HCC heterogeneity: Molecular pathogenesis and clinical implications

Emilia Fransvea [a], Angelo Paradiso [b], Salvatore Antonaci [a] and Gianluigi Giannelli [a,*]

[a] *Department of Internal Medicine, Immunology, and Infectious Diseases, Section of Internal Medicine, University of Bari Medical School, Bari, Italy*
[b] *Clinical Experimental Oncology Laboratory, National Cancer Institute, Bari, Italy*

Abstract. *Background*: Hepatocellular carcinoma (HCC) poses a major challenge because of the extreme variability of the clinical outcome, which makes it difficult to properly stage the disease and thereby estimate the prognosis. There is growing evidence that this heterogeneous clinical behavior is attributable to several different biological pathways. A novel approach to mapping these differences is by investigating the epigenetics associated with certain clinical aspects.

Design: Herein, the relevance of these molecular differences in combination with the biological and molecular pathways regulating the clinical outcome will be discussed. Use of a mechanistic and pathogenic approach to clarify the natural history of HCC is not just an academic speculation but should help to develop new therapies and to tailor these therapies to each individual patient.

Conclusion: New biological therapies targeting components of the tumoral or peritumoral microenvironment are crucial to the fight against HCC. However, biological redundancies and the presence of several growth factors, hormones, cytokines, etc., potentially involved in HCC tumor progression make it difficult to assess the best target. Sorafenib, a multi-tyrosine kinase inhibitor, blocks the functions of different growth factors present in the tissue microenvironment. The use of Sorafenib in patients with HCC offers a new approach to the therapy of this disease, stimulating research focusing on the development of drugs based on new molecular and pathogenic insights.

Keywords: Biological therapies, HCC, molecular pathogenesis, TGF-β1, tissue microenvironment, TK-receptors, tumor progression, heterogeneity

1. Introduction

Hepatocellular carcinoma (HCC) has long been considered simply as the last stage of chronic progressive liver damage. Initially, the total lack of therapies discouraged clinicians from performing instrumental diagnoses. In the last twenty years, considerable success has been achieved in terms of improved diagnosis, therapies and hence life expectancy. Nevertheless, with this better knowledge has come the realization that we are still only scraping the surface. Despite improved diagnostic tools, clinicians still complain of the unacceptable number of cases diagnosed too late and, therefore, not suitable for "curative" therapies. In addition, even in the case of patients apparently successfully treated with radio-frequency ablation, or better with liver transplantation or surgical resection of the tumor, HCC recurrence is a common event and still poses a difficult challenge. Not all the reported features of HCC apply to all patients, each of whom presents a different clinical outcome without any apparent explanation.

An important issue that might contribute to elucidate the heterogeneity of HCC is the different etiologies. It has recently been reported that non-alcoholic steatohepatitis may develop into HCC through a MYC-independent mechanism, while viral and alcohol-dependent diseases display up-regulation of MYC, suggesting that at least some genetic alteration may depend on the different etiology of the underlying liver disease [44]. The oncogene has been reported to be responsible for cancer dormancy in a murine model, in which its inactivation led to a switch to a non-invasive but still cancer-transformed HCC phenotype [45]. This could contribute to explain the irregular clinical out-

*Corresponding author: Gianluigi Giannelli, Dipartimento di Clinica Medica, Immunologia e Malattie Infettive, Sezione di Medicina Interna, Policlinico, Piazza G. Cesare 11, 70124 Bari, Italy. Tel.: +39 080 5478 127; Fax: +39 080 5478 126; E-mail: g.giannelli@intmed.uniba.it.

come of patients with HCC, who show slow or fast periods of disease progression for no apparent reason.

2. HCC is a heterogeneous cancer

HCC is a peculiar type of cancer because in Western and North American countries it commonly develops in livers damaged by the pre-existing underlying chronic liver disease, while all other malignancies occur in otherwise healthy organs. This has to be taken into due account in the management of therapies, partly because it is not possible to rule out the possibility that the liver damage could affect the clinical outcome of these patients [9]. However, this does not seem to suffice to explain the different clinical outcome, prognosis and life span of patients with HCC. Unlike all other malignancies, in which it is essential to classify patients according to the TNM or other specific criteria in order to proper stage the tumor and so choose the optimal therapy, no widely accepted validated classification is yet available for HCC. Different systems including the TNM, Okuda, CLIP and Barcelona criteria have been used, but none is able to satisfactorily predict patients prognosis and survival [26,30,37,40].

What makes it so hard to define a reliable staging for HCC? It is probably the difficulty in gating patients according to common characteristics, because what HCC patients actually have in common is the great heterogeneity of their disease [6,31]. This hypothesis, although difficult to accept in view of the better knowledge of the cancer biology of all other malignancies, is gradually gaining strong support in the case of HCC. A better understanding of this issue is of paramount importance because of its clinical implications, in terms of which therapy to choose and in a wider sense, how to predict which patients with LC will likely develop HCC, and so change the natural history of chronic liver disease.

3. Genetic and epigenetic differences

Recently, a more technological approach has been used to classify patients according to genetic characteristics: grouping patients according to common molecular features derived from gene expression profile studies. In theory, such a classification should help to tailor targeted therapies. In the last few years, various studies have explored this hypothesis, reporting that an altered gene expression is suggestive of clinical aspects such as tumor growth rate, etiologic differences, tumor recurrence and the development of metastases [21,24,39].

However, these data are not conclusive because common and reproducible expression patterns have not yet been identified in the different studies, likely because of different sample collection and different methodologic approaches. In addition, the biological relevance of these findings remains to be clarified, considering that a number of alterations may occur during hepatocarcinogenesis that have no biologic role, and that common biologic redundancy can lead down alternative pathways. It must also be borne in mind that different phenotypic alterations of HCC can occur according to the stage of the disease, and reverse during tumor progression, thus further confusing the issue. Integration of the genomic profile with protein-based studies and with biological demonstrations of proposed mechanisms should provide seminal information for the clinical management of HCC.

A fascinating hypothesis that could contribute to explain the heterogeneity observed in HCC patients has been made in a recent study investigating differences at the transcriptional level between human HCC samples and fetal hepatoblasts. This study identified a cluster of patients with a higher growth rate and poorer prognosis who shared a common gene expression, likely derived from the adult cancer stem cell, with fetal hepatoblasts [25]. This was the first study to propose cancer stem cells as a discriminating factor in the clinical outcome of patients with HCC. However, in this case all the tumor samples were obtained from Chinese patients, who have a very different type of HCC from the disease observed in Western countries. On the other hand, the possibility that HCC cells can de-differentiate as a result of the molecular pathways originated by the surrounding tumor microenvironment should also be taken into consideration. This represents a further genetic alteration occurring in the course of HCC, that again stresses the complex, multi-step process of hepatocarcinogenesis.

4. Biological mechanisms

The biological functions of all epithelial cells, such as proliferation, migration, invasion, differentiation, apoptosis, etc., depend on cross talk among extracellular matrix (ECM) proteins, growth factors, hormones, and on cellular receptors that ensure "inside–outside" signalling. In the case of HCC, it seems likely that

different clinical outcomes may be modulated by different biological pathways, because the tumor cells are embedded in tissue enriched by ECM proteins, deposited as a consequence of the underlying liver cirrhosis. This tissue undergoes constant proteolytic remodelling, so that new cryptic sites of these molecules are unveiled and/or others are degraded. Consequently, some biological pathways can be switched on and others switched off, this dynamic balance being finely regulated by the proteolytic balance of matrix metalloproteases (MMPs), as degrading enzymes, and tissue inhibitors of MMPs (TIMPs), as their specific inhibitors [16,38,53]. These modifications are involved in carcinogenesis as well as in determining the aggressiveness of the cancer phenotype. In HCC metastatic spread, there is up-regulation of the transcription factor, Snail, regulating the expression of a tissue membrane MMP, MT1-MMP, that ultimately correlates with portal invasion and poorer survival [35]. These mechanisms are closely related to the epigenetic modifications discussed above, so that it is impossible to establish a chronological hierarchy.

In this context, cancer epithelial cells acquire a less differentiated mesenchymal phenotype characterized by an up-regulation of the zinc finger transcription factors Snail, Slug and SIP-1 that down-regulate the expression of E-cadherin, translocate β-catenin into the nuclei and promote a change in the cellular morphology, that assumes a more elongated shape [36,49]. All these modifications are known as the epithelial to mesenchymal transition (EMT). Interestingly, the opposite process, known as the mesenchymal to epithelial transition (MET), also occurs at the same time in malignancies, so that a dynamic situation named "plasticity" has been described [8,27,49]. In colorectal carcinoma, continuous EMT/MET processes lead to tumor progression and cancer stem cell formation (reviewed in [4]). These data further suggest that tumor progression is a dynamic process, so that clustering the patients only on the basis of transcriptional data will reflect the real status of the malignancy at that given moment but fail to interpret the whole behavior of the disease.

5. Heterogeneity of the tissue microenvironment

In HCC, the transforming growth factor beta-1 (TGF-β1) is an example of how proteolytic tissue remodelling commonly occurring in cancer modulates cancer behavior by changing the biological properties of tissue microenvironment components. TGF-β1 is secreted in latent form and stored in the extracellular space, becoming active after proteolytic processing by MMP-9 and MMP-2 [17,54]. These enzymes are abundantly secreted in several malignancies, including HCC where an imbalance between MMP-2 and TIMP-2 has been reported [16,38,48,53]. Active TGF-β1 has an opposite role in tumors, behaving as a tumor suppressor inhibiting proliferation and stimulating apoptosis, but also as a tumor promoter triggering metastatic spread (reviewed in [11,46,51]). HCC cells are refractory to tumor suppression but sensitive to the tumor promoter function [5,42]. Therefore, activated TGF-β1 induces an EMT phenotypic transformation of HCC cells via an up-regulation of the zinc-finger transcription factors Snail, Slug and Sip-1 with a consequent down-regulation of E-cadherin and nuclear translocation of β-catenin [22,33,35,47,49].

A situation that can be used as a model of cooperation among different molecular pathways has recently been described. HCC invasive cells, in which the TGF-β1 pathway is active, constitutively and partially EMT-transformed, acquire a fully aggressive phenotype in the presence of Laminin-5 (Ln-5), a member of the Laminin family [7]. In addition, other HCC non-invasive cells undergo a complete EMT transformation following TGF-β1 and Ln-5 stimulation in a dual-step process [15]. Consistent with this hypothesis, selective inhibition of the TGF-β1 pathway by the LY2109761 compound, which competes with ATP for the phosphorylation site of the intracellular tail of TGF-β receptor I, reduces HCC cell migration and invasion via the up-regulation of E-cadherin. Therefore, this inhibitor induces a MET of the more invasive and aggressive HCC cells [12].

These, data that represent the first mechanistic evidence of the role of TGF-β1 in HCC, combined with a number of studies reporting increased levels of TGF-β1 in patients' biological fluids as the hallmark of the disease [2,50], present TGF-β1 as a potential target for clinical therapies to prevent cancer spread.

6. Different tumor growth

The epidermal growth factor (EGF), like other growth factors, activates a number of tyrosine-kinase (TK) receptors sharing common signalling pathways and promoting cell proliferation. This has led to the use of anti-EGFR drugs such as Iressa, that blocks cancer growth and metastases in the treatment of lung and

colon cancer [10,18,23], as an alternative therapy in advanced tumors [19].

In HCC, highly differential rates of tumor growth (ranging from 1 to 20 months [9]), likely depend on an altered regulation of growth factor signalling pathways. For instance, an alternative pathway promoting HCC proliferation is regulated by Ln-5 but not other ECM proteins. This effect requires phosphorylation of the Erk1/2 pathway, as it does for EGF. This pathway requires the presence of the receptors for Ln-5, $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, that cooperate in a sort of "inside–outside" signalling. In HCC tissue both $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins receptors are only expressed in the area where Ln-5 and Ki-67 are also expressed, but HCC cells do not express both integrins in all cases, which helps to explain why some tumors grow faster than others (Fig. 1) [3]. In contrast, TGF-$\beta 1$ induces strong expression of integrin $\alpha 3\beta 1$ on HCC cells, modulating the phenotype of these cells even during the natural history of the disease [17]. This observation could help to explain why after some years a dormant tumor suddenly displays an increased rate of tumor growth. A better knowledge of these biological pathways is crucial in order to be able to choose the most

suitable drug-based therapies and evaluate their efficacy.

From this perspective, preclinical studies of Iressa represent a paradigm. In an "*in vivo*" orthotopic model Iressa reduces HCC growth and metastases via inhibition of MMPs activity in a cirrhotic rat model [34,43]. Consistently, in an "*in vitro*" model Iressa inhibits HCC cell proliferation and survival; however, in the presence of Ln-5 but not of Collagen I, Collagen IV, Fibrinogen, Fibronectin, Vitronectin or Laminin-1, the therapeutic effectiveness is abolished [13]. This effect was not due to a direct phosphorylation of the EGFR receptor, but likely to a proliferative function of Ln-5, reducing the efficacy of Iressa. Therefore, an analytical study of the molecular and biological role of the tissue surrounding HCC is crucial not only to predict the clinical outcome of the disease but also to be able to better tailor the therapy to each individual patient.

7. Therapies based on biological targets

We are at the dawn of a new era of drug-based therapies in patients with HCC. This is obviously of para-
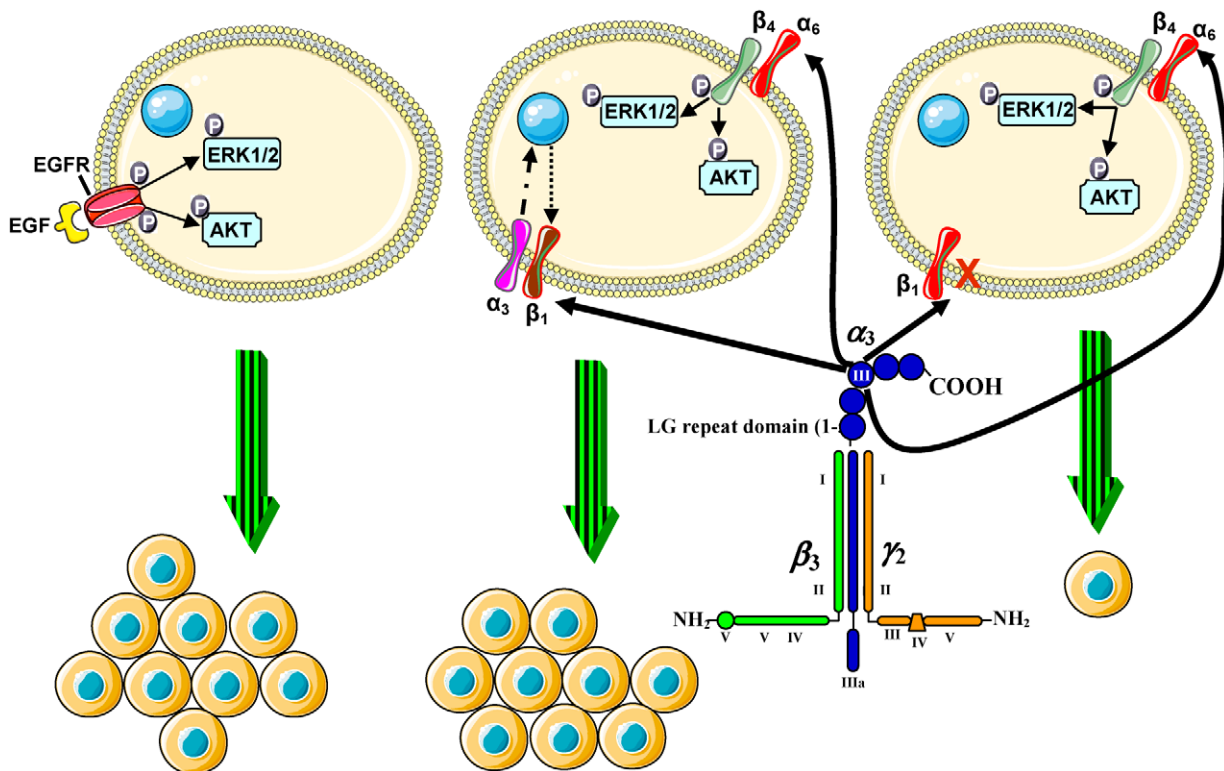

Fig. 1. Schematic representation of HCC tumor growth.

mount importance considering that at the time of diagnosis, nearly 40% of HCC patients are ineligible for surgical therapies [29]. The idea of extending the knowledge of biological therapies gained in other malignancies to HCC has already been discussed in the literature. A small TK quinazolamine inhibitor such as Iressa reduces HCC cell proliferation "*in vitro*", enhancing the cytostatic or cytotoxic effect of doxorubicin and cisplatin [20,55]. In a clinical phase II study, Erlotonib, a molecule pharmacologically similar to Iressa, induced a progression-free period of six months in about 30% of the patients [41]. Nevertheless, in the same period, the NIH stopped a clinical trial in HCC patients using Iressa due to poor efficacy, and the original promising study by Philip had no successor in the literature. Other agents with inhibitory effects against a larger range of TK receptors have been developed and tested in preclinical studies. These include SU5416 and ZD6474, inhibitors of EGFR and vascular endothelial growth factor receptors showing promising results thanks to their multi-functional activity [14,52].

Sorafenib, a broader multikinase inhibitor with activity against Raf kinase and other TK receptors, showed a strong antitumoral activity in HCC preclinical studies in "*in vitro*" and "*in vivo*" models [28]. Based on these promising data, a phase II clinical trial was completed with encouraging results in terms of low toxicity and a phase III randomized study was then completed, showing a significant increase in life expectancy by about 34 weeks [1,32]. Sorafenib will likely become the gold standard therapy for all patients not eligible for other invasive treatments, and the treatment could also be extended to those patients receiving so-called "curative" therapies. Furthermore, even if Sorafenib therapy, although encouraging does not seem to dramatically change the clinical outcome of patients with HCC, it opens new perspectives for developing new drugs against biological targets.

8. Conclusions

The heterogeneity of HCC is the main challenge we need to face. There is no doubt that the combination of genetic and biological studies will rewrite the natural history of HCC, enabling earlier identification of cirrhotic patients at higher risk of cancer development. A better knowledge of the molecular and biological mechanisms underlying HCC progression is critical to the design of targeted drug-based therapies and tailored, individualized therapies. Sorafenib currently offers new hope for HCC patients and even if it does not dramatically change the long term prognosis, may pave the way for the coming "era" of individualized, biological therapies for HCC.

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