

Transforming Growth Factor- β as a Therapeutic Target in Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma arises in patients as a consequence of long-standing preexisting liver illnesses, including viral hepatitis, alcohol abuse, or metabolic disease. In such preexisting liver diseases, TGF- β plays an important role in orchestrating a favorable microenvironment for tumor cell growth and promoting epithelial–mesenchymal transition (EMT). TGF- β signaling promotes hepatocellular carcinoma progression by two mechanisms: first, via an intrinsic activity as an autocrine or paracrine growth factor and, second, via an extrinsic activity by inducing microenvironment changes, including cancer-associated fibroblasts, T regulatory cells, and inflammatory mediators. Although there is an increasing understanding on how TGF- β signaling is associated with tumor progression in hepatocellular carcinoma, it is not clear whether TGF- β signaling is limited to a certain subgroup of patients with hepatocellular carcinoma or is a key driver of hepatocellular carcinoma during the entire tumorigenesis of hepatocellular carcinoma. Inhibitors of the TGF- β signaling have been shown to block hepatocellular carcinoma growth and progression by modulating EMT in different experimental models, leading to the clinical investigation of the TGF- β inhibitor LY2157299 monohydrate in hepatocellular carcinoma. Preliminary results from a phase II clinical trial have shown improved clinical outcome and also changes consistent with a reduction of EMT. *Cancer Res*; 74(7); 1890–4. ©2014 AACR.

Introduction

Since its discovery in the early 1980s, TGF- β signaling has been increasingly recognized as a key driver in cancer (1, 2). Unlike its tumor suppressor function in normal tissue, TGF- β activation causes tumor promotion in cancer tissue. The switch from tumor suppression to promotion is not well understood, but intrinsic and extrinsic factors seem to play important roles. The loss of cell polarity, acquisition of motile properties, and a mesenchymal phenotype during epithelial–mesenchymal transition (EMT) are considered crucial intrinsic changes of the tumor cells (3). These tumor promotion changes mediated by TGF- β signaling are also accompanied by extrinsic factors originating from the tumor microenvironment, such as angiogenesis, inflammation, and fibroblast activation. In addition to changes in the tumor tissue, alterations in the TGF- β signaling pathway can also contribute to tumor growth. The TGF- β signaling occurs via a canonical and a noncanonical pathway. The canonical TGF- β signaling pathway is activated

when one of the three ligands (TGF- β 1, TGF- β 2, TGF- β 3) binds to the TGF- β receptor II (TGF- β RII), heterodimerizes with the TGF- β -receptor I (TGF- β RI or ALK5), and transphosphorylates the kinase domain of both receptors. This phosphorylation step leads to a recruitment and phosphorylation of SMAD2 and SMAD3. After this phosphorylation, a SMAD signaling cascade is initiated and it results in nuclear translocation and gene transcription for a wide range of tumor-promoting mediators (4). In some tumors (including hepatocellular carcinoma), SMAD4 is mutated, which alters the TGF- β signaling pathway, rendering the pathway inactive. The less-known noncanonical activation pathway is associated with several intracellular phosphorylation of proteins, such as jun N-terminal kinase (JNK), p38 MAPK, ERK, or MEKK. For example, the TGF- β inhibitor LY2109761, a surrogate compound of LY2157299, has shown inhibitory activity by blocking dephosphorylating FAK, β ₁-integrin, MEK, ERK, AKT, mTOR, and PTEN but not p-38-MAPK-kinase (5). This suggests that TGF- β signaling has several mechanisms to activate either the tumor cell or cells of the microenvironment.

TGF- β Activation in Liver Disease Predisposing Hepatocellular Carcinoma

A growing number of studies suggest the hepatocellular carcinoma onset and progression is not just dependent on the tumor cells or injured hepatocyte, but on other factors as well. Using a more holistic view, it is becoming increasingly clear that the cross-talk between tumor cells and host stroma plays a key role in tumor growth. In such a context, TGF- β

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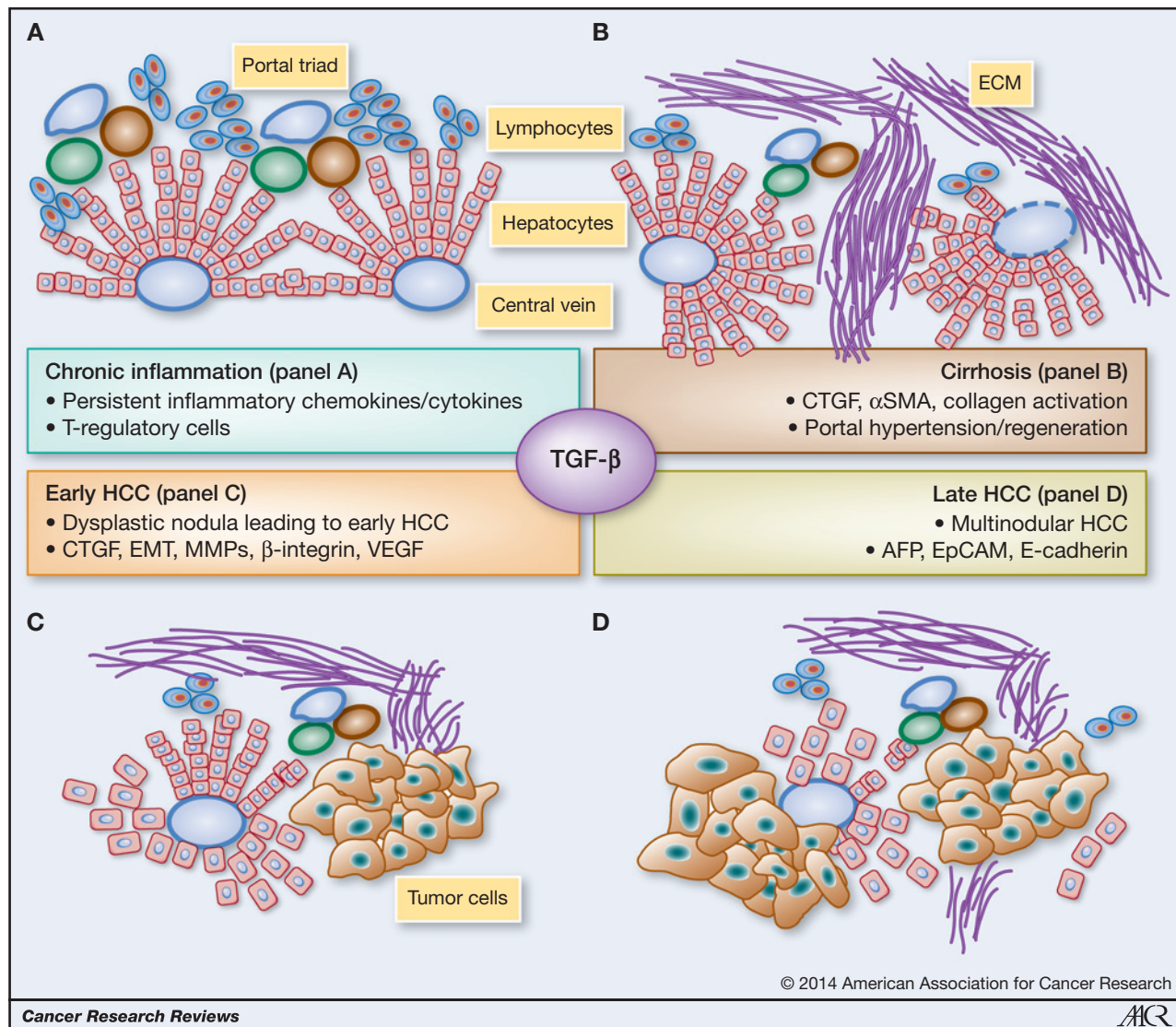


Figure 1. Development from chronic inflammation to hepatocellular carcinoma (HCC; red circle, artery; green circle, bile duct; blue circle, vein). Hepatocellular carcinoma develops from preexisting chronic inflammation (A) that progresses to cirrhosis (B), followed by early (C) and late tumor lesions (D). CTGF, connective tissue growth factor; α -SMA, α -smooth muscle actin; MMP, matrix metalloproteinase.

orchestrates the homeostasis of microenvironment components and tumor cells by balancing the activity of inflammatory cells or fibroblast with tumor cell growth or progression. For example, in treatment-resistant viral hepatitis, TGF- β and IL-10 secretions are increased and associated with increased T regulatory cells; these have a local immunosuppressive effect and consequently contribute to a persistent viral infection. With continued inflammation of the liver, reversible and irreversible remodeling are often observed, including fibrosis and early cirrhosis (6). This remodeling is characterized by accumulation of extracellular matrix (ECM) proteins. The rich ECM deposition is driven in large part by activation of TGF- β ; if not stopped, this leads to cirrhosis and eventually to liver failure. The accumulation of ECM proteins leading to fibrosis is still a reversible step. This phase would be particularly important for therapeutic interventions aimed at blocking the progression of liver disease, thus

preventing hepatocellular carcinoma occurrence. Unfortunately, such therapies are not yet available. As cirrhotic process progresses inside the liver, dysplastic nodules with initial chromosomal changes are often observed. These dysplastic nodules can progress to the early stages of hepatocellular carcinoma. In the liver tissue, cancer cells grow embedded in an environment enriched with ECM and mediated by TGF- β and connective tissue growth factor (CTGF; refs. 7, 8). This tight interaction between the tumor and the surrounding cirrhotic liver is perhaps the most remarkable hallmark of hepatocellular carcinoma (Fig. 1).

TGF- β Signaling in Hepatocellular Carcinoma

A seminal gene expression study by Coulouarn and colleagues demonstrated in a mouse model and later also in human tissue (9), that there are two different TGF- β

signaling responses, one called an "early" and another termed "late" TGF- β signaling response. Intriguingly, the early response pattern is associated with longer and the late response pattern with shorter survival. It is possible that the "early response" pattern reflects the physiologic inflammatory response, while the "late response" pattern is associated with a long-term TGF- β activation similar to the one described in colorectal cancer (10). The role of TGF- β is also recognized in a molecular classification of hepatocellular carcinoma, where the Wnt signaling pathway is regulated by TGF- β . In one subgroup of hepatocellular carcinoma tumors, TGF- β signaling appears to control tumor cell growth; in a second subgroup, it is associated with larger tumors, low α -fetoprotein (AFP) expression and poor prognosis (11). The identification of such a subgroup may represent a first attempt to identify patients with hepatocellular carcinoma likely to respond to TGF- β signaling inhibition. Because of the differences between transcriptome-based studies, a review of such data is being conducted to obtain a common classification and to better understand the pathophysiologic pathways driving hepatocellular carcinoma. Upon review of these transcriptome-based reports, TGF- β signaling may be associated with expression of AFP and EpCAM (12). On the basis of this concomitant expression, TGF- β signaling may be present in 25% of all early hepatocellular carcinoma. It is important to remember that transcriptome-based evaluations are derived from surgical specimen after local resections and are not from advanced hepatocellular carcinoma. This has implications when interpreting the effect of TGF- β signaling in later stages of hepatocellular carcinoma or blocking this pathway with pharmacologic agents in more advanced hepatocellular carcinoma stages.

The extrinsic effect of TGF- β signaling is a result of tumor cell growth embedded in an ECM-enriched environment. This condition is characterized by secretion of TGF- β and other associated factors, such as CTGF, which affects cancer-associated fibroblasts (7, 8). In addition to activation of fibroblasts, TGF- β signaling has been associated with T regulatory cells, for example by activation of chemokines (such as CCL22) or by immune presentation of AFP (13, 14). More recently, TGF- β signaling has also been associated with tumor-initiating cells (15). Ongoing stimulation of liver progenitor cells by TGF- β leads to their transformation into tumor-initiating cells as shown by TGF- β -induced changes in CD90 and CD133 expression (15).

The intrinsic effects of TGF- β signaling are mostly observed in highly invasive tumor conditions. TGF- β signaling is associated with loss of cell polarity, acquisition of cellular motility, and increased tumor invasion (5, 16–22). A main change in tumor cells is related to EMT as measured by E-cadherin expression, a marker of EMT. In the presence of TGF- β , E-cadherin is shed from tumor cells and renders tumor cells more invasive and migratory. In association with other components of the tissue microenvironment such as laminin-5 (Ln-5) or CD44, TGF- β induces the transcription factor Snail, another component of EMT; this induction is also associated with poor prognosis (19, 23). Taken together, it appears that TGF- β signaling causes EMT and renders tumors more invasive.

Barriers for Developing TGF- β Inhibitors in Hepatocellular Carcinoma

One of the main challenges for developing an effective therapy against hepatocellular carcinoma is related to the underlying cirrhosis, which is not only associated with a reduced liver function, but also provides the support for tumor cell growth. Because of this unique microenvironment, new drugs should not only treat the tumor cells, but also prevent new tumoral lesions. Hence, the ideal drug should have a low toxicity profile, antitumor activity, and chemopreventive activity. Such a drug may have activity in later lines of treatment, but also in the adjuvant setting together with other ablative therapies.

For example, multikinase inhibitors have toxicity profiles that limit their use to later lines of treatment. Sunitinib was discontinued because of serious adverse events; other antiangiogenic kinase inhibitors carried a high risk of bleeding in patients with compromised coagulation. With the exception of sorafenib that has been used in conjunction with transarterial chemoembolization (24), few kinase inhibitors have been evaluated in adjuvant treatment regimens primarily because of their toxicity profile.

The antitumor activity of the ideal drug candidate would also have to show a relatively immediate antitumor effect. Sorafenib blocks multiple signaling pathways and has not only in an antiangiogenic effect, but also induces tumor cell apoptosis (25). However, several attempts to develop similarly active agents have thus far failed.

Finally, the ideal drug candidate would need to block processes associated with liver cirrhosis. By targeting this microenvironment signal, it is possible to inhibit the stimulus for tumor cell growth and thus prevent new tumor lesion. This aspect of drug development may have been overlooked during recent years; only recently have agents demonstrated this activity.

Unlike previously investigated agents, the inhibition of TGF- β signaling affects hepatocellular carcinoma in different stages of development and progression and may offer a wider therapeutic application. This is due to the presence of TGF- β signaling throughout the development of hepatocellular carcinoma. However, a modulator of microenvironment may not have an immediate antitumor effect as observed for chemotherapeutic treatments. Rather, the impact on the molecular and cellular tumor biology may require longer treatment times to "restore" the host tissue to a more physiologic phenotype.

Blocking TGF- β in Hepatocellular Carcinoma Using a Specific TGF- β Inhibitor

Given the hypothesized activity of TGF- β signaling in hepatocellular carcinoma, small-molecule inhibitors targeting the TGF- β RI serine/threonine kinase have been developed, including LY2157299 monohydrate (LY2157299; ref. 16). LY2157299 is now in clinical evaluation and has shown antitumor effects in patients with glioblastoma and hepatocellular carcinoma (26). Studying the antitumor activity *in vitro* and *in vivo* remains a challenge for LY2157299. Hence, LY2157299 and the surrogate compound LY2109761 have been used in some select *in vitro*

and *in vivo* models to better understand its activity in hepatocellular carcinoma. Using *in vitro* assays first, we characterized the role of TGF- β signaling in the migration and invasion of hepatocellular carcinoma cells. These studies suggested that LY2157299 did not have an apoptotic effect at the intended pharmacologic levels for patients, but rather blocked invasion and migration of hepatocellular carcinoma cells (17). At the same time, the levels of E-cadherin were altered increasing the E-cadherin tissue expression and reducing the secretion of E-cadherin in the supernatant. LY2157299 seems to also block the CTGF production with the consequent reduction of stromal reaction. This reduces tumor growth in experimental models and will likely have a beneficial impact on the cirrhotic process (17, 20). LY2157299 also reduces hepatocellular carcinoma growth and progression by inhibiting neoangiogenesis. Indirectly, LY2157299 blocks VEGF production with better biologic activity than the antiangiogenic drug bevacizumab. Moreover, LY2157299 blocks β 1-integrin activation in cancer cells and consequently blocks intravasation of hepatocellular carcinoma cells into the blood vessels (18, 21).

Overall, the encouraging antitumor activities observed with LY2157299 formed the rationale for investigating LY2157299 in a phase II clinical trial of patients who either failed previous sorafenib treatment or were ineligible to receive sorafenib (NCT01246986, <http://clinicaltrials.gov>). On the basis of the previous observations that high AFP levels correlate with high TGF- β 1 levels (27), a study in patients with elevated AFP was initiated to investigate the activity of a TGF- β inhibitor in patients with hepatocellular carcinoma, where EMT is a key driver. In these patients, LY2157299 reduced not only TGF- β 1, but also E-cadherin in plasma of patients (28). This reduction is consistent with the *in vitro* studies, where LY2157299 also reduced the secretion of E-cadherin while inhibiting the TGF- β signaling (17). So far, no other drug treatment has been

associated with changes in plasma TGF- β 1 and E-cadherin levels. This is remarkable because biomarker responses are rarely observed in hepatocellular carcinoma treatments. In approximately 23% of patients treated with LY2157299, serum AFP levels were reduced by more than 20% compared with baseline. These patients had a benefit assessed by time-to-tumor progression (18.6 weeks) and also by overall survival (93.1 weeks) compared with patients who had no AFP responses (29). Although AFP is a tumor marker of hepatocellular carcinoma, the responses in patients with elevated AFP may perhaps mean more than just an overall tumor response. Currently, the relationship of AFP to E-cadherin is being investigated to better understand the significance of AFP responses.

The use of LY2157299 to block TGF- β signaling appears to modulate EMT and has a clinically meaningful benefit to patients. The measurement of E-cadherin and TGF- β 1 levels may also open new opportunities in novel drug development approaches; ongoing clinical studies will determine whether LY2157299 will provide a new treatment option for patients. Meanwhile, it is gratifying that some of the *in vitro* observations have resulted in clinical observations.

Disclosure of Potential Conflicts of Interest

M. Lahn is a medical fellow and has ownership interest (including patents) in Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

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References

- Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
- Roberts AB, Anzano MA, Lamb LC, Smith JM, Sporn MB. New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues. *Proc Natl Acad Sci U S A* 1981; 78:5339–43.
- Thiery JP. Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002;2:442–54.
- Heldin CH, Moustakas A. Role of Smads in TGF β signaling. *Cell Tissue Res* 2012;347:21–36.
- Fransvea E, Mazzocca A, Santamato A, Azzariti A, Antonaci S, Giannelli G. Kinase activation profile associated with TGF- β -dependent migration of HCC cells: a preclinical study. *Cancer Chemother Pharmacol* 2011;68:79–86.
- Brenner C, Galluzzi L, Kepp O, Kroemer G. Decoding cell death signals in liver inflammation. *J Hepatol* 2013;59:583–94.
- Bissell D. The origin of hepatic myofibroblasts. Mak KM, Leo MA, Lieber CS. Alcoholic liver injury in baboons: transformation of lipocytes to transitional cells [Gastroenterology 1984;87:188–200]. *J Hepatol* 2002;37:298.
- Gressner OA, Lahme B, Siluschek M, Rehbein K, Weiskirchen R, Gressner AM. Intracrine signalling of activin A in hepatocytes upregulates connective tissue growth factor (CTGF/CCN2) expression. *Liver Int* 2008;28:1207–16.
- Coulouarn C, Factor VM, Thorgeirsson SS. Transforming growth factor- β gene expression signature in mouse hepatocytes predicts clinical outcome in human cancer. *Hepatology* 2008;47:2059–67.
- Calon A, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Céspedes MV, et al. Dependency of colorectal cancer on a TGF- β -driven program in stromal cells for metastasis initiation. *Cancer Cell* 2012;22:571–84.
- Hoshida Y, Nijman SM, Kobayashi M, Chan JA, Brunet JP, Chiang DY, et al. Integrative transcriptome analysis reveals common molecular subclasses of human hepatocellular carcinoma. *Cancer Res* 2009;69: 7385–92.
- Yamashita T, Forgues M, Wang W, Kim JW, Ye Q, Jia H, et al. EpCAM and alpha-fetoprotein expression defines novel prognostic subtypes of hepatocellular carcinoma. *Cancer Res* 2008;68:1451–61.
- Behboudi S, Pereira SP. Alpha-fetoprotein specific CD4 and CD8 T cell responses in patients with hepatocellular carcinoma. *World J Hepatology* 2010;2:256–60.
- Yang P, Li QJ, Feng Y, Zhang Y, Markowitz GJ, Ning S, et al. TGF- β -miR-34a-CCL22 signaling-induced Treg cell recruitment promotes venous metastases of HBV-positive hepatocellular carcinoma. *Cancer Cell* 2012;22 291–303.
- Wu K, Ding J, Chen C, Sun W, Ning BF, Wen W, et al. Hepatic transforming growth factor beta gives rise to tumor-initiating cells and promotes liver cancer development. *Hepatology* 2012;56:2255–67.

16. Dituri F, Mazzocca A, Peidrò FJ, Papappicco P, Fabregat I, De Santis F, et al. Differential Inhibition of the TGF- β signaling pathway in HCC cells using the small molecule inhibitor LY2157299 and the D10 monoclonal antibody against TGF- β receptor type II. *PLoS ONE* 2013;8:e67109.
17. Fransvea E, Angelotti U, Antonaci S, Giannelli G. Blocking transforming growth factor- β up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells. *Hepatology* 2008;47:1557–66.
18. Fransvea E, Angelotti U, Antonaci S, Giannelli G. Targeting transforming growth factor (TGF)- β RI inhibits activation of β 1 integrin and blocks vascular invasion in hepatocellular carcinoma. *Hepatology* 2009;49:839–50.
19. Giannelli G, Bergamini C, Fransvea E, Sgarra C, Antonaci S. Laminin-5 with transforming growth factor- β 1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. *Gastroenterology* 2005;129:1375–83.
20. Mazzocca A, Fransvea E, Dituri F, Lupo L, Antonaci S, Giannelli G. Down-regulation of connective tissue growth factor by inhibition of transforming growth factor β blocks the tumor-stroma cross-talk and tumor progression in hepatocellular carcinoma. *Hepatology* 2010;51:523–34.
21. Mazzocca A, Fransvea E, Lavezzari G, Antonaci S, Giannelli G. Inhibition of transforming growth factor β receptor I kinase blocks hepatocellular carcinoma growth through neo-angiogenesis regulation. *Hepatology* 2009;50:1140–51.
22. Serova M, Tijeras-Raballand A, Dos Santos C, Muller N, Benhadji KA, Paradis V, et al. Effects of TGF- β signaling inhibition with LY2157299 in hepatocarcinoma models and in ex vivo whole tumor tissue samples from patient specimen [abstract]. In: Proceedings of the 104th Annual Meeting of the American Association for Cancer Research; 2013 Apr 6–10; Washington, DC. Philadelphia (PA):AACR; 2013. Abstract 2094.
23. Mima K, Hayashi H, Imai K, Kuroki H, Nakagawa S, Okabe H, et al. High CD44s expression is associated with the EMT expression profile and intrahepatic dissemination of hepatocellular carcinoma after local ablation therapy. *J Hepatobiliary Pancreat Sci* 2013;20:429–34.
24. Cui HZ, Dai GH, Shi Y, Chen L. Sorafenib combined with TACE in advanced primary hepatocellular carcinoma. *Hepatogastroenterology* 2013;60:305–10.
25. Abou-Alfa GK, Schwartz L, Ricci S, Amadori D, Santoro A, Figer A, et al. Phase II study of sorafenib in patients with advanced hepatocellular carcinoma. *J Clin Oncol* 2006;24:4293–300.
26. Rodon J, Carducci MA, Sepúlveda JM, Azaro A, Calvo E, Seoane J, et al. Integrated data review of the first-in-human dose (FHD) study evaluating safety, pharmacokinetics (PK), and pharmacodynamics (PD) of the oral transforming growth factor- β (TGF- β) receptor I kinase inhibitor, LY2157299 monohydrate (LY). *J Clin Oncol* 31, 2013 (suppl; abstr 2016).
27. Giannelli G, Mazzocca A, Fransvea E, Lahn M, Antonaci S. Inhibiting TGF- β signaling in hepatocellular carcinoma. *Biochim Biophys Acta* 2011;1815:214–23.
28. Giannelli G, Santoro A, Kelley RK, Merle P, Gane E, Douillard JY, et al. Phase 2 study of the oral transforming growth factor- β (TGF- β) receptor I kinase inhibitor LY2157299 [abstract]. In: Proceedings of the 7th ILCA (International Liver Cancer Association) Annual Conference; 2013; Washington, DC. Abstract 0-029.
29. Faivre SJ, Santoro A, Kelley RK, Merle P, Gane E, Douillard JY, et al. A phase 2 study of a novel transforming growth factor- β (TGF- β 1) receptor I kinase inhibitor, LY2157299 monohydrate, in patients with advanced hepatocellular carcinoma (HCC). *Gastrointestinal Cancers Symposium* 2014. San Francisco, California. Abstract LBA173.