro*[25,26]. Among the multitude of soluble factors triggering the persistent activation of CAFs, TGF-β, fibroblast growth factor and, again, several PDGF family members, undoubtedly play a pivotal role[6,12].

Evidence for the pro-neoplastic effects exerted by CAFs

In CCA samples, the expression of α-SMA is barely detectable in fibroblasts populating the peritumoral areas, whereas most, if not all, fibroblasts embedded in the tumor stroma are α-SMA$^+$[27]. Consistently, in a hamster model of CCA, the density of α-SMA$^+$ fibroblasts within liver tissue was clearly shown to increase during cholangiocarcinogenesis[28]. There is a strong evidence that an increased density of CAFs within the TRS correlates with increased tumor growth and poor outcome in CCA patients. Indeed, high stromal expression of α-SMA was reported as an independent prognostic factor for overall and disease-free survival[27,29]. In line with these findings, both incubation of CCA cells with CAF conditioned medium and co-culture of CCA cells with CAFs resulted in increased cancer cell proliferation and migration, *in vitro*[27,30]. On the contrary, slighter pro-tumorigenic effects were elicited by liver fibroblasts isolated from the peritumoral areas, arguing for a deep biological gap between CAFs and their naïve counterpart (see below)[27]. Of further interest, Campbell *et al*[31] developed a three-dimensional organotypic culture model of CCA by embedding to-

gether within a collagen gel matrix clonal strains of CCA cells and CAFs, both derived from a syngeneic rat model of CCA generated by orthotopic inoculation of spontaneously transformed cholangiocytes. Clearly, these culture conditions more accurately reproduce the complex biological interactions occurring *in vivo* within the desmoplastic tumor. Interestingly, the authors observed that CCA cells co-cultured with CAFs exhibited markedly distinct growth features as compared to CCA cells cultured alone. In particular, the number of duct-like structures formed in the gel matrix by CCA cells dramatically increased in direct proportion to initial CAFs plating density. The *in vitro* ability of primary and established HSCs (that is, major CAF precursors) to boost CCA proliferation, survival and migration/invasion has been widely reported as well[25,29,32-36]. Moreover, it was shown that co-transplantation of CCA cells with either HSCs or liver myofibroblasts in immunodeficient mice resulted in accelerated tumor growth, compared with mice inoculated with cancer cells alone[25,26]. On the other hand, in a syngeneic rat model of CCA, selective CAF depletion in the tumor microenvironment, obtained by unleashing the specific CAF pro-apoptotic protein Bax by navitoclax, suppressed tumor growth and improved host survival[37]. Overall, these data indicate that myofibroblastic-like cells populating the tumor stroma are leading actors in fueling CCA progression.

Molecular players underlying the tumor-promoting effects of CAFs

Gene expression profiling of CAFs from human CCA samples revealed profound genetic changes as compared to normal liver fibroblasts. Most of the differentially expressed genes are involved in cell metabolism, likely reflecting the biologically active role of CAFs in supporting tumor growth. In addition, some of the up-regulated genes encode secreted proteins exerting pro-tumorigenic functions in multiple carcinomas (*i.e.*, amphiregulin, epiregulin, Jagged 1, PDGF-AA, periostin, secretogranin 2 and ADAM metallopeptidase domain 12), thus emerging as potential candidates underlying the harmful cross-talk between CAFs and CCA cells[38]. Below, we will summarize the most prominent CAF-derived molecules fostering CCA aggressiveness. It is also worth noting that, beyond paracrine soluble factors, extracellular vesicles, especially exosomes, nano-sized molecular shuttles of about 40-100 nm of diameter, are also claimed to mediate the paracrine communications between cancer cells and neighboring stromal components. Indeed, exosomes can transfer functional proteins, lipids and nucleic acids from one cell to another, thereby modulating gene expression programs[11,19]. In this regard, it was recently showed that microRNA-loaded vesicles derived from myofibroblastic-like cells can selectively target CCA cells, thus influencing their neoplastic properties, both *in vitro* and *in vivo*[39]. However, a detailed characterization of their cargo is still missing, thus further studies are needed to better elucidate their role in tumor progression.

IL-1β: Chemokines can be secreted by many cell types, such as epithelial cells, fibroblasts and endothelial cells, either constitutively or upon inflammatory conditions. Besides their role in the immune system, chemokines are also implicated in tumor biology, owing to their ability to recruit specific subsets of leukocytes, stimulate angiogenesis, and directly promote cancer cell proliferation and invasiveness in an autocrine or paracrine fashion[40]. In particular, a mass spectrometry analysis of conditioned media from co-cultures of CCA cells and HSCs revealed a striking increase in C-X-C chemokine ligand (CXCL)5 production by cancer cells, as compared to mono-culture media. Consistently, CXCL5 expression by neoplastic bile ducts positively correlated with stromal expression of α-SMA, overall suggesting its active role in the interplay between tumor and stroma. In particular, IL-1β, a paramount inflammatory cytokine, has been pointed out as the most likely HSC-derived inducer of CXCL5 production. Interestingly, IL-1β secretion by HSCs can be further enhanced by CCA cells themselves through paracrine signals. Autocrine production of CXCL5 promotes CCA cell proliferation, migration and invasion, by activating phosphatidylinositol 3-kinase (PI3K)/Akt and extracellular signal-regulated kinases (ERK)1/2 pathways in a CXCR2-dependent manner, *in vitro*[41]. Moreover, CXCL5 provides cancer cells with the ability to massively recruit tumor-infiltrating neutrophils, which in turn enhance CCA growth and invasiveness, *in vivo*[42]. In line with these findings, high CXCL5 expression negatively affected the overall survival of CCA patients[41,42].

Stromal cell-derived factor 1: Stromal cell-derived factor (SDF)-1, also known as CXCL12, acts as ligand for the G protein-coupled receptors CXCR4 and CXCR7. SDF-1 binding to its receptors triggers a variety of downstream signaling pathways, governing cell proliferation, survival and chemotaxis. Besides its well-established role in embryogenesis and tissue homeostasis, the SDF-1/CXCR4 axis is also diffusely implicated in the pathogenesis of autoimmune and inflammatory diseases, as well as in cancer progression[43]. In CCA, SDF-1 is solely expressed by CAFs, and not by cancer cells, which overexpress its cognate receptor CXCR4. In contrast, fibroblasts in the peritumoral stroma weakly express SDF-1, suggesting that SDF-1 expression may markedly increase following their recruitment to the TRS, likely upon angiotensin Ⅱ stimulation[33]. SDF-1 secretion by cultured HSCs was demonstrated to enhance CCA cell survival and invasiveness (along with EMT-like changes), *via* up-regulation of the anti-apoptotic protein Bcl-2, and activation of ERK1/2 and PI3K/Akt pathways, respectively[32,33]. In addition, SDF-1 could also promote the activation and proliferation of HSCs in an autocrine fashion, thus supporting further CAF enrichment. Consistent with these data, high stromal expression of SDF-1 predicted poor prognosis in CCA patients[33]. Noteworthy, CCA cells become hyper-

responsive to SDF-1 due to the overexpression of CXCR4, likely induced by either TNF-α released from TAMs[44] or hepatocyte growth factor produced by CAFs[31]. This clearly outlines the wide web of communications sustaining the pro-tumorigenic function of the TRS, allowing multidirectional paracrine loops among its different cellular components, which support each other in speeding up tumor progression.

PDGF-BB: PDGF family includes five dimeric ligands (PDGF-AA, -BB, -AB, -CC, -DD), acting *via* two receptor tyrosine kinases (PDGFRα and PDGFRβ). The PDGF/PDGFR system is involved in various biological processes requiring mesenchymal cell activation, mostly related to tissue repair and wound healing. Moreover, overexpression of PDGF ligands and receptors has been documented in a huge variety of epithelial cancers, and usually predicts poor outcome[45]. Among growth factors commonly produced by cultured HSCs, PDGF-BB is one of the most abundantly expressed. HSCs secrete PDGF-BB at much higher levels compared with CCA cells, which, from their side, express its cognate receptor PDGFRβ. Co-culture experiments demonstrated that HSC-derived PDGF-BB promoted CCA cell resistance to TNF-related apoptosis-inducing ligand-mediated apoptosis, by activating the Hedgehog (Hh) signaling cascade[35,36], a morphogen pathway directing several cholangiocyte functions critical for liver repair[46,47]. Specifically, PDGF-BB binding to PDGFRβ increases intracellular levels of cyclic adenosine monophosphate, resulting in a protein kinase A-dependent translocation of the Hh signaling activator Smoothened (SMO) to the plasma membrane, which eventually leads to the activation of GLI transcription factors[35]. Importantly, both cyclopamine (SMO inhibitor) and imatinib mesylate (PDGFRβ inhibitor) were able to reduce tumor growth by promoting cancer cell apoptosis in an orthotopic syngeneic rat model of CCA[35,36]. Kim *et al*[34] further confirmed that paracrine signals from HSCs (which, actually, may include Sonic Hh as well) are of paramount importance for the activation of Hh signaling within CCA cells, whereas autocrine activation only plays a minor role. Furthermore, they also outlined the involvement of Hh signaling in CCA cell proliferation, migration and invasiveness.

Heparin-binding epidermal growth factor: In CCA, overexpression of epidermal growth factor receptor (EGFR) is one of the most common genetic aberrations, and, most relevantly, it was associated with poor survival and tumor recurrence after resection[48]. In CCA xenografts derived from subcutaneous co-injection of cancer cells and liver myofibroblasts, EGFR activation was shown to promote tumor growth and metastasis, and, above all, to be strictly dependent on the presence of activated fibroblasts. Indeed, cultured myofibroblasts secrete high amounts of heparin-binding epidermal growth factor (HB-EGF), a well-known EGFR ligand, thereby triggering the activation of EGFR signaling in CCA cells, *in vitro*. The HB-EGF/EGFR axis promotes CCA cell proliferation, migration and invasion, along with EMT-like changes, through activation of signal transducer and activator of transcription (STAT)-3 and ERK1/2 pathways. Of note, HB-EGF expression in fibroblasts can be further enhanced by TGF-β1 released from CCA cells, whose production is in turn triggered by EGFR activation, thus outlining the presence of a self-perpetuating paracrine loop[26].

The deleterious interplay between CAFs and endothelial cells: emerging evidence

Importantly, the paracrine signals released by CAFs not only directly exacerbate the malignancy of cancer cells, but also participate in the recruitment of other stromal components, including inflammatory cells and endothelial cells, thereby further supporting cancer growth and progression[6]. In particular, we recently unveiled that CAFs may cooperate with CCA cells in driving the development of a rich lymphatic vasculature within the tumor stroma. CCA is characterized by a striking expansion of the intratumoral and peritumoral lymphatic vessels, which represents a key determinant of the early metastasization to the regional lymph nodes, often precluding curative surgery[6]. Consistently, a high lymphatic microvessel density in CCA tissues correlated with significantly reduced overall and disease-free survival of patients[49]. Our recent findings demonstrated that, within the TRS, lymphatic endothelial cells (LECs) localize in close spatial relationship with either CCA cells or CAFs. Indeed, besides recruiting fibroblasts around the neoplastic ducts, PDGF-DD produced by CCA cells can also provide CAFs with the ability to secrete lymphangiogenic growth factors, namely vascular endothelial growth factor (VEGF)-A and VEGF-C, which eventually promote the recruitment of LECs, along with their tubular assembly in highly anastomosed structures[50]. Overall, these observations are consistent with the concept that CAFs are able to generate a pro-invasive microenvironment conducive to the lymphatic metastatic behavior of CCA.

It is important to note that in CCA, the large expansion of the lymphatic vasculature is not paralleled by an equal increase in blood vessels[6]. Nevertheless, angiogenesis has been also associated with a high risk of recurrence after surgery[51]. In this regard, it is likely that CAFs, especially those originated from HSCs, contribute to generate a pro-angiogenic microenvironment, as reported in other cancer types[52]. Indeed, HSCs likely behave as liver-specific pericytes, participating to vascular remodeling during both liver regeneration and tumor-associated angiogenesis. In this context, PDGF has been pinpointed as a relevant player. Specifically, PDGF ligands (especially PDGF-BB) released from the vascular endothelium are able to drive the recruitment of PDGFR-β-expressing HSCs, along with their subsequent adhesion to the vessel wall, similar to what occurring in embryogenesis. From their side, activated HSCs promote vascular tube formation by secreting VEGF ligands and angiopoietins under the effect of hypoxia, and mecha-

nically stabilize the sprouting vessels by providing a tight envelope around the sinusoidal endothelial cell layer[53-56].

TUMOR-ASSOCIATED MACROPHAGES

Among the several immune cell types populating the TRS, macrophages are the most represented. Tumor-associated macrophages (TAMs) are mainly derived from circulating monocytes ($CD14^+/CD16^+$), rather than from resident macrophages ($CD68^+$) or Kupffer cells in the liver. They are efficiently recruited to the tumor mass by a range of chemoattractants variably secreted by neoplastic and stromal cells, including C-C motif ligand (CCL) chemokines [*e.g*., monocyte chemoattractant protein (MCP)-1, also known as CCL2], colony stimulating factor (CSF)-1 and VEGF[6,57,58]. For instance, CAFs, especially FAP^+ CAFs, are a major source of MCP-1[59]. In contrast to T cells, which can exert both tumor-promoting and tumor-suppressive functions, TAMs are almost exclusively implicated in boosting cancer aggressiveness, a function exemplified by their predominant localization at the tumor front. TAMs mostly display a M2 (or alternatively activated) phenotype, manipulated by paracrine signals originating from both malignant cells and specific subsets of T cells (including IL-10, CSF-1 and TGF-β), as well as by tumor hypoxia. Pro-tumorigenic effects of the M2 phenotype rely on a range of properties, including limited antigen-presenting functions, strong tissue remodeling and immune tolerance abilities, and production of pro-angiogenic and pro-lymphangiogenic growth factors; furthermore, TAMs directly provide cancer cells with pro-migratory inputs. TAMs are characterized by low expression of major histocompatibility complex class Ⅱ molecules and IL-12, and high expression of IL-10, arginase-1 and multiple scavenging, mannose, and galactose receptors. Conversely, the so-called classically activated M1 macrophages, which are usually less represented within the TRS, possess strong antigen-presenting abilities, prime tissue destruction and anti-tumor immune responses, and possess tumoricidal activities[10,12,58,60-63].

Conditioned medium from CCA cells fostered the emergence of the M2 phenotype in cultured macrophages, which actually up-regulated the expression of the M2 specific marker CD163, as well as of the M2-related molecules IL-10 (immunosuppressive cytokine), TGF-β (pro-fibrotic cytokine), VEGF-A (pro-angiogenic growth factor) and matrix metalloproteinase (MMP)-2[64]. Growing interest has also been drawn on the interplay between TAMs and CAFs, as it was recently found that conditioned medium from HSCs affected the differentiation of macrophages, stimulating the production of pro-inflammatory (IL-6) and pro-fibrotic cytokines (TGF-β)[65]. Furthermore, within the CCA stroma, the density of M2 TAMs positively correlated with the number of regulatory T cells, suggesting that they contribute to macrophage polarization toward the pro-neoplastic phenotype[64]. Interestingly, cholangiocyte ability to finely orchestrate a macrophage-centric inflammatory response was also reported by our group in a mouse model of congenital hepatic fibrosis, a disease of the biliary epithelium at increased risk for CCA development. In this model, dysfunctional biliary epithelial cells (due to a genetic defect in the ciliary protein fibrocystin) secrete a range of chemokines (CXCL1, CXCL10, CXCL12) able to recruit and activate bone marrow-derived macrophages, which then progressively switch from an M1 to an M2 phenotype as the disease progresses[66]. However, it is worth considering that the macrophage phenotype is extremely plastic, showing a continuum of activation states, in which M1 and M2 types only represent the extreme points[62]. In line with this concept, many tumor-promoting cytokines that are actually M1 cytokines, such as IL-6, are even produced by TAMs[61]. Recently, Raggi *et al*[67] revealed that the CCA stem-like compartment is actively involved in both the recruitment of circulating monocytes and their differentiation into TAMs, owing to the release of IL-13, IL-34 and osteoactivin. Of note, cancer stem cell (CSC)-associated TAMs display unique phenotypic and functional features, namely mixed expression of M1 and M2 markers (*e.g.*, M1-related chemokines CXCL9 and CXCL10, and M2-related chemokines CCL17 and CCL18), increased adhesive and invasive abilities, *in vitro*, and enhanced tumor-promoting functions, *in vivo*. This clearly highlights the existence of different TAM subsets within the tumor, depending on the multitude of microenvironmental cues originating from various cell niches.

Evidence for the pro-neoplastic effects exerted by TAMs

In CCA tissues, M2 macrophages are definitely much more abundant than in the peritumoral areas, and TAM are mostly located at the leading edge of the tumor[67]. Consistently, immunohistochemical analyses in *Opisthorchis viverrini*-associated CCA in a hamster model revealed a progressive, dramatic increase in M2 macrophages through carcinogenesis[28]. Studies from different groups showed that a high density of TAMs at the invasive front correlated with poor survival of CCA patients after resection[64,68,69]. However, it is important to underline that not all of these studies provided evidence that the observed TAM actually exhibited the pro-neoplastic, M2 phenotype. Whereas Subimerb *et al*[68] evaluated the expression of MAC387, a marker of recently infiltrated, bone marrow-derived, macrophages, Atanasov *et al*[69] evaluated the expression of resident, $CD68^+$ macrophages. On the contrary, Hasita *et al*[64] sought to distinguish M2 TAMs from total resident macrophages based on their expression of CD163, in order to highlight their specific contribution to tumor progression. They found that, in CCA tissues, the number of $CD163^+$ M2 cells was, as expected, lower than the number of $CD68^+$ cells, and that high infiltration of M2 macrophages, but not of total macrophages, was significantly associated with poor disease-free survival of patients. Of further interest, the density of M2 macrophages within CCA stroma also correlated with the presence of extrahepatic metastases[28], the tumor pathological grade[67], and the microvascular density[64]. Although these findings are

based on the evaluation of different phenotypic markers, overall, they suggest that TAMs strongly influence CCA progression, with a major role played by M2 TAMs, thus confirming what observed in other cancer types. In accordance with these immunohistochemical findings, conditioned medium from M2 macrophages boosted the migratory abilities of CCA cells by inducing EMT-like changes, *in vitro*[28].

As previously mentioned, recruitment of circulating monocytes, rather than proliferation of resident macrophages, is the mechanism responsible for TAM accumulation in the TRS[57]. In fact, in CCA patients, levels of circulating $CD14^+/CD16^+$ monocytes were increased, and correlated with high density of $MAC387^+$ TAMs, and with poor survival rates. $CD14^+/CD16^+$ monocytes represent a minor subset of total monocytes, whose expansion is usually associated with acute or chronic inflammation. They are classically regarded as more mature cells than $CD14^+/CD16^-$ monocytes, and thought to be the major precursors of tissue macrophages. Besides expressing a larger number of adhesion molecules, enabling them to strongly adhere to vascular endothelium, $CD14^+/CD16^+$ monocytes also up-regulate the EGFR ligand epiregulin, and the angiogenic chemokine CXCL3. Overall, these features are consistent with the adoption of a pro-tumorigenic phenotype, likely induced by tumor-derived molecules, which may also drive their recruitment into the TRS[70].

Molecular players underlying the tumor-promoting effects of TAMs

MMP-9: MMPs, in particular MMP-9, are the most important proteolytic enzymes in the context of tumor spread, and their overexpression tends to be predictive of worst outcome in human cancers. Besides underpinning cancer cell invasion through the selective deletion of ECM integrity, MMPs can also elicit the post-translational activation of growth factors and cytokines, thereby influencing key cellular processes[71]. Subimerb *et al*[68] found that TAMs (especially those located at the tumor-host interface) rather than cancer cells represent the main source of MMP-9 in CCA. Moreover, CCA patients with high numbers of $MMP\text{-}9^+$ TAMs displayed significantly shorter survival times than those with low numbers, thus pointing out MMP-9 production as a key driver of CCA progression promoted by TAMs. Furthermore, a broad expression of other pivotal ECM remodeling-related genes, namely *MMP-2*, *ADAM10*, and *ADAM17* was reported in CSC-associated TAMs[67].

TNF-α: TNF-α is a pleiotropic cytokine, acting as a central pro-inflammatory mediator in human carcinogenesis, wherein it was reported to play both anti-tumoral and pro-tumoral effects[72]. In CCA, as well as in the majority of carcinomas, TNF-α is widely expressed by macrophages located at the tumor edge, whereas it is only focally expressed by cancer cells[44]. Lipopolysaccharide (LPS)-activated macrophages were able to elicit EMT-like phenotypic changes in CCA cells (namely, down-regulation of the epithelial markers E-cadherin and cytokeratin 19, along with up-regulation of the mesenchymal markers S100A4 and MMP-9), probably mediated by a TNF-α-induced activation of Snail and ZEB2 transcription factors[73-75]. Consistently, upon TNF-α stimulation, CCA cells gained increased migratory functions in conjunction with the activation of ERK, Akt and nuclear factor (NF)-κB[74,76].

IL-6: Aberrant activation of the IL-6 classical downstream effector STAT3 is described in many epithelial cancers, and is currently regarded as a major oncogenic event[77]. For instance, in CCA patients, high expression of STAT3 by cancer cells was associated with poorly differentiated tumor phenotypes, as well as with low survival rates[78]. In particular, in CCA cells, increased cell survival by up-regulation of the anti-apoptotic protein myeloid cell leukemia-1 is the fundamental mechanism triggered by the IL-6/STAT3 axis[79]. In the hamster experimental model of *Opisthorchis viverrini*-induced CCA, STAT3 activation peaked at the pre-cancerous stage, in association with a high degree of inflammation. Consistently, conditioned medium from LPS-activated macrophages led to a robust STAT3 activation in CCA cells[78], likely mediated by IL-6, whose secretion is potently stimulated by LPS[73]. Although IL-6 can be secreted even by CCA cells themselves, paracrine signaling is probably essential to reach broad STAT3 activation[79], and TAMs may actually be central in this process.

YKL-40: YKL-40, also called chitinase 3-like 1, is a secreted glycoprotein, which is supposed to play key roles in different aspects of tumorigenesis, from cell proliferation and survival, to angiogenesis and ECM remodeling. Interestingly, YKL-40 serum levels are dramatically increased in patients with multiple chronic inflammatory diseases, such as liver cirrhosis, as well as in patients with several malignancies, including breast, lung and colorectal carcinomas[80]. In CCA patients, YKL-40 serum levels were actually much higher than those of healthy subjects, and also negatively correlated with overall survival. Importantly, within the tumoral area, CCA cells represent only minor contributors to YKL-40 production, which is primarily caused by infiltrating inflammatory cells, especially TAMs. Of further interest, exogenous YKL-40 stimulated CCA cell growth and migration, by triggering Akt and ERK1/2 activation[81].

Wnt3: Involvement of Wnt/β-catenin pathway in CCA pathogenesis and progression is a well-established concept for many years[82,83]. Wnt family ligands are secreted glycoproteins modulating fundamental transcriptional programs by stimulating the nuclear translocation of β-catenin. In the basal conditions, β-catenin is mainly located at the cell-cell junctions, whereas a minor pool is sequestered in the cytoplasm by a destruction complex, where phosphorylation at specific residues (Ser 33/37 and Thr 41) is a pre-requisite to allow its inactivation

and proteasomal degradation. Binding of Wnt ligands to Frizzled receptors lets β-catenin to detach from the membrane, accumulate within the cytoplasm, and then translocate into the nucleus, where it interacts with several co-activators, among which T-cell factor and lymphoid enhancer-binding factor 1 are the main partner in gene regulation. β-catenin target genes encompass well-known proto-oncogenes relevant for CCA growth, such as c-Myc, cyclin D1 and ZEB1[84,85]. In CCA tissues, β-catenin is constitutively expressed at high levels either in the cytoplasm or in the nucleus of cancer cells, whereas its membranous expression is decreased, consistent with the activation of the Wnt signaling. Among Wnt ligands, whereas Wnt5a and Wnt7b are overexpressed by neoplastic bile ducts, TAMs represent a major source of Wnt3. Notably, conditioned medium from LPS-activated macrophages elicited β-catenin nuclear translocation in CCA cells, resulting in enhanced cell growth[86].

MSCs

MSCs are non-hematopoietic stem cells primarily resident in the bone marrow, where they are recruited from by chemotactic signals mainly originating from injured tissues and inflammatory sites. Indeed, MSCs are multipotent cells able to differentiate in a variety of cell types, thus being classically regarded as a valuable source of tissue replacement. However, under the influence of cancer-derived chemokines, MSCs can also home to primary tumor sites, wherein they eventually become an additional component of the tumor microenvironment. Tumor-resident MSCs have been also reported to perform several activities supporting cancer progression. For instance, they can interfere with anti-tumor immunity, promote angiogenesis, and directly enhance the aggressiveness of malignant cells through secreted factors[8,87,88].

In nude mice bearing subcutaneous human CCA xenografts, it was shown that, upon infusion into the venous circulation, MSCs were able to selectively reach both the primary tumor and the metastatic liver, thus confirming their pronounced tumor tropism. Furthermore, exposure of CCA cell to conditioned medium from MSCs resulted in increased proliferation, apoptosis resistance, and invasiveness, likely due to the activation of the Wnt/β-catenin signaling. Consistently, subcutaneous co-injection of CCA cells with MSCs in immunodeficient mice led to accelerated tumor growth, and higher incidence of liver metastases, compared with mice inoculated with cancer cells alone[87]. Interestingly, the ability of MSCs to promote CCA cell proliferation was further strengthened by preliminary exposure of MSCs to cancer cell-derived extracellular vesicles (with features consistent with exosomes). Indeed, these vesicles induced profound changes in the MSC secretome, including increased secretion of IL-6, CCL2/MCP-1, CXCL1/GRO-α, CX3CL1/Fractalkine and PDGF-AA. Besides directly favoring tumor cell growth, MSCs may also represent an additional (although minor) source of CAFs, as conditioned medium from CCA cells prompted a phenotypic switch from MSCs into myofibroblastic-like cells[88].

ECM

Besides providing a physical support to cells, the ECM (mainly consisting of collagens, glycoproteins and proteoglycans) also communicates straight with them, thereby modulating a variety of cellular functions, and acts as a paramount reservoir of cell-derived soluble factors[6]. Throughout carcinogenesis, the ECM gradually undergoes stiffening and profound compositional changes, resulting from the accumulation of secreted structural and non-structural proteins, in particular collagen type Ⅰ and fibronectin, as well as of matrix modifying enzymes[19,22,89]. An abnormal ECM leads to a dysregulated behavior of both cancer and stromal cells, thereby affecting several processes related to tumor biology, including cancerous fibrogenesis, inflammation and angiogenesis[11,90]. Interestingly, ECM stiffness is emerging as a driving force behind cancer progression. As previously mentioned, tumor-associated ECM is typically stiffer than the normal matrix, due to a pathological remodeling mainly driven by neoplastic cells and CAFs. This stiff, collagen enriched ECM can signal to cells through specific mechanosensors, thus activating intracellular pathways regulating the acquisition of malignant phenotypic traits[90,91]. Among the intracellular sensors of ECM-driven mechanical stress, the transcriptional co-activator yes-associated protein (YAP) and its paralog, transcriptional co-activator with PDZ-binding motif (TAZ), are emerging as master directors of cancer cell reprogramming and enhanced invasiveness[92]. Indeed, high levels of cytoskeletal contractility, resulting from increased ECM rigidity, are generally coupled with the activation of YAP/TAZ, which can profoundly affect epithelial cell behavior, including the balance between proliferation and apoptosis[93,94]. Interestingly, in CCA, YAP overexpression was reported to enhance cancer cell proliferation, invasion (*via* EMT-like changes) and resistance to chemotherapeutic drugs, both *in vitro* and in tumor xenografts[95]. Therefore, it is tempting to speculate that, after being recruited by CCA cells through PDGF-DD[24], CAFs may gradually manipulate ECM stiffening within the TRS, thereby inducing YAP/TAZ activation in cancer cells, leading to the emergence of a particularly aggressive tumor phenotype.

In CCA, interactions between tumor cells and specific molecular components of the ECM may trigger additional pathways of tumor invasiveness. In fact, CCA cells cultured on a reconstituted basement membrane preparation (mainly composed of collagen type Ⅳ and laminin), showed enhanced invasive properties compared with cells grown on uncoated culture plates. This was dependent on the dysregulated expression of a wide range of proteins, especially L-plastin, which is an actin-bundling protein supporting cell motility and adhesion. L-plastin is dramatically up-regulated in many types of malignant cells and, in CCA tissue, it is primarily expressed at the tumor front, thereby indicating its involvement in tumor invasion[96]. The ability of the TRS-associated ECM to

support cancer aggressiveness is also well exemplified by three fundamental non-structural ECM proteins, namely tenascin, periostin and osteopontin, reported as poor prognostic biomarkers for CCA patients. In CCA samples, tenascin is abnormally expressed in the intratumoral stroma, as well as at the tumor leading edge. Although CAFs undoubtedly represent the main source of tenascin, carcinoma cells can contribute to its biosynthesis. In CCA patients, aberrant deposition of tenascin at the invasive front positively correlated with tumor size and lymph node metastasis, and also predicted poor survival. It is worth noting that the expression pattern of tenascin roughly parallels that of EGFR, which tenascin can bind to, likely underpinning its tumor-promoting functions[97]. Similarly, high expression of periostin within the TRS, which is solely due to CAFs, was an independent prognostic factor for overall survival of CCA patients. Moreover, serum periostin levels were significantly higher in CCA patients compared with both healthy subjects and patients with other hepatic malignancies. Consistent with these findings, exogenous periostin induced CCA cell proliferation and invasion through its interaction with integrin receptors $\alpha5\beta1$ and $\alpha6\beta4$, leading to the activation of the PI3K/Akt pathway, *in vitro*[38,98,99]. High stromal expression of osteopontin is also an independent risk factor for reduced overall and disease-free survival in CCA patients, positively correlating with both tumor size and the presence of lymph node or macrovascular invasion[20].

THE TRS AS POTENTIAL THERAPEUTIC TARGET

Classically, anticancer therapies aim at targeting intrinsic traits of neoplastic cells, which, until recently, were actually seen as the only players deserving attention in the context of clinical management. However, in CCA, a lethal malignancy paradigmatic of the strong resistance to conventional chemotherapy, mounting evidence supports the role of tumor microenvironment in dictating tumor growth, progression and metastatic dissemination. Indeed, CCA cells establish intense, mutual, paracrine communications with neighboring stromal components, in particular CAFs and TAMs, which are a rich source of signals promoting malignancy (Figure 1). Therefore, combinatorial therapies that both directly tackle tumor growth and turn off the tumor-promoting functions of the TRS might represent an important step forward in anticancer treatment, especially in CCA. In addition to provide a number of druggable targets, TRS may help to identify (by gene expression profiling) molecular signatures serving as novel prognostic biomarkers, useful for predicting therapeutic response or monitoring tumor recurrence, as it could be the case with periostin[8,63,89]. It is worth noting that, unlike cancer cells, which undergo multiple genetic/epigenetic changes giving rise to a tremendously heterogeneous population, stromal cells represent a genetically stable, more uniform compartment, and thus stand out as viable and compelling therapeutic targets[12,37]. Basically, TRS-oriented therapeutic approaches should aim at: (1) hampering the recruitment of reactive stromal cells by counteracting tumor-derived chemokines; (2) promoting TRS depletion by eliciting apoptosis of its cellular components; (3) interfering with the intracellular pro-oncogenic pathways triggered by the TRS within the cancer cell; and (4) interfering with the paracrine communications between stromal and cancer cells, by neutralizing specific soluble factors or antagonizing their cognate receptors[6]. The study performed by Mertens *et al*[37] is an archetype of these potential new strategies. Using the BH3 mimetic navitoclax (a small molecule mimicking the pro-apoptotic protein Bad), the authors were able to selectively induce Bax-dependent apoptosis in CCA-derived CAFs, but not in normal fibroblasts or CCA cells, *in vitro*. By translating these findings in an *in vivo*, orthotopic syngeneic rat model of CCA, navitoclax markedly reduced tumor growth and metastasis, and significantly improved survival, an effect related to a quantitative depletion of CAFs from the stroma. Taking a different approach, the mammalian target of rapamycin inhibitor everolimus, in addition to directly reduce CCA cell proliferation and invasion[100], was reported to hamper the cross-talk between CAFs and CCA cells, by both impairing the activation of CAF-induced motogenic pathways in cancer cells, and inhibiting the secretion of tumor-promoting cyto/chemokines by CAFs[30]. Interestingly, everolimus is already an FDA-approved drug for the treatment of breast, neuroendocrine and renal cell carcinomas[101]. By turning to TAMs, it was shown that liposome-encapsulated clodronate, a selective macrophage-depleting agent, as well as GW2580 or AZD7507, small molecules preventing monocyte-to-macrophage differentiation, significantly reduced the growth of subcutaneous human CCA xenografts. Moreover, the tumor-suppressive effect of liposomal clodronate was also confirmed in a non-transgenic, thioacetamide-induced rat model of CCA, which faithfully reproduces the inflammatory and desmoplastic microenvironment associated with human CCA[102]. Noteworthy, besides priming TAMs for apoptosis or blocking monocyte recruitment, it might be possible to harness the inherent plasticity of macrophages in order to revert their polarization from the pro-neoplastic M2 phenotype to the anti-tumoral M1 phenotype[12,57]. However, the development of combinatorial therapies targeting both tumor and stromal cells must be rooted in a deep knowledge of the epithelial-mesenchymal interactions occurring within the CCA microenvironment, which is not possible without proper experimental models. In this regard, two-dimensional co-culture systems and, even more, three-dimensional organotypic culture models represent powerful tools for investigation, but, of course, they cannot fully reproduce the complexity of the TRS, which integrate a multitude of cell elements. On the other hand, rodent models of CCA more closely mimic the structural and functional heterogeneity of the TRS, even though the murine environment may not accurately

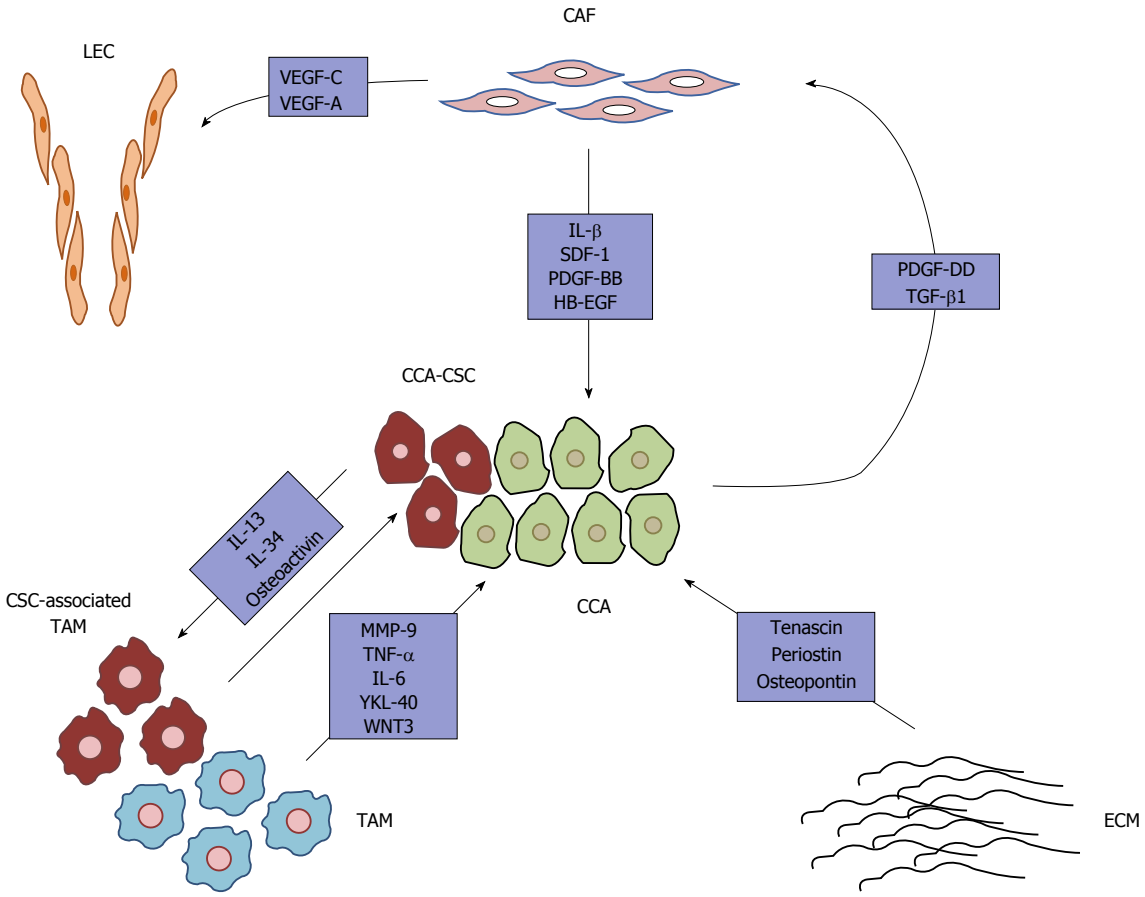

Figure 1 Soluble factors derived from reactive stromal cells, along with tumor-associated extracellular matrix, sustain cholangiocarcinoma cell malignancy. CCA cells shape the surrounding microenvironment to meet their highly demanding needs, thus providing CAFs and TAMs with the ability to secrete a broad range of cyto/chemokines, growth factors, morphogens and proteinases, which boost cancer cell proliferation, survival and invasiveness. In this model, CAFs are recruited by PDGF-DD released by CCA cells. In addition, TGF-β1, also derived by CCA cells, stimulates CAFs to produce the EGFR ligand, HB-EGF, which triggers the acquisition of malignant behaviors (*i.e.*, EMT-like changes) by cancer cells. CAF-derived tumor-promoting molecules also include IL-1β, SDF-1 and PDGF-BB. Moreover, PDGF-DD induces CAFs to acquire pro-lymphangiogenic functions (exerted by VEGF-A and VEGF-C). On the other hand, TAMs, displaying a predominant M2 phenotype, also support tumor survival and invasiveness by secreting several soluble factors, including MMP-9, TNF-α, IL-6, YKL-40 and Wnt3. Of note, the CCA stem-like compartment molds a specific subset of TAMs (through secretion of IL-13, IL-34 and osteoactivin), displaying a mixed M1/M2 phenotype, to promote self-renewal and drug-resistance properties. In addition, non-structural proteins expressed by the abnormally remodeled ECM (tenascin, periostin, osteopontin) further enhance CCA aggressiveness. CAF: Cancer-associated fibroblast; CCA: Cholangiocarcinoma; CSC: Cancer stem cell; ECM: Extracellular matrix; LEC: Lymphatic endothelial cell; TAM: Tumor-associated macrophage; IL: Interleukin; TGF: Transforming growth factor; TNF: Tumor necrosis factor; PDGF: Platelet-derived growth factor; EGFR: Epidermal growth factor receptor; MMP-9: Matrix metalloproteinase 9.

reproduce the wide range of paracrine communications occurring in the human disease setting, all the more so in xenograft models, where the host is immunodeficient[8,11].

CONCLUSION

Unravelling the complex mechanisms underlying the mutual interactions between the tumoral and stromal compartments is indeed a topic of great translational significance, worth being pursued further in the next future. Based on the data discussed above, specific targeting of the signals operating in the tumor microenvironment, coupled with conventional anticancer treatments, could actually open new promising and feasible therapeutic avenues in CCA, hopefully expandable to other aggressive desmoplastic epithelial malignancies, such as pancreas and breast carcinomas. It is tempting to speculate that these innovative, multitargeted therapies might more effectively eradicate tumor cells, owing to concurrent switching-off actions on intrinsic (cancer cell-dependent) as well as extrinsic (TRS-derived) tumor-promoting mechanisms, eventually leading to improved patient outcomes.

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ORIGINAL ARTICLE

Case Control Study

Annexin A2 as a biomarker for hepatocellular carcinoma in Egyptian patients

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Abstract

AIM

To investigate the clinical utility of serum annexin A2 (ANXA2) as a diagnostic marker for early hepatocellular carcinoma (HCC).

METHODS

This study was performed in HCC Clinic of Ain Shams University Hospitals, Cairo, Egypt and included: Group 1: Fifty patients with early stage HCC (Barcelona Clinic Liver Cancer stage A); Group 2: Twenty five patients with chronic liver disease; and Control Group: Fifteen healthy, age- and sex-matched subjects who were seronegative for viral hepatitis markers. The following

laboratory investigations were done: Viral hepatitis markers [hepatitis B surface antigen and hepatitis C virus (HCV) antibodies], HCV RNA in HCV antibody-positive patients, serum alpha fetoprotein (AFP), and serum ANXA2 levels.

RESULTS

In this study, 88% of HCC patients (n = 44) were HCV-positive, while HBV infection represented only 8% of all HCC patients (n = 4); and two patients were negative for both viral markers. A highly significant difference was found between patients with HCC and chronic liver disease as well as controls with regard to serum ANXA2 levels (130, IQR 15-240; 15, IQR 15-17; and 17, IQR 15-30 ng/mL, respectively). The area under the curve of ANXA2 was 0.865; the cut-off value was established to be 18 ng/mL with a diagnostic sensitivity of 74% and a specificity of 88%, while the sensitivity and specificity of AFP at the cut-off value of 200 ng/dL were 20% and 100%, respectively.

CONCLUSION

Serum ANXA2 may serve as a biomarker for the early detection of HCC.

Key words: Hepatocellular carcinoma; Hepatitis C virus; Annexin A2; Alpha-fetoprotein; Tumor markers

Core tip: Thirty percent of hepatocellular carcinoma (HCC) patients present with normal serum alpha fetoprotein, which highlights the need for new biomarkers for HCC. In the present study, a highly significant difference was observed among patients with HCC and chronic liver disease as well as controls with regard to serum annexin A2 (ANXA2) levels (130, IQR 15-240; 15, IQR 15-17; and 17, IQR 15-30 ng/mL, respectively). The area under the curve of ANXA2 was 0.865; the cut-off value was 18 ng/mL with a diagnostic sensitivity of 74% and specificity of 88%. Thus, ANXA2 may serve as a useful biomarker for the early detection of HCC.

Shaker MK, Abdel Fattah HI, Sabbour GS, Montasser IF, Abdelhakam SM, El Hadidy E, Yousry R, El Dorry AK. Annexin A2 as a biomarker for hepatocellular carcinoma in Egyptian patients. *World J Hepatol* 2017; 9(9): 469-476 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i9/469.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i9.469

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. According to the National Institute of Cancer in Egypt, HCC is considered one of the commonest malignancies in Egypt as a result of the high prevalence of hepatitis B and C infections, since these represent approximately 45.3% of all new cases of this type of cancer[1].

Because of the asymptomatic nature of early HCC as well as the lack of its effective screening strategies; most patients (> 80%) present with an overt advanced disease[2]. Approximately 30% of HCC cases with normal serum alpha fetoprotein (AFP) levels are diagnosed before the appearance of clinical manifestations, and this highlights the need for new and early reliable biomarkers for the detection of HCC[3].

Annexins are a family of proteins that bind anionic phospholipids in a calcium-dependent manner. Annexins were first discovered in animal cells and were named for their ability to "annex" or aggregate membranes. The annexins are expressed in vertebrates (ANXA), invertebrates (ANXB), fungi and protozoa (ANXC), plants (ANXD) and protists [*e.g.*, algae (ANXE)][4,5].

Annexin A2 (ANXA2) is primarily expressed in human endothelial cells, mononuclear cells, macrophages, marrow cells and some tumor cells[6]. Moreover, ANXA2 is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies and has emerged as an attractive candidate receptor for increased plasmin generation on the tumor cell surface[7]. It plays multiple roles in the regulation of cellular functions including angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion[8,9].

ANXA2 is almost undetectable in the normal liver and in chronic hepatitis tissues, while it is highly expressed in HCC[10]; moreover, serum levels of ANXA2 are elevated in patients with early stage HCC who are AFP-negative[11]. ANXA2 was reported to promote HCC metastasis and invasion through its interaction with HAb18G/CD147 (a member of the immunoglobulin family of proteins)[6]. Nevertheless, the importance of the change in serum levels of ANXA2 in the early stages of HCC has yet to be elucidated.

This study aimed to determine the clinical utility of the serum level of ANXA2 as a diagnostic biomarker of HCC and to correlate its level with that of AFP.

MATERIALS AND METHODS

This prospective case control study was conducted at the HCC clinic, Departments of Tropical Medicine and Clinical Pathology; Ain Shams University Hospitals (Cairo, Egypt), after approval from the Research and Ethics Committee of Ain Shams University was obtained in accordance with local research governance requirements. This study was performed in accordance with the 1964 Declaration of Helsinki and all subsequent revisions.

This study included two patient groups. Group 1: Fifty patients with early stage HCC on top of chronic liver disease (CLD) Child-Pugh class A and B. They were diagnosed according to the Barcelona Clinic Liver Cancer (BCLC) staging system (BCLC A)[12]; and Group 2: Twenty-five patients with CLD (without HCC), their diagnosis was based on clinical, laboratory, and ultrasonographic findings.

CLD in this study represented patients with: (1) persistent viral infection [hepatitis C virus (HCV) and/or hepatitis B virus (HBV)] or affected liver functions for more than 6 mo; and (2) ultrasound features suggestive of CLD: Coarse liver echo-texture, dilated portal vein, attenuated hepatic veins, splenomegaly and/or ascites.

Inclusion criteria for HCC group: (1) confirmed diagnosis of HCC according to the European Association for the Study of Liver Diseases[12]; (2) rarly stage HCC (Stage A) according to the BCLC staging system (single or 3 nodules < 3 cm; Performance Status 0)[12]; and (3) informed consent from all participants before enrollment in the study.

Exclusion criteria for HCC group: (1) intermediate or advanced stage HCC as defined by the BCLC staging system; (2) major vascular tumor invasion or metastasis as confirmed by radiological imaging studies; (3) patients with other suspected solid malignancies or liver metastasis; and (4) other types of CLD as autoimmune hepatitis.

In addition, fifteen age- and sex-matched healthy persons were enrolled, constituting the control group. The healthy controls were collected from the outpatient clinics among those coming for pre-employment screenings. Liver and systemic diseases were excluded by history, physical examination, laboratory and radiologic assessment.

All included patients and control subjects were subjected to the following: (1) full medical history and thorough clinical examination; and (2) laboratory investigations included: Liver function tests to determine the levels of aspartate aminotransferase, alanine aminotransferase, serum bilirubin (total and conjugated), serum albumin, and prothrombin time; viral markers hepatitis B surface antigen, and HCV by enzyme linked immunosorbent assay (ELISA) and detection of HCV RNA by real-time polymerase chain reaction; detection of the serum AFP level by electro-chemiluminescence; and determination of the serum concentration of ANXA2 by ELISA.

Samples

A total of 5 mL of venous blood was withdrawn from each subject under complete aseptic conditions. Then, 1.8 mL was placed into sodium citrate (3.2%) tubes in a ratio of 9:1 (blood: Citrate) for the PT assay. The remainder of the blood was placed in sterile vacutainers with a clot activator and was left to clot for 30 min. The serum was then separated by centrifugation at 1000 × *g* for 15 min and was divided into two aliquots: One aliquot was used for the immediate routine liver function tests and serum AFP detection, while the remaining portion of sera was stored as an aliquot at -20 ℃ until future use (*i.e.*, the detection of the serum level of ANXA2). Frozen samples were allowed to thaw to room temperature just prior to the analysis. Hemolyzed samples were discarded, and repeated freezing and thawing was avoided.

Analytical methods

AFP: Quantitative determination of AFP was conducted in an Immulite immunoassay auto analyzer using an AFP kit supplied by DPC (DIAGNOSTICA Product Corporation, Los Angeles, CA, United States). This assay is based on an electro-chemiluminescence immunoassay technique. The antigen (sample), a biotinylated monoclonal AFP-specific antibody and a monoclonal AFP-specific antibody labeled with a ruthenium complex react to form a sandwich complex. Streptavidin-coated microparticles were added, and the complex was then bound to a solid phase *via* the interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of the electrode. Unbound substances were then removed with ProCell. The application of a voltage to the electrode then induced chemiluminescent emission, which was measured by a photomultiplier. The results were determined *via* a calibration curve that was specifically generated by 2-point calibration and a master curve provided by the reagent barcode.

ANXA2 assay: This assay was performed with a commercially available ELISA kit supplied by Glory Science (Glory Science Co., Del Rio, TX, United States). This assay employs a quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ANXA2 is pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ANXA2 that is present becomes bound by the immobilized antibody. After any unbound substances are washed away, an enzyme-linked monoclonal antibody specific for ANXA2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ANXA2 bound in the initial step. The color development is then stopped and the intensity of the color is measured at 450 nm[13].

Radiological investigations included abdominal ultrasound and triphasic spiral abdominal computed tomography (CT) to confirm the diagnosis and staging of HCC.

Statistical analysis

IBM SPSS Statistics for Windows, Version 19.0 (IBM Corp., Armonk, NY, United States) was used for the data analysis. The quantitative variables were presented as the mean and the standard deviation, while the qualitative variables were presented as frequencies and percentages. The values of skewed parameters were expressed as the median and IQR (25^{th}-75^{th}). An unpaired *t* test (*t* value) was used to compare a quantitative variable between two independent groups for parametric data, while a Mann-Whitney test (*Z* value)

Table 1 Descriptive statistical data of the various parameters in the three studied groups[1]

Parameter	HCC (n = 50)	CLD (n = 25)	Control (n = 15)
ALT (U/L)	43 (31-72.5)[2]	31 (22.5-41)[2]	25 (17-29)[2]
AST (U/L)	60 (42.25-97.25)[2]	45 (41-61.5)[2]	26 (21-35)[2]
PT (s)	13.8 ± 1.53	16 ± 3.37	12 ± 0.1
Alb (g/dL)	3.25 ± 0.53	2.75 ± 0.65	3.8 ± 0.28
T.Bil (mg/dL)	1.33 (1-2.2)[2]	2 (1.15-2.9)[2]	0.8 (0.6-0.9)[2]
D.Bil (mg/dL)	0.55 (0.29-0.9)[2]	0.6 (0.4-1.3)[2]	0.1 (0.1-0.2)[2]
AFP (ng/mL)	41.5 (8.4-191.25)[2]	8.5 (4.1-12.5)[2]	3.1 (2.3-4.6)[2]
ANXA2 (ng/mL)	130 (15-240)[2]	15 (15-17)[2]	17 (15-30)[2]

[1]Values are given as the mean ± SD; [2]Values are given as the median (IQR). HCC: Hepatocellular carcinoma; CLD: Chronic liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; INR: International normalized ratio; Alb: Albumin; T.Bil: Total bilirubin; D.Bil: Direct bilirubin; AFP: Alpha-fetoprotein; ANXA2: Annexin A2; IQR: Interquartile range.

Table 2 Comparison of the different studied groups with regard to alpha-fetoprotein and annexin A2[1]

Parameter	HCC *vs* control		CLD *vs* control		HCC *vs* CLD	
	Z value	*P* value	*Z* value	*P* value	*Z* value	*P* value
AFP (ng/mL)	-5.006	< 0.01[2]	-3.4	< 0.01[2]	-4.17	< 0.01[2]
ANXA2 (ng/mL)	-3.5	< 0.01[2]	-1.6	> 0.05	-4.8	< 0.01[2]

[1]Wilcoxon Rank Sum test (non-parametric data); [2]Statistically significant difference. AFP: Alpha-fetoprotein; ANXA2: Annexin A2; HCC: Hepatocellular carcinoma; CLD: Chronic liver disease.

was used instead of the *t* test to compare a quantitative variable between two independent groups when the data were non parametric (SD > 25% of mean). A χ^2 test (χ^2 value) was used to compare a qualitative variable between two independent groups. The Spearman correlation test (rho value) was used to rank different non parametric variables against each other, either positively or inversely.

A P value < 0.05 was considered significant. The diagnostic accuracy of AFP and ANXA2 was determined by a receiver operating characteristic (ROC) curve analysis, the area under the curve (AUC) and its 95% confidence interval. The diagnostic cut-off, the related sensitivity and specificity, and the positive and negative predictive values (PPV, NPV) were also determined.

The statistical methods of this study were reviewed by Ahmed Mohamed Kamal, consultant in Biostatistics, Ain Shams University; Cairo, Egypt.

RESULTS

The demographic features of the included population were as follows: 33 males (66%) and 17 females (34%) with an age range from 28 to 62 years in Group 1 and 13 males (52%) and 12 (48%) females with an age range from 22 to 68 years in Group 2. The control group included 15 healthy subjects (11 males and 4 females) with an age range between 22 and 60 years.

Group 1 included 50 patients with early stage HCC on top of CLD. Among them, 44 patients (88%) were HCV-positive, 4 patients (8%) had HBV infection and two patients (4%) were negative for both viral markers and were diagnosed as cryptogenic cirrhosis.

Group 2 (CLD group) included 25 patients with CLD only (without HCC). All patients (100%) in this group were having HCV infection.

Descriptive statistics of the different laboratory parameters in all of the patient groups and the controls are shown in Table 1, Figures 1 and 2.

Comparative statistics between various groups in terms of AFP and ANXA2 using the Wilcoxon Rank Sum test (non-parametric data) are shown in Table 2. In regard to AFP, highly significant increases were observed in patients with HCC compared with patients with CLD (P < 0.01) and compared with controls (P < 0.01). In addition, the level of AFP was significantly higher in patients with CLD compared with the control group (P < 0.01). In regard to ANXA2, highly significant increases were observed in patients with HCC compared with patients with CLD (P < 0.01) and compared with controls (P < 0.01). However, a non-significant difference was observed between patients with CLD and controls with respect to ANXA2 (P > 0.05).

The correlation analysis between ANXA2 and AFP in both HCC (r = 0.22; P = 0.124) and CLD (r = 0.28; P = 0.173) patients using Spearman's rank correlation test revealed no statistically significant differences.

ROC curve analysis was performed to assess the diagnostic performance of AFP and ANXA2 in the discrimination of patients with HCC from those with CLD. This analysis revealed that the best cut-off value for AFP was 19.8 ng/mL, with a diagnostic sensitivity of 70%, a specificity of 96%, a PPV of 97.2%, a NPV of 61.5% and an efficacy of 78.7%. While the sensitivity and specificity of AFP at the cut-off value 200 ng/mL (the standard cut-off value used to diagnose HCC) was 20% and 100%, respectively, and the PPV was 100% and the NPV was 50%. The best cut-off value for ANXA2 was 18 ng/mL,

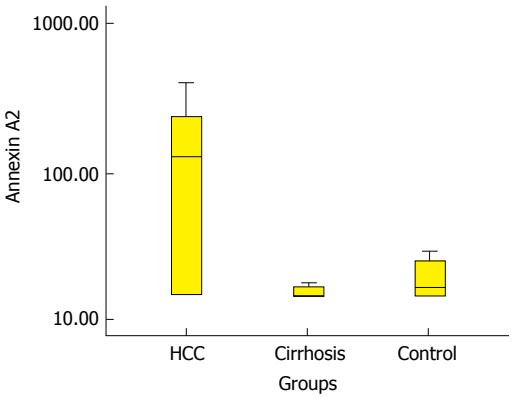

Figure 1 Box-plot diagram that shows the annexin A2 level in the three groups. HCC: Hepatocellular carcinoma.

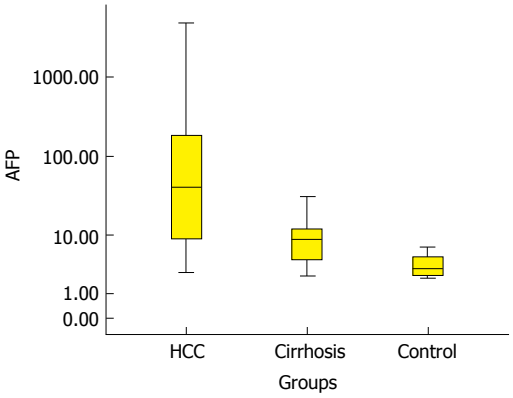

Figure 2 Box-plot diagram shows the alpha-fetoprotein level in the three groups. AFP: Alpha-fetoprotein; HCC: Hepatocellular carcinoma.

the diagnostic sensitivity was 74%, the specificity was 88%, the PPV was 92.5%, the NPV was 62.9% and the efficacy was 78.7% (Figure 3 and Table 3).

DISCUSSION

HCC is the fifth most common cancer and is the third leading cause of cancer death worldwide[14]. Unlike other solid malignancies, the coexistence of inflammation and cirrhosis makes an early diagnosis and prognostic assessment of HCC much more difficult[15]. In addition, the conventional tests of hepatic function do not distinguish HCC from cirrhosis, and thus they contribute little to the diagnosis of such tumor[16].

Detection of circulating markers is the most effective method because it is simple, accurate and cost-effective, but no ideal biomarker has been found thus far[7]. For this reason, early diagnosis of HCC is critical to ensure a good prognosis. Worldwide, ongoing and continuous studies will determine and evaluate sensitive and specific new diagnostic markers for HCC.

The imaging-based diagnosis of small tumors is relatively inaccurate, as cirrhotic and dysplastic nodules can resemble HCC, and hence, a given imaging modality cannot always differentiate between benign hepatic

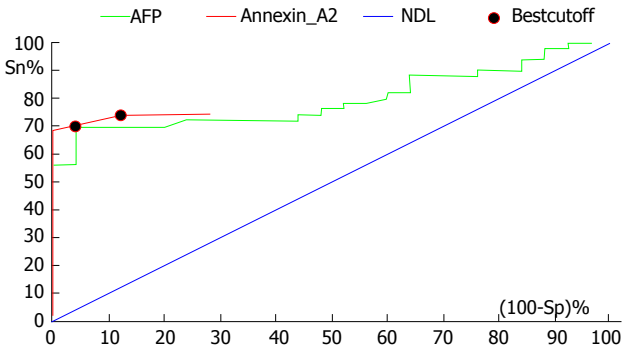

Figure 3 Receiver operating characteristic curve analysis shows the diagnostic performance of alpha-fetoprotein and annexin A2 in the discrimination of patients with hepatocellular carcinoma from those with chronic liver disease. AFP: Alpha-fetoprotein; NDL: Non-discriminating line (Diagonal line).

lesions and HCC; as a result, early and small lesions might be overlooked[17].

Since AFP was discovered in the serum of individuals with HCC in 1964, it has been regarded as the most useful serum protein marker for patients at risk for HCC. However, its sensitivity for the detection of HCC ranges between 25%-60%, and its specificity is also low since serum AFP can also be detected in patients with cirrhosis (11%-47%) and chronic hepatitis (15%-58%)[15]. In addition, highly and poorly differentiated HCC cells usually produce little AFP in contrast to the high levels that are synthesized by moderately differentiated HCC cells[18]. Therefore, the positive rate of AFP in the diagnosis of HCC is generally only 60%-70%. The availability of a more sensitive serological marker that distinguishes between HCC and benign hepatic lesions would therefore be very useful and essential for early and specific diagnosis[19]. Unfortunately, surveillance programs are hindered by the poor performance of the commonly used serum markers, namely AFP[20], even in combination with abdominal ultrasound. A great effort has been put forth and continues to be applied in the search for better HCC biomarkers.

ANXA2 is a 36-kDa calcium and phospholipid binding cytoskeletal protein of the Annexin superfamily that is localized to the extracellular surface of endothelial cells and various types of tumor cells[21]. Many reports have shown that ANXA2 is differentially expressed between normal and malignant tissues and is potentially involved in tumor progression[22]. The increased expression of ANXA2 was reported in cancers of the breast, liver, prostate and pancreas. ANXA2 has also been demonstrated to play a role in processes that are essential for cancer metastasis, such as tumor cell migration, invasion, and adhesion[5].

The purpose of this study was to determine the clinical utility of the serum level of ANXA2 as a diagnostic marker of HCC and to correlate its level with that of alpha fetoprotein, which is currently the most widely used marker for HCC.

Our study revealed that 88% of patients with HCC were HCV-positive, while only 8% of patients with

Table 3 Diagnostic performance of serum alpha-fetoprotein and annexin A2 in the discrimination of patients with hepatocellular carcinoma from those with chronic liver disease

Variable	Cut-off	Sn (%)	Sp (%)	NPV (%)	PPV (%)	Efficacy
AFP (ng/mL)	19.8	70	96	61.5	97.2	78.7
ANXA2 (ng/mL)	18	74	88	62.9	92.5	78.7

AFP: Alpha-fetoprotein; ANXA2: Annexin A2; Sn: Sensitivity; Sp: Specificity; NPV: Negative predictive value; PPV: Positive predictive value.

HCC were HBV-positive. This was in agreement with the results of El-Serag[2], Zidan *et al*[23] and Zekri *et al*[24] and reflects the close relationship between HCV and HCC. The prevalence of HCV infection in Egypt is high and its percentage in patients who develop HCC is higher than that in patients in other countries[25,26].

Our results revealed highly significant increases in the levels of AFP in patients with HCC compared with patients with CLD and subjects in the control group; this result was in agreement with that of El-Tayeh *et al*[27] and Awadallah *et al*[28]. They explained their results by an increase in the selective transcriptional activation of the AFP gene in malignant hepatocytes, which resulted in the increased secretion of AFP during the development of HCC.

Additionally, a highly statistically significant difference was observed between patients with CLD and control subjects with respect to AFP; this was in agreement with the result of Page *et al*[14], who declared that one of the limitations in the use of AFP for the diagnosis of HCC is its increase in patients who have hepatitis and CLD but who do not have HCC. El-Serag[2], stated that hepatic injury and regeneration alone (such as during active hepatitis C virus infection) can increase the serum levels of AFP in patients who do not have HCC. In 2011, AASLD guidelines[29] omitted AFP from the algorithm for surveillance and diagnosis of HCC.

By contrast, we found that ANXA2 levels were highly and significantly increased in patients with HCC compared with the levels in patients with CLD and in controls; however, no statistical significance was found between patients with CLD and the controls with respect to ANXA2 expression. This was in agreement with that of Zhang *et al*[6], Liu *et al*[22], Ibrahim *et al*[30] and Wang and Lin[31].

This was explained by Zhang *et al*[6] who stated that the ANXA2 gene is up-regulated in HBV- and/or HCV-associated HCC. In addition, Mohammad *et al*[32], stated that ANXA2 is rarely detected in either normal or chronic hepatic tissues but is over expressed at both the mRNA and protein levels in tumor and non-tumor regions of HCC (primarily localized within cancer cells).

It has been shown that the increased ANXA2 in HCC featured phosphorylation of its tyrosine residues. Interestingly, the tyrosine phosphorylation of ANXA2 was detected in HCC but not in cirrhotic tissue. These data suggest that tyrosine phosphorylation is an important event in hepatocarcinogenesis. It has been reported that ANXA2 is an excellent substrate for Src kinase. Mohammad *et al*[32] reported that the level of Src kinase activity in HCC is higher than that in cirrhotic tissues. These data suggest that ANXA2 in HCC may be tyrosine-phosphorylated *via* the elevated tyrosine kinase activity of Src or other kinases, and that increased levels of ANXA2 and the phosphorylation of its tyrosine residues may be related to human hepatocarcinogenesis.

The present study revealed no significant correlation between ANXA2 and AFP in either the CLD or the HCC group; this agrees with the study by Sun *et al*[9].

The clinical utility of AFP and ANXA2 in the discrimination of patients with HCC from those with CLD was assessed by ROC curve analysis. This revealed that the best diagnostic cut-off value of AFP for the discrimination of patients with HCC from those with CLD was 19.8 ng/mL. This had a diagnostic sensitivity of 70%, a specificity of 96%, a PPV of 97.2%, a NPV of 61.5% and an efficacy of 78.7% (AUC = 0.822). In regards to ANXA2, the best cut-off value was 18 ng/mL. This had a diagnostic sensitivity of 74%, a specificity of 88%, a PPV of 92.5%, a NPV of 62.9% and an efficacy of 78.7% (AUC = 0.873). In accordance with our results, Ibrahim *et al*[30] found that the AUC for AFP was 0.84 and that for ANXA2 was 0.89. In another Egyptian study, AUC for ANXA2 was 0.910 (95%CI: 0.84-0.97). Combining both ANXA2 and AFP increased the diagnostic efficiency (98% specificity and 97.6% PPV)[33].

In conclusion, our data show that the serum level of ANXA2 might be a good biomarker for the early detection of HCC since it had a higher sensitivity, specificity, and positive and negative predictive values than AFP. ANXA2 could differentiate between HCC and CLD since we found that the levels of ANXA2 were significantly higher in patients with HCC than in CLD patients and in controls. An in-depth analysis of the dynamic changes in serum ANXA2 in both normal and disease conditions as well as a future trial that includes a larger number of patients are emphasized.

ACKNOWLEDGMENTS

The authors thank all of the staff members of the HCC clinic (Hepatoma group) at Ain Shams University Hospitals, Cairo, Egypt.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the fifth most common malignancy in the world. Approximately 30% of individuals with HCC present with normal levels of

serum alpha fetoprotein (AFP), and therefore, this highlights the need for new biomarkers for HCC.

Research frontiers

This research study was conducted at the HCC clinic, Ain Shams University Hospitals, Faculty of Medicine, Cairo, Egypt to investigate the potential role of annexin A2 (ANXA2) as a new biomarker for HCC. Compared with AFP, the results were encouraging. A future trial that involves a larger number of patients and the combination of both markers to increase the diagnostic accuracy is strongly recommended.

Innovations and breakthroughs

The literature suggests a benefit for ANXA2 as a potential tumor marker for HCC. The current trial adds that the level of ANXA2 in patients with chronic liver disease and healthy controls was significantly lower than that in patients with HCC.

Applications

The authors' data show that the serum level of ANXA2 might be a good marker for HCC because it has a higher sensitivity, specificity, and positive and negative predictive values than AFP. ANXA2 may serve as a marker for the early detection of HCC and for the differential diagnosis between HCC and CLD because they found that the levels of ANXA2 were significantly higher in patients with HCC than in patients with CLD and in controls. An in-depth analysis of the dynamic changes in serum ANXA2 in both normal and disease conditions is therefore warranted.

Terminology

ANXA2 is an inducible, calcium-dependent phospholipid-binding protein that is overexpressed in a variety of human malignancies and has emerged as an attractive candidate receptor for increased plasmin generation on the tumor cell surface. It plays multiple roles in the cellular angiogenesis, proliferation, apoptosis, cell migration, invasion and adhesion.

Peer-review

The authors prospective case control study investigated 50 early stage HCC, 25 CLD and 15 healthy age-, sex- matched subjects with seronegative viral markers of hepatitis and normal liver function. AFP and ANXA2 were measured from each subject. Authors concluded that ANXA2 at cut-off value of 18 ng/mL was a good diagnostic marker for early HCC.

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ORIGINAL ARTICLE

Observational Study

Characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in South Egypt

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Abstract

AIM

To investigate the prevalence and virological characteristics of occult hepatitis B virus (HBV) infections in patients with hematological malignancies in South Egypt.

METHODS
Serum samples were collected from 165 patients with hematological malignancies to monitor titers of HBV DNA, hepatitis B surface antigen (HBsAg), and antibodies to HBV core (anti-HBc) and surface antigens. Serum samples negative for HBsAg and positive for anti-HBc were subjected to nucleic acid extraction and HBV DNA detection by real-time polymerase chain reaction. DNA sequences spanning the S region were analyzed in cases with occult HBV infection. *In vitro* comparative study of constructed 1.24-fold wild type and S protein mutant HBV genotype D clones was further performed.

RESULTS
HBV DNA was detected in 23 (42.6%) of 54 patients with hematological malignancies who were HBsAg negative, but anti-HBc positive, suggesting the presence of occult HBV infection. The complete HBV genome was retrieved from 6 occult HBV patients, and P120T and S143L were detected in 3 and 2 cases, respectively. Site directed mutagenesis was done to produce 1.24-fold genotype D clones with amino acid mutations T120 and L143. The *in vitro* analyses revealed that a lower level of extracellular HBsAg was detected by chemiluminescence enzyme immunoassay (CLEIA) with the clone containing T120 mutation, compared with the wild type or the clone with S143L mutation despite the similar levels of extracellular and intracellular HBsAg detected by Western blot. Southern blot experiments showed that the levels of intracellular HBV DNA were not different between these clones.

CONCLUSION
Occult HBV infection is common in patients with hematological malignancies and associated with P120T and S143L mutations. 120T mutation impairs the detection of HBsAg by CLEIA.

Key words: Occult hepatitis B infection; Hematological malignancies; Escape mutation

Core tip: The present study was conducted to investigate the prevalence and virological characteristics of occult hepatitis B virus (HBV) infections in patients with hematological malignancies in Egypt. Serum samples were collected from 165 patients with different hematological malignancies and screened for occult HBV infection. In the present study, occult HBV infection was detected in 23 (42.6%) of 54 patients with hematological malignancies who were hepatitis B surface antigen (HBsAg) negative, but antibodies to HBV core (anti-HBc) positive. Based on *in vitro* study of clones inserted with 120T and 143L, it was found that the 120T mutation could impair HBsAg detection by changing its conformation. Patients with hematological malignancies should be screened and closely monitored for anti-HBc and HBV DNA.

Elkady A, Iijima S, Aboulfotuh S, Mostafa Ali E, Sayed D, Abdel-Aziz NM, Ali AM, Murakami S, Isogawa M, Tanaka Y. Characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in South Egypt. *World J Hepatol* 2017; 9(9): 477-486 Available from: URL: http://www.wjgnet.com/1948-5182/full/v9/i9/477.htm DOI: http://dx.doi.org/10.4254/wjh.v9.i9.477

INTRODUCTION

Occult hepatitis B virus (HBV) infection is defined by the presence of HBV DNA in the liver (with or without HBV DNA in the serum) in hepatitis B surface antigen (HBsAg) negative individuals[1]. Apart from posing diagnostic challenges, several studies indicated that occult HBV infection also associates with flares of liver disease in hepatitis C virus (HCV) infected patients who do not exhibit changes in HCV RNA levels and reduces the response rate to interferon therapy[2]. Furthermore, occult HBV infection is frequently detected in cryptogenic liver diseases and autoimmune hepatitis[3-5]. HBV reactivation is a well-known complication in patients with occult infection under immune suppression, such as anticancer therapy, and human immunodeficiency virus (HIV) infection[6,7]. In addition, occult HBV infection increases the risk of HBV transmission through blood transfusion[8].

Significant advances have been achieved in understanding the molecular basis for occult HBV infection, and several factors have been implicated in the pathogenesis of occult HBV infection[9,10]. A variety of mutations in HBsAg have been reported to affect *in vitro* antigen detection, *in vivo* immune recognition, HBV infectivity, cell tropism and virion morphology[11-14]. The aim of this study was to determine the prevalence of occult HBV infection in patients with hematological malignancies in South Egypt. An *in vitro* study was performed to assess the virological characteristics of prevalent HBsAg mutations detected in patients with occult HBV infection.

MATERIALS AND METHODS

Patients

Serum samples were collected consecutively from 165 patients with hematological malignancies hospitalized in the Oncology Department of the Sohag Faculty of Medicine and South Egypt Cancer Institution from November 2010 to October 2011. All patients started their treatment regimen at the time of conduction of the study.

The serum samples were stored at -80 ℃ for future examination of HBsAg, antibodies to HBsAg (anti-HBs), antibodies to HBV core (anti-HBc), and HBV DNA.

Serological markers of HBV infection

HBsAg was measured by enzyme immunoassay (AxSYM; Abbott Japan, Tokyo, Japan) and chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan).

The IgG class of anti-HBc was determined by radioimmunoassay (Abbott Japan). Anti-HBs was tested by enzyme immunoassay (AxSYM; Abbott Japan, Tokyo Japan). Anti-HCV was tested by CLEIA (Fujirebio, Tokyo, Japan). All serologic assays were performed according to the manufacturer's instructions.

DNA extraction

DNA was extracted from serum samples (200 μL) using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and re-suspended in 100 μL of the storage buffer provided by the kit manufacturer.

Quantitation of serum HBV DNA

HBV DNA sequences spanning the entire S gene were amplified by real-time polymerase chain reaction (PCR) according to a previously described protocol with a slight modification[15]. The detection limit of the assay was 100 copies/mL (equivalent to 20 IU/mL).

HBV genomic amplification, sequencing and molecular evolutionary analysis of HBV

Extracted DNA was subjected to PCR for amplifying the complete genome of HBV as previously described[16]. Amplicons were sequenced directly using the ABI Prism Big Dye ver. 3.1 kit on the AMI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, United States). All sequences were analyzed in both forward and reverse directions. HBV genotypes were determined by molecular evolution analysis. Target sequences were aligned by CLUSTALX with reference sequences retrieved from the DDBJ/EMBL/GenBank databases, and genetic distances were estimated by the 6-parameter method in the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/)[17]. Based on the obtained distances, phylogenetic trees were constructed by neighbor-joining method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times for analysis by the ODEN program of the National Institute of Genetics.

Plasmid constructs of HBV DNA and sequencing

Various plasmids were constructed based on a consensus clone named D-IND 60 in which 1.24-fold HBV DNA genome of wild type genotype D (nt 1413-3215/1-2185) was inserted into a pUC19 vector (Invitrogen Corp., Carlsbad, CA, United States)[18].

For site-directed mutagenesis, plasmid D-IND 60 was digested by HindⅢ and EcoO65I, and ligated with the fragments carrying P120T and S143L amino acid mutations to produce the 1.24-fold HBV genome. Cloned HBV DNA sequences were confirmed using ABI Prism Big-Dye (Applied Biosystems, Foster City, CA, United States) on an ABI 3100 automated sequencer.

Cell culture and DNA transfection

The hepatoma derived cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For the standard replication assay, 1×10^6 Huh-7 cells were seeded onto a 10-cm-diamater dish, and 16 h later, transfected with 5 μg DNA constructs using a Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN, United States). Transfection efficiency was monitored by cotransfecting 0.5 μg of a reporter plasmid expressing secreted alkaline phosphatase (SEAP) and measuring SEAP activity in the culture supernatant. The supernatants and cell lysates of the transfected cells were collected 3 d after the transfection to analyze HBV markers. Three independent experiments were conducted for each clone[19].

Determination of HBV markers

HBsAg and HBcAg were determined by CLEIA using commercial assay kits (Fujirebio Inc., Tokyo, Japan). To detect the intracellular replicative intermediates of HBV, the core particle-associated HBV DNA in the cells was isolated and measured by Southern blot[20,21]. Briefly, cells were harvested and lysed in 1.5 mL of lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA and 1% IGEPAL CA-630 (Sigma-Aldrich, Japan G.K.). Total cell lysate was treated with 120 μg/mL of RNase A and 30 μg/mL of DNase Ⅰ for 3 h at 37 ℃, in the presence of 6 mmol/L magnesium acetate. HBV DNA was then released by proteinase K digestion, extracted with phenol, and ethanol precipitated. DNA was separated on a 1.2% w/v agarose gel and then transferred to a positive-charged nylon membrane (Roche Diagnostics). The membrane was hybridized with digoxigenin (DIG)-dUTP-labeled full-length HBV genotype C fragment, which was generated using the DIG High Prime DNA Labeling and Detection Starter Kit Ⅱ (Roche Diagnostics GmbH), and then detected by alkaline phosphatase-labelled anti-DIG antibody according to the manufacturer's instructions. Signals were analyzed using an ImageQuant LAS 4000 mini (GE Healthcare United Kingdom Ltd).

Western blot

Huh7 cells (1×10^6) were transfected with genotype D wild type clone or its mutants using Fugene6 (Promega, Madison, WI, United States). Transfected Huh7 cells and culture supernatants were harvested 72 h post transfection. Cells were washed with phosphate-buffered saline twice and lysed with lysis buffer (CelLytic M Cell lysis reagent; Sigma). The culture supernatants and cell lysates were quantified for HBsAg and HBcrAg by CLEIA (Fujirebio, Japan). The protocol of Western blot was previously described[22]. To detect HBsAg and HBcAg in cell lysates and viral particles, we used monoclonal antibodies specific for PreS1 and Core (Institute of Immunology, Japan), respectively. Immunoreactive proteins were visualized using chemiluminescence reagents (Immobilon; Millipore).

Statistical analysis

Statistical analyses were performed by Fisher's exact probability test and independent *t*-test for continuous

Table 1 Characteristics of patients with hematological malignant disease included in the study *n* (%)

Characteristic	Total (*n* = 165)
Age (mean ± SD)	36.1 ± 23.1
Gender (M)	89 (55.6)
ALT (mean ± SD)	29.7 ± 32.8
AST (mean ± SD)	38.2 ± 49.2
Anti-HCV(+)	39 (23.6)
HBsAg(+)	13 (7.9)
Anti-HBc(+)/HBsAg(-)	54 (32.7)
Clinical characteristics	
Malignant lymphoma	88 (53.3)
Hodgkin's disease	8 (4.8)
Acute leukemia	43 (26.1)
Chronic leukemia	23 (13.9)
Multiple myeloma	3 (1.8)
Steroid containing treatment	110 (66.7)

ALT: Alanine transaminase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antibody; HBc: Hepatitis B virus core; M: Male.

variables using SPSS software (SPSS, Chicago, IL, United States). *P*-values (two-tailed) less than 0.05 were considered statistically significant.

Ethical consideration

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from all patients.

RESULTS

Patient characteristics

The baseline characteristics of 165 patients with hematological malignant diseases are shown in Table 1. The mean age of the studied cohort was 36.1 ± 23.1 years old. Among the 165 patients with hematological malignancies, 13 (7.9%) were found positive for HBsAg, 39 (23.6%) positive for anti-HCV, and 152 (92.1%) negative for HBsAg, of whom 54 (35.5%) were serologically positive for anti-HBc. Male predominance was observed in the studied cohort (55.6%). Overt HBV and HCV co-infections were not detected in the studied cohort. Eighty-eight (53.3%) patients were diagnosed with malignant lymphoma, 43 (26.1%) diagnosed with acute leukemia, 23 (13.9%) with chronic leukemia, 8 (4.8%) with Hodgkin's disease, and 3 (1.8%) with multiple myeloma.

Occult HBV infection in the studied cohort

HBV DNA was detected in 42.6% (23/54) of the patients with hematological malignancies who were negative for HBsAg, but positive for anti-HBc, suggesting the presence of occult HBV infection. Anti-HCV was detected in 26.1% (6/23) of occult hepatitis B cases. The complete genome of HBV was successfully obtained from 6 cases with occult HBV infection. The clinical and HBV virological aspects of these 6 patients are summarized in Table 2. Four of these 6 patients were diagnosed with malignant lymphoma, and two patients were diagnosed with Hodgkin's disease and ALL each (Table 2). Their age ranged between 5 and 80 years. Two patients were serologically anti-HBs positive (Samples ID; Egl6 and EGL4). Three patients were serologically negative for anti-HBs (samples ID; U79, D1 and D14). There was insufficient serum sample to measure anti-HBs levels in one case (sample ID; A79).

Phylogenetic analysis of the retrieved complete HBV genomic sequences indicated that all isolates were of genotype D subtype D1 (Figure 1). Further analysis was applied to the major hydrophilic region (MHR) of the HBV genome, revealing the presence of two prevalent amino acid substitutions; P120T in patients Egl4, A79, D1, and D79 and S143L in U31 and Egl4 (Table 2).

HBV genomic short sequences encompassing the "a" determinant region were available in 12 of 17 samples. Of the 12 samples, escape mutation Q129R was present in 7, while P120T was detected in 4 and S143L was present in 1, suggesting that the clones with 129R mutation were from a minor population with relatively low HBV-DNA levels.

In vitro analysis of P120T and S143L mutations

To elucidate the virological characteristics of P120T and S143L mutations obtained from the complete sequences in this study, these mutations were individually inserted by site mutagenesis into a 1.24-fold replication competent HBV clone based on genotype D. Wild type genotype D clone, and two variants containing P120T and S143L mutations were transfected to Huh7 cells, and supernatants (extracellular) and cell lysates (intracellular) were collected to compare HBsAg and HBcAg levels. As shown in Figure 2A, a lower concentration of HBsAg was detected in the supernatant of Huh7 cells transfected with the P120T variant compared with wild type or S143L variant transfected cells. No significant difference was observed in extracellular or intracellular HBcrAg levels between these three clones (Figure 2B and C). Furthermore, Southern blot experiments showed that the levels of intracellular HBV single stranded DNA were not different between these clones (Figure 2D). Taken together, these results indicate that P120T strongly reduces HBsAg levels as detected by CLEIA without affecting the HBV DNA replicative capacity.

P120T mutation reduces in vitro antigenicity of HBsAg

The above data suggest that the P120T mutation either prevents the secretion of HBsAg or reduces the antigenicity of HBsAg. To distinguish between these alternatives, we examined the extracellular and intracellular expression levels of denatured HBsAg by Western blot after transfecting Huh7 cell with the wild type genotype D clone, and both the P120T, and S143L mutants. As controls, we also monitored the extracellular and intracellular expression levels of the denatured HBcAg by Western blot. As shown in Figure 3, the extracellular

Table 2 Clinical characteristics of patients with occult hepatitis B and detectable full hepatitis B virus genome

Sample ID	Age	Gender	Diagnosis	Anti-HBs (mIU/L)	HBV DNA Log (copies/mL)	Mutation[1]
EGL6	38	M	Malignant lymphoma	(+)/15	3.5	-
U31	55	F	Malignant lymphoma	(-)	3.8	S143L
EGL4	80	F	Malignant lymphoma	(+)/18.9	2.4	P120S, S143L
A79	5	M	ALL	NT	4.4	P120T
D1	70	M	Malignant lymphoma	(-)	3.9	P120T
D14	5	M	HD	(-)	3.6	P120T

[1]Mutation in the "a" determinant region of the S gene product. ALL: Acute lymphoblastic leukemia; HD: Hodgkin disease; HBV: Hepatitis B virus; NT: Not tested; M: Male; F: Female.

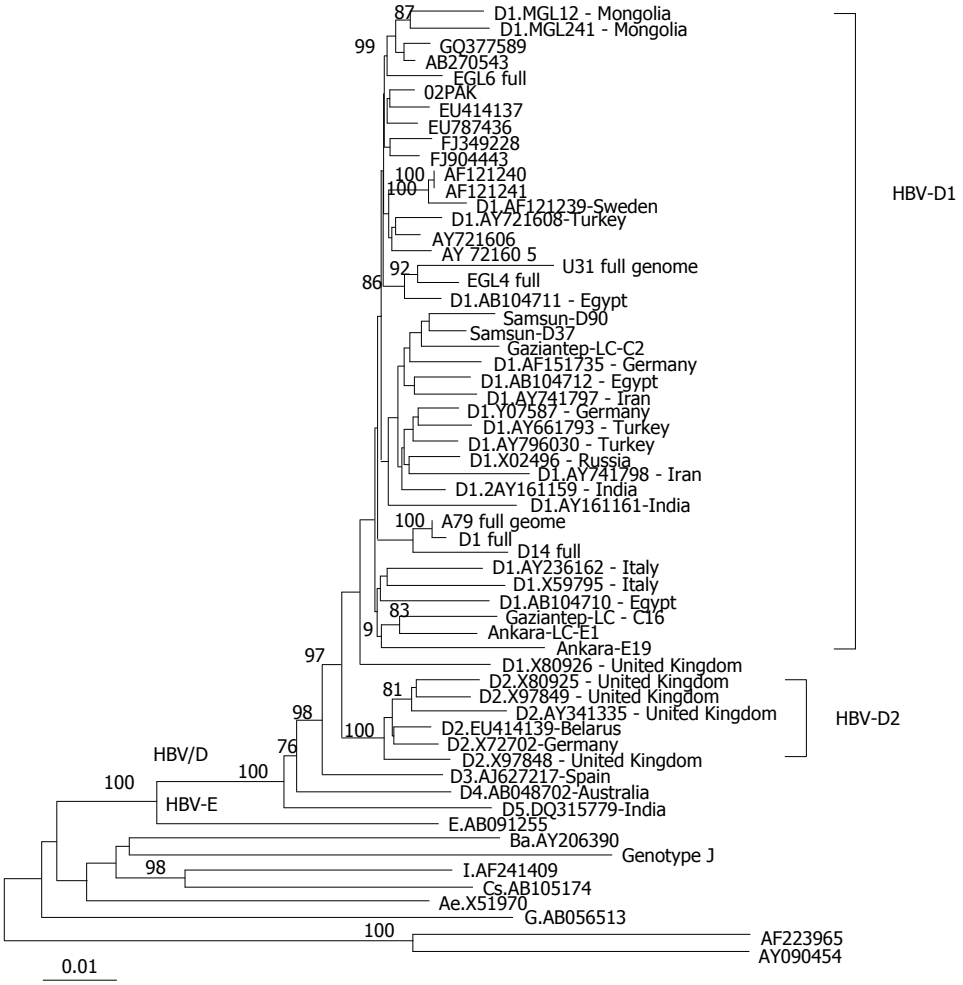


Figure 1 Phylogenetic tree constructed from the nucleotide sequences of the full hepatitis B virus genome. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (> 75%) are indicated in the tree roots. HBV sequences isolated from the studied cohort are indicated in bold. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted by their accession numbers. The country origin of the reference sequences is indicated in brackets. HBV genotypes A-H are indicated in the cluster roots. HBV: Hepatitis B virus.

expression levels of denatured HBsAg were not different between the wild type and mutants. The result suggests that the reduced HBsAg level detected by CLEIA in the supernatant of P120T mutant transfected cells was caused by reduced antigenicity *in vitro* rather than intracellular retention of the mutant HBsAg. In line with this notion, a similar amount of intracellular HBsAg was detected in cells transfected with either of the three clones (Figure 4). Collectively, these results suggest that the P120T mutation reduces the antigenicity of HBsAg *in vitro*.

DISCUSSION

Few studies have reported the characteristics of occult HBV infection with genotype D compared to reports of genotypes B and C. In the present study, we investigated the characteristics of occult HBV infections among patients with hematological malignancies in a population where genotype D infection is prevalent[23,24].

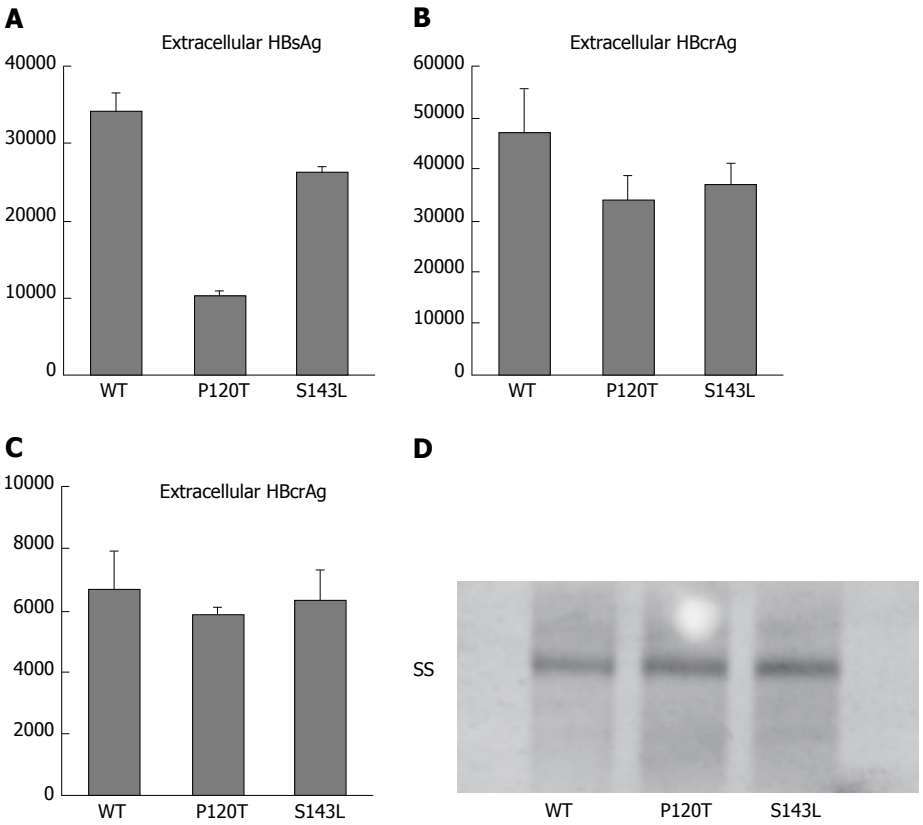


Figure 2 Expression of hepatitis B virus DNA and antigens after transfection of Huh 7 cells with wild type hepatitis B virus genotype D clone (WT) and mutants with S gene mutations P120T (120T) and S143L (143L). A and B represent the extracellular expression of HBsAg and HBcrAg, respectively, by WT and S gene mutants (120T and 143L); C represents the intracellular expression of the core gene product (HBcrAg) by WT and S gene mutant clones; D: The density of the single-stranded DNA in Southern blot analysis of cell lysates of Huh7 cells transfected with WT and mutants with S gene mutations.

We detected two previously reported escape mutations P120T and S143L that were associated with occult HBV infection[25-28], and investigated the mechanism by which these mutations cause occult infection using an *in vitro* system.

No accurate estimate regarding the prevalence of occult HBV infections in different Egyptian cohorts has been reported, perhaps due to the high expense of PCR and low budget for scientific research in Egypt[29]. However, a high prevalence rate (17.2%; 52/303) of occult HBV was reported in Egyptian blood donors positive for anti-HBc[30]. Our results indicated that the frequency of occult HBV infection among patients with hematological malignancies was even higher. Therefore, patients with hematological malignancies might be more likely to develop occult HBV infections than those without. The highest prevalence of occult HBV was reported in patients with hepatocellular carcinoma (62.5%; 25/40) in tissue samples[8,31].

HCV infection was not uncommon in patients with occult HBV. In a similar Egyptian cohort (children with hematological malignancy), 38% (15/49) were HCV coinfected patients[32]. HCV and HBV viruses shared many risk factors and routes of transmission, and more likely parenteral antischistosomal therapy was responsible for transmission of HBV and HCV in many Egyptians[33].

The presence of anti-HBs in patients with occult HBV was in line with a recent study conducted on patients treated for TB, which showed that half of patients with occult HBV infection had both anti-HBc and anti-HBs[34]. Occult hepatitis B was also detected in blood units from healthy volunteer blood donors showing an adequate level of anti-HBs[30]. Possible explanation for the presence of such cases (anti-HBc+/anti-HBs+/HBV DNA+) in HBsAg- individuals is that anti-HBs antibody is poorly neutralizing due to loss of recognition, allowing these mutant viruses to escape neutralization even when antibody is present at protective levels[35-37].

Isolated anti-HBc positive individuals with undetectable anti-HBs or HBV DNA were observed in the studied cohort. In a cohort of blood donors with anti-HBc only, the observation of the anti-HBs kinetics after administration of Engerix HBV vaccine allowed the discrimination between the naïve HBV infection with likely false positive anti-HBc, subjects with resolved HBV infection, and subjects with persistent low level replication[38]. Coffin *et al*[39] believed that the long-term presence of anticore antibodies alone is a consequence of sustained restimulation of the immune system by virus nucleocapsid produced during low-level hepadnaviral assembly.

Several studies reported the higher prevalence of mutations in the MHR region of the S gene product in HBV isolates retrieved from occult HBV cases compared to overt HBV cases[40]. In addition, Martin *et al*[41] re-

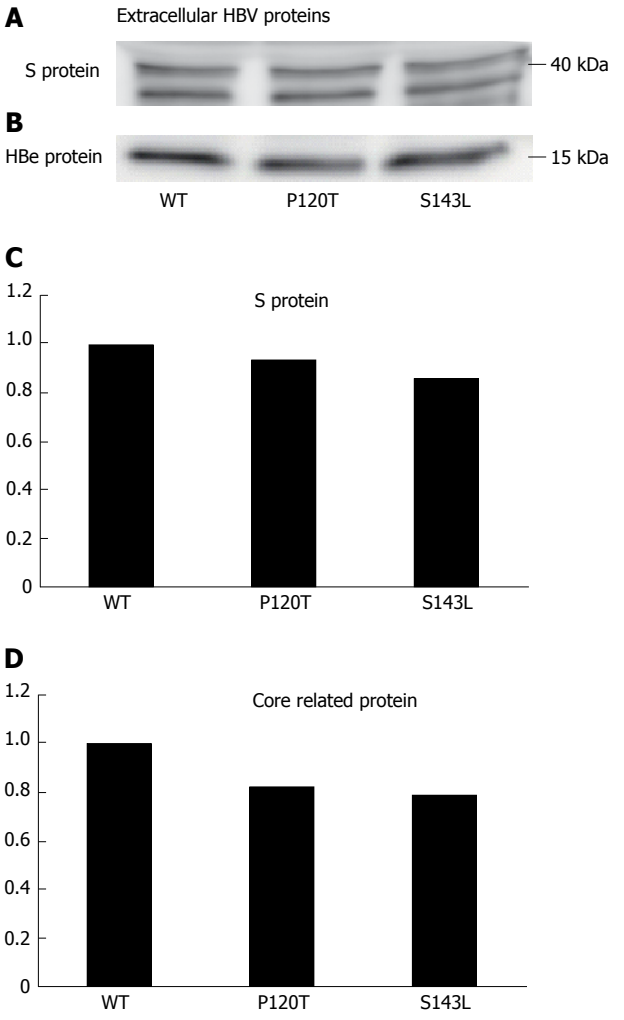


Figure 3 Western blot analysis of hepatitis B surface antigen (A, C), hepatitis B virus core (B), core related proteins (D) expressed by hepatitis B virus genotype D clones (WT) and hepatitis B surface antigen mutant clones (120T and 143L) in the supernatant of Huh 7 cells. The results for the wild type clones in the three independent transfection experiments were similar and their mean value is set at 1.0. The hepatitis B surface antigen and hepatitis B virus core proteins levels are expressed relative to this value in (C) and (D). HBV: Hepatitis B virus.

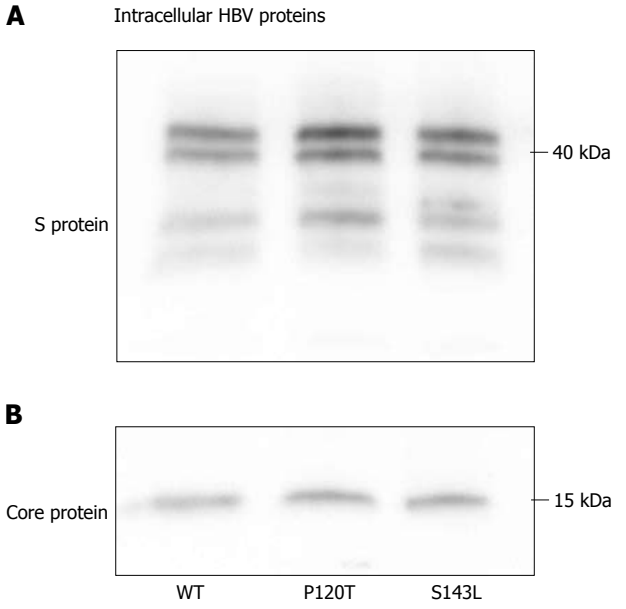


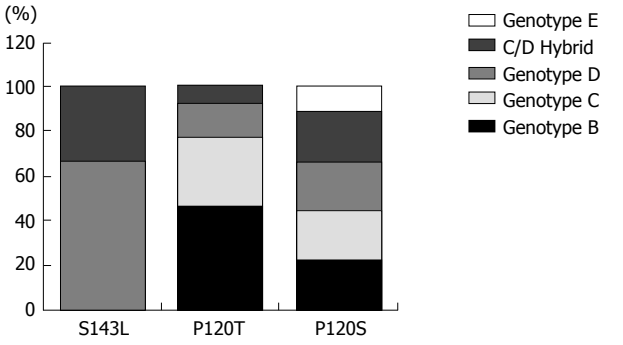
Figure 4 Western blot analysis of hepatitis B surface antigen (A) and hepatitis B virus core (B) proteins expressed by hepatitis B virus genotype D clones (WT) and hepatitis B surface antigen mutant clones (120T and 143L) in the cell lysate of Huh 7 cells. HBV: hepatitis B virus.

ported the potential virological differences between chronic HBV and occult HBV in HIV coinfected individuals and that positive selection immune pressures are acting against Pre-S and S regions in occult HBV, resulting in mutations that may adversely affect the production and/or detection of HBsAg. In concordance with previous findings, the high prevalence of mutations was observed in another immunocompromised group (patients with hematological malignancy).

The HBV complete genome was retrieved from 6 patients, and sequence and phylogenic analysis revealed that they were of genotype D1, the prevalent HBV genotype in Egypt regardless of the studied cohort. Two amino acid substitutions, P120T and S143L, were found in HBsAg.

Inspection of 2846 HBV strains currently available in DDBJ, EMBL and GenBank genetic databases indicated that amino acid substitutions 120T and 143L are present in 0.5% (13/2846) and 0.4% (9/2846), respectively, of the total isolates examined. The 143L substitution is common in genotype D strains, while 120T is common in genotypes B, C, D, C/D hybrid, and E. Collectively, these results suggest that 143L specifically occurs in genotype D, while 120T is a genotype independent substitution (Figure 5). Interestingly, 15% (2/13) and 25% (3/12) of cases infected with T/S120 and 143L variants were occult HBV infection, respectively (Figure 5). In contrast, 129R was mainly found in the short sequences (low HBV DNA) in this study as well as genotypes A, B and C isolates as previously reported[26,42,43]. Taking previous data together, further *in vitro* analysis of 120T and 143L was applied to the current study.

Both mutations are located in the MHR that extends from amino acids 99 to 169 of the HBsAg. The MHR is exposed on the surface of the antigen and is the principal binding site of anti-HBs following natural infection, and after immunization[44]. In many cases, mutations in the MHR are frequently associated with occult HBV infection[45,46], because they can change immunogenicity and render the HBsAg unrecognizable by commercially available detection assays. Clones with such mutations are often referred to as "diagnostic-escape" mutants[47]. Consistent with this, the results obtained by *in vitro* experiments showed that extracellular HBsAg with P120T was less detectable than wild type HBsAg or the mutant with S143L by CLEIA. The lower extracellular HBsAg levels observed were not due to reduced HBV replication or the intracellular retention of HBsAg. Rather, the P120T mutation appeared to reduce the antigenicity of HBsAg. A similar result was also reported by Yen *et al*[48], but their study did not address the impact of 120T mutation on HBV replication or HBsAg secretion. *In vitro* studies described the impairment of virion and/or S protein



Total (n = 2846)	n	%	Incidence of occult HBV in these variants
P120T	13	0.5	2/13 (15.4%)
P120S	9	0.3	1/9 (11.1%)
S143L	12	0.4	3/12 (25%)

Figure 5 Genotype distribution of the detected S gene product mutation among the database reference sequences. HBV: Hepatitis B virus.

secretion in both Huh7 cells and hydrodynamic injected mice by Q129R MHR mutation[49].

Our previous study demonstrated that HBV reactivation frequently occurs among patients with hematological malignancies under chemotherapy[50]. One important risk factor for the development of HBV reactivation in this critical group is the presence of occult HBV infection[50,51]. The present data suggest that patients with hematological malignancies should be screened and closely monitored for anti-HBc and HBV DNA.

In conclusion, the prevalence of occult hepatitis B was detected in patients with hematological malignancies in South Egypt in association with two mutations in the HBsAg, P120T and S143L. Neither of these mutations affected the replication activity, virion or S protein secretion but one of the mutations, P120T, interfered with detection by current commercial assays probably by inducing a conformational change. Our results highlight a challenge for detecting occult strains in developing countries.

COMMENTS

Background

Occult hepatitis B (OBI) is defined by the presence of hepatitis B virus (HBV) DNA in the serum or the liver in the absence of hepatitis B surface antigen (HBsAg) with or without anti-HBc or antibodies to HBsAg. Prevalence of OBI is different according to the endemicity of HBV. OBI is implicated in different clinical contexts including the progression of liver disease, the development of hepatocellular carcinoma, the risk for HBV reactivation, and the transmission of HBV infection. Both viral and host factors are implicated in the pathogenesis of OBI. Major hydrophilic region in genomic HBV extending from aa99 to aa169, clustered with a highly conformational epitope, is critical to the antigenicity of HBsAg and may affect the diagnosis of HBV in HBV screening tests.

Research frontiers

In a cohort of 165 patients with hematological malignancies receiving cancer chemotherapy who were negative for HBsAg, 54 patients (35.5%) were serologically positive for antibodies to HBV core (anti-HBc). Occult HBV infection was determined in 42.6% (23/54) of patients with hematological malignancies who were negative for HBsAg, but positive for anti-HBc. The complete genome of HBV was successfully obtained from 6 cases with occult HBV infection and all were of genotype D subtype D1. Two prevalent amino acid substitutions P120T and S143L were associated with OBI in the present study. *In vitro* analysis of these two amino acid mutations revealed that P120T mutation reduces the antigenicity of HBsAg *in vitro* without affecting the HBV DNA replication capacity.

Innovations and breakthroughs

This study records the high prevalence of occult HBV infection in patients with hematological malignancies. Occult HBV infection is associated with 120T and 143L mutations, and 120T mutation might impair HBsAg detection by changing its conformation.

Applications

The study strongly recommends mandatory serological screening for anti-HBc and HBV DNA in patients with hematological malignancies.

Peer-review

In this manuscript, Abeer Elkady *et al* argue characteristics of escape mutations from occult hepatitis B virus infected patients with hematological malignancies in Egypt. The authors concluded that occult HBV infection is associated with P120T and S143L mutations and 120T mutation impairs the detection of HBsAg by chemiluminescence enzyme immunoassay. The aim of this study might be interesting and important.

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CASE REPORT

Case of hepatocellular carcinoma in a patient with hereditary tyrosinemia in the post-newborn screening era

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Abstract

Hereditary tyrosinemia type 1 (HT-1) is a metabolic disorder caused by a defect in tyrosine degradation. Without treatment, symptoms of hepatomegaly, renal tubular dysfunction, growth failure, neurologic crises resembling porphyrias, rickets and possible hepatocellular carcinoma can develop. The use of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione and early diagnosis through newborn screening initiatives have resulted in a sharp decline in morbidity and mortality associated with this disease. We present a case report of a 7-year-old patient with HT-1 who was born prior to the addition of tyrosinemia to the newborn screening in her birth area. At her time of diagnosis, the patient had developed many of the symptoms associated with her disease, including chronic kidney disease, rickets, and myopathy that left her non-ambulatory. During her initial evaluation, she was also noted to have hepatocellular carcinoma. With cadaveric liver transplantation and nutritional support, her symptoms all either resolved or stabilized. Her case illustrates the severity of the disease if left untreated, the need for vigilance in populations who do not routinely receive newborn screens, and the markedly improved outcomes in patients following transplant.

Key words: Tyrosinemia; Screening; Hepatocellular carcinoma; Liver transplantation

Core tip: Hereditary tyrosinemia type 1 is a metabolic defect resulting in several disease manifestations including life threatening hepatorenal disease, neurologic disease, and rickets. Although neonatal screening for this disorder has allowed early identification and medical treatment with nitisinone, the need for recognition of this disorder in older individuals remains since aggressive intervention, including medical treatment and possible liver transplantation, may be lifesaving and have profound effects on morbidity and mortality.

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INTRODUCTION

Hereditary tyrosinemia type 1 (HT-1) is a metabolic disorder caused by a defect in tyrosine degradation due to a deficiency of fumarylacetoacetate hydrolase (FAH). In 1956, Baber provided one of the earliest descriptions of the disorder in her report of a 9 mo old child with cirrhosis, renal tubular dysfunction, and rickets[1]. As a result of advancements in amino acid analysis, a number of reports followed over the next decade in which infants with similar findings were noted to have elevations of plasma tyrosine and aminoaciduria[2-4]. In 1977, Lindblad was able to identify decreased activity of FAH as the enzyme defect responsible for the disorder[5].

The major disease manifestations of HT-1 are now well-defined and include hepatic dysfunction, renal tubular dysfunction, and peripheral nerve injury. The mechanism is most likely a result of increases in toxic metabolites, including fumarylacetoacetate, maleylacetoacetate, and succinoacetylacetate. Most affected children present in infancy with an acute form of the disease associated with failure to thrive, severe liver dysfunction, and death in infancy if untreated. A chronic form may also occur with hepatomegaly, renal tubular dysfunction, growth failure, neurologic crises resembling porphyrias, and rickets. Hepatocellular carcinoma appears to be a common finding in both forms of untreated disease and may be noted in up to 37% of children that survive beyond 2 years of age. The incidence of this tumor increases with age and is reported to be the cause of death in over 50% of untreated individuals[6].

We report our recent experience in a 7-year-old female who underwent liver transplantation for advanced hepatocellular carcinoma in association with a delayed diagnosis of HT-1.

CASE REPORT

A 7-year-old Hispanic female with an uneventful birth history presented to our referral institution for workup and management of balance and gait disturbance and an inability to walk that was first noted at 3 ½ years of age. She was noted to have increasing "clumsiness" in the months leading to her inability to walk. She was also noted to have chronic renal disease and liver disease with hepatomegaly and enlarged kidneys in the first 4 years of life. Her inability to walk was previously attributed to severe pain throughout her body.

At 6 years of age, she developed bilateral lower extremity fractures and an upper extremity fracture while attempting to stand. She was noted to have bowing of her lower extremities and elevated alkaline phosphatase and was subsequently diagnosed with rickets that was believed to be the result of chronic kidney disease. Shortly thereafter, she was referred to a local medical center where she underwent liver biopsy, kidney biopsy, and muscle biopsy. Her liver biopsy revealed cirrhosis with minimal chronic inflammation and her kidney biopsy revealed nonspecific glomerular and tubular changes with some parenchymal fibrosis. Her muscle biopsies revealed severe myopathic changes with myofiber atrophy. She was noted to have mitochondrial DNA quantification on her muscle biopsy which was reduced and less than 29% of controls. She was subsequently diagnosed with mitochondrial depletion syndrome.

When her weakness and strength worsened further, she was referred to our institution's mitochondrial clinic where she underwent additional workup. Extensive metabolic evaluation revealed an elevated succinylacetone which led to a diagnosis of HT-1. Analysis of the *FAH* gene revealed a homozygous splice mutation known to be associated with HT-1. She ultimately underwent treatment with dietary modification and 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), but she was immediately referred for cadaveric liver transplantation after further workup revealed imaging findings consistent with hepatocellular carcinoma. Following this initial pre-transplant treatment, her condition improved substantially with significant improvement in her amino acid profile, improvement in her mental status, and reduction in her alpha-fetoprotein from 2320 ng/mL to 1585 ng/mL. Despite this metabolic improvement, she required liver transplantation as a result of her hepatocellular carcinoma. Since this lesion was felt to be adjacent to major blood vessels and liver transplantation was not ideal without improved nutrition and rehabilitation, she underwent transarterial chemoembolization for a 1.5 cm × 1.6 cm lesion in her liver (Figure 1) after which time she had further reduction in her alpha-fetoprotein to 511 ng/mL. After continued improvement in nutrition and physical conditioning, she ultimately underwent liver transplantation 4 mo after her diagnosis was made. Explant of her liver revealed a yellow-orange and nodular liver, and histology revealed cirrhosis and multinodular well-differentiated hepatocellular carcinoma with bile duct proliferation felt to be consistent with tyrosinemia (Figure 2).

Her immediate postoperative course was unremarkable and, following transplant, she had further improvements in her cognitive function, nutrition, and physical conditioning.

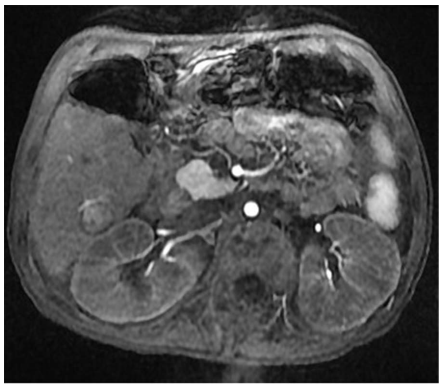

Figure 1 Magnetic resonance imaging of the liver with a large 1.5 cm × 1.6 cm lesion with arterial enhancement characterizing hepatocellular carcinoma and requiring chemoembolization.

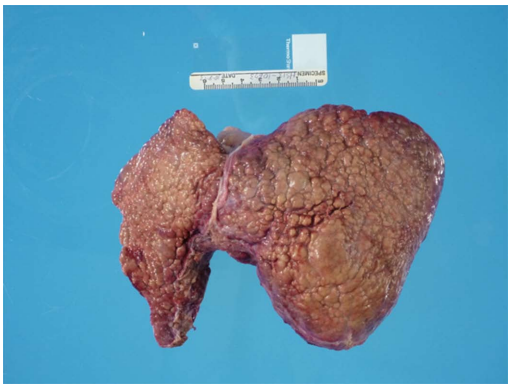

Figure 2 Explant of nodular and shrunken tyrosinemic liver obtained at the time of liver transplantation.

Within 6 mo, she was ambulating without assistance and attending regular school without difficulty. Her rickets had also improved and resolved with her bone mineral density Z-score in her hip improving from -4.1 prior to transplant to -1.1 fifteen months following transplant. Her renal function improved with her estimated GFR before and 6 mo after transplant noted to be 75 and 121 mL/min per 1.73 m^2 respectively. She had no recurrence of tumor on follow-up imaging obtained 2 years following her transplantation, and her alpha-fetoprotein was normal during this time.

DISCUSSION

While HT-1 can result in a variety of multisystem and life-threatening complications, including hepatic and renal disease, recent approaches to management over the past few decades have resulted in a sharp decline in morbidity and mortality associated with this disease. Since Lindstedt first published his experience using NTBC in 5 patients with HT-1 in 1992, its incorporation into the standard treatment for HT-1 has resulted in improved effects on the long term outcome of individuals with this disease with improved metabolic control[7]. In addition, newborn screening for HT-1 is a common practice in most Western countries allowing early identification and treatment of affected individuals as early as the first month of life. In the large Quebec experience, infants beginning treatment with NTBC in the first month of life had no detectable liver lesions after more than 5 years of follow-up and no need for liver transplantation[8]. The combined effects of NTBC use and newborn screening have resulted in a significant reduction in the need for liver transplantation noted over the past decade[9].

Since our patient was born in 2006, one year prior to initiation of newborn screening in her region, her diagnosis was not identified early in life. Her presenting symptoms of developmental delay and weakness which were first noted at 3 years of age led to an underlying diagnosis of mitochondrial depletion syndrome. Interestingly, electron microscopy of tissue from individuals with HT-1 has revealed mitochondrial abnormalities with a relative loss of matrical bodies and decreased matrix density[10]. Like tyrosinemia, mitochondrial depletion syndromes may involve a variety of organs, including the liver, kidney, and peripheral nervous system. Her abnormal movements and unusual behavior could also be attributed to an underlying mitochondrial or primary neuromuscular disorder although these resolved after dietary and NTBC were started.

Marked elevations in alpha-fetoprotein were noted in our patient. Elevated alpha-fetoprotein is a typical finding in HT-1, even in the absence of hepatocellular carcinoma. Improvement in alpha-fetoprotein is also noted with improvement in metabolic control in HT-1 patients as we saw in our patient. Nevertheless, our patient required extensive workup to rule out hepatocellular carcinoma given the obvious risk of hepatocellular carcinoma in individuals with tyrosinemia, her continued markedly elevation alpha-fetoprotein, and her delayed age at diagnosis.

The presence of renal dysfunction in individuals with HT-1 requires consideration for combined liver-kidney transplantation in some individuals. Continued exposure to calcineurin inhibitors may cause significant deterioration in kidney function in some individuals receiving liver transplantation. In the Quebec experience, combined liver-kidney transplant may be warranted when the GFR < 40 mL/min per 1.73 m^2[11]. Despite her late age at diagnosis, our patient had a GFR which was slightly below normal but still sufficient enough to make isolated liver transplant a reasonable option.

This case highlights a number of important points. The presence of liver disease in association with other uncharacteristic organ pathology warrants consideration for an underlying metabolic disorder. Despite great advancement in newborn screening, metabolic disorders such as HT-1 are still possible due to imperfection of neonatal screening and potential for missed populations. Recent political and economic upheaval has introduced a large number of migrants into Western countries who often may not have had screening for a variety of metabolic or genetic disorders. In addition, children

receiving transplantation for metabolic liver disease have improved outcomes compared to children transplanted for other disorders, such as biliary atresia, with 1- and 5-year survival of 95% and 89%[12]. Finally, the presence of severe extrahepatic disease such as severe neuromuscular disease in our patient should not deter consideration for transplantation since liver transplantation can result in remarkable improvement and reversal in neurologic and cognitive dysfunction, particularly in children.

COMMENTS

Clinical diagnosis

The symptoms of hereditary tyrosinemia include hepatorenal dysfunction, neuromuscular symptoms, and rickets.

Differential diagnosis

The differential diagnosis of hereditary tyrosinemia includes disorders of carbohydrate metabolism, mitochondrial disorders, and Wilson's disease.

Laboratory diagnosis

Laboratory diagnosis of tyrosinemia includes confirmation by noting the presence of succinylacetone in urine specimens or body fluids, identification of causative mutations, or enzyme analysis revealing decreased fumarylacetoacetate hydrolase.

Imaging diagnosis

Imaging in tyrosinemia may reveal complictations of this disorder, including cirrhosis, hepatomegaly, hepatocellular carcinoma, renal abnormalities, and rickets.

Pathological diagnosis

Pathological diagnosis of this disorder is obtained by laboratory methods described above.

Treatment

Treatment of tyrosinemia involved use of nitisinone and a protein restricted diet in conjunction with long term management by a specialist. Liver transplantation may be required for severe disease or complications of this disorder.

Experiences and lessons

A diagnosis of hereditary tyrosinemia should be considered in individuals with hepatorenal disease, rickets, and neuromuscular weakness since rapid initiation of aggressive treatment may be lifesaving.

Peer-review

Imseis E *et al* reported about a case of hepatocellular carcinoma in a patient with hereditary tyrosinemia, a kind of rare lesion, in the post-newborn screening era. This case report is very interesting.

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